Genotypic profiles of emerging multidrug-resistant *Staphylococcus capitis* isolates from an ongoing outbreak in critically ill patients

BAREND J MYNHARDT

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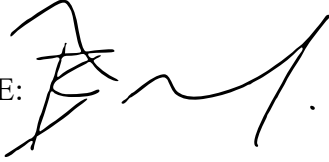Submitted in partial fulfilment of the requirements for the degree

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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 own original work and has not previously, in its entirety or in part, been submitted to any university or degree. I further declare that all sources cited are acknowledged using a list of references.

SIGNATURE: _____ DATE: 18 May 2023

DEDICATION

I dedicate this dissertation to my parents for nurturing me with affections and love and their dedicated partnership for success in my life.

"What's the bravest thing you've ever said? asked the boy.

'Help,' said the horse.

'Asking for help isn't giving up,' said the horse. 'It's refusing to give up.''

~ Charlie Macksey, The Boy, the Mole, the Fox and the Horse

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS

α	alpha
β	beta
μ	micro
%	percentage
°C	degree Celsius

ABBREVIATIONS

ABC	Adenosine triphosphate-binding cassettes
AC	Arterial catheters
ACME	Arginine catabolic mobile element
AMR	Antimicrobial resistance
AOLC	Arcidine orange leukocyte cytospin
API	Analytical profile index
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
bp	Base pair
BSI	Bloodstream infection
CA	Community-acquired
CAN	Colistin-nalidixic acid
CARD	Comprehensive Antibiotic Resistance Database
CAUTI	Catheter-associated urinary tract infection
CDC	Centers for Disease Control and Prevention
CDER	Center for Drug Evaluation and Research
CFU	Colony forming unit
CGE	Center of Genomic Epidemiology
CLABSI	Central line-associated bloodstream infection
CONS	Coagulase-negative *Staphylococcus*
CoPS	Coagulase-positive *Staphylococcus*
CRBSI	Catheter related bloodstream infection

CVC	Central venous catheters
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
ECDC	European centre for disease prevention and control
ECM	Extracellular matrix
EOS	Early-onset sepsis
ER	Emergency room
FBRI	Foreign body related infection
FDA	Food and Drug Administration
FISH	Fluorescent *in situ* hybridisation
g	Gram
g	Relative centrifugal force
Gb	Gigabase
GDP	Guanosine diphosphate
GP	Gram-positive
GTP	Guanosine-5'-triphosphate
HAI	Healthcare-associated infection
HAP	Healthcare-associated pneumonia
HCl	Hydrochloric acid
HGT	Horisontal gene transfer
HRMA	High resolution melting curve analysis
HVR	Hypervariable region
ICU	Intensive care unit
IF	Initiation factor
IMD	Invasive medical devices
IV	Intravenous
IVC	Intravascular catheter
IS	Insertion sequence
KCl	Potassium Chloride
LMIC	Low and middle income countries
LOS	Late-onset sepsis
LZR	Linezolid resistance

M	Molar
M-PCR	Multiplex Polymerase Chain Reaction
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
Mbp	Mega base pair
MDR	Multidrug-resistance
MIC	Minimum inhibitory concentration
MSF	Médecins Sans Frontières
MgCl$_2$	Magnesium Chloride
MGE	Mobile genetic elements
min	Minutes
mL	Millilitre
MLST	Multi locus sequence typing
MLVA	Multiple locus variable number tandem repeat analysis
mM	Millimolar
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar
µmol	Micromole
MR-CONS	Methicillin-resistant coagulase-negative *Staphylococcus*
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MSA	Mannitol salt agar
MSCRAMM	Microbial surface components recognising adhesive matrix molecule/s
NaCl	Sodium Chloride
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
NHLS	National Health Laboratory Service
NICD	National Institute of Communicable Diseases
NICU	Neonatal intensive care unit
NRCS-A	New Reduced-complexity Sequence Type A clone
ORF	Open reading frame

PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEA	Phenylethyl alcohol
PFGE	Pulsed-field gel electrophoresis
PGA	Poly-γ-glutamic acid
PIA	Polysaccharide intercellular adhesin
PICC	Peripherally inserted central venous catheters
pmol	Pico molar
PNAG	Poly-N-acetylglucosamine
PSM	Phenol-soluble modulins
PTC	Peptide-transferase centre
rpm	Revolutions per minite
rRNA	ribosomal-Ribonucleic acid
SCC*mec*	Staphylococcal cassette chromosome methicillin
sec	Seconds
SMRT	Single Molecule Real Time
SOP	Standard operating procedure
ST	Sequence Type
STG	Standard Treatment Guideline
TBE	Tris Borate Ethylenediaminetetraacetic acid Buffer
UHN	University Health Network
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UTI	Urinary tract infection
UV	Ultraviolet
v/v	Volume per volume
VAD-RBSI	Ventricular-assist device related bloodstream infection
VAP	Ventilator-associated pneumonia
VISA	Vancomycin intermediate *Staphylococcus aureus*
VNTR	Variable number tandem repeat
VRSA	Vancomycin resistant *Staphylococcus aureus*
wgMLST	Whole genome multi locus sequence typing
WGS	Whole genome sequencing

WHO   World Health Organization

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

MANUSCRIPTS IN PREPARATION

Mynhardt BJ, Hoosien E, Laurent F, Kock MM & Ehlers MM (2023) Genotypic profiles of emerging multidrug-resistant *Staphylococcus capitis* isolates from an ongoing outbreak in critically ill patients. To be submitted to the *Journal of Clinical Microbiology and Infection*.

CONFERENCE PRESENTATIONS IN PREPARATION

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GENOTYPIC PROFILES OF EMERGING MULTIDRUG-RESISTANT *STAPHYLOCOCCUS CAPITIS* ISOLATES FROM AN ONGOING OUTBREAK IN CRITICALLY ILL PATIENTS

by

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SUMMARY

Staphylococcus capitis, a type of coagulase-negative *Staphylococcus* (CONS), is frequently found on human skin. However, under certain conditions, it can lead to a range of illnesses including skin infections, bloodstream infections and sepsis. Linezolid is a last resort treatment for serious Gram-positive infections and multidrug-resistant (MDR) infections. This study aimed to examine the genetic characteristics of *S. capitis* isolates that are both linezolid-resistant (LZR) and MDR and were obtained from an ongoing outbreak since September 2014 among critically ill patients in private sector hospitals in Gauteng.

A total of 119 *S. Capitis* isolates were obtained from a private diagnostic laboratory. The *S. capitis* isolates were isolated from critically ill patients in intensive care units. Antimicrobial susceptibility testing (AST) was performed by the VITEK® 2 automated system (bioMérieux, France) and showed that all *S. capitis* isolates were LZR and resistant to at least two different antimicrobial classes. Out of the 119 isolates included in the panel, one isolate demonstrated resistance to all 12 antimicrobial agents while six isolates showed resistance to nine different antimicrobial agents, 23 isolates demonstrated resistance to eight antimicrobial agents.

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Additionally, 63 isolates were resistant to seven antimicrobial agents, 18 isolates were resistant to six antimicrobial agents, seven isolates were resistant to five antimicrobial agents while one isolates was resistant to one antimicrobial agent with other antimicrobial agents not reported. The ETEST® strip (bioMérieux, France) used for linezolid susceptibility testing showed a wide range of minimum inhibitory concentration (MIC) values from 8 μg/mL to >256 μg/mL. The M-PCR assays showed the *cfr* gene was only present in one isolate while the *optrA* and *poxtA* genes were absent in all isolates. These genes are reported to mediate resistance to linezolid by acquisition via plasmid or composite transposons.

The pulsed-field gel electrophoresis (PFGE) showed that the majority of the LZR MDR *S. capitis* isolates were closely related and were grouped into one major pulsotype (76 isolates), three minor pulsotypes and 10 singletons. After whole-genome sequencing (WGS) of five representative isolates, analysis through several online pipelines revealed the presence of antimicrobial resistant genes such as: *tetK*, *aac*(6')-le-*aph*(2")-la, *fusB*, *sepA*, *sdrM*, *mupA*, *mdeA*, *mecA*, *blaZ*, *ermC*, *dfrC*, *gyrB*, *qacA*, *qacD* and *cat*. All five representative isolates showed a point mutation at G2604T and T173A on the rRNA gene conferring resistance to linezolid. No virulence genes were detected using the VirulenceFinder (CGE, Denmark). Plasmids detected with PlasmidFinder included: [repL (pDLK1), repA (pLW043), repA (SAP016A), ORF (pKH1), rep (pSSAP1) and repI (pGB354)]. With SCCmecFinder, only one type was detected in all five isolates namely SCC*mec* type Vb(5C2&5). This observation represents the only correlation between the results of this study and the characteristics of the NRCS-A clone. Isolate 143 with the *cfr* gene was not sequenced as the MIC value (8 μg/mL) did not show any significance. The majority of the LZR MDR *S. capitis* isolates were closely related and harboured similar antimicrobial resistance genes (ARGs), plasmids and *SCCmec* elements which suggests the establishment of a successful clone based on the pulsotype cluster. These findings emphasize the necessity of surveillance to track the dissemination of successful LZR MDR *S. capitis* clones in hospitals in the greater Gauteng region.

Keywords: *Staphylococcus capitis*, linezolid resistance (LZR), multidrug-resistance (MDR)

DISSERTATION OUTLINE

This dissertation is written in the format of a manuscript for submission to a relevant journal. The dissertation comprises of four chapters as shown below:

Chapter 1: Introduction

The evolution of *Staphylococcus capitis* from contaminant of clinical samples or human microflora to a colonising agent in healthcare-associated infections in recent years are introduced. The aim and objectives of the study is to investigate the genotypic profiles of emerging multidrug-resistant *Staphylococcus capitis* isolates from an ongoing outbreak in critically ill patients in South Africa's private sectors.

Chapter 2: Literature Review

An elaborate explanation of *S. capitis* infections are discussed in the literature review. This chapter begins with a section on how the knowledge of catheters and catheter-related infections contribute to understanding the pathogenicity of CONS, more specifically *S. capitis*. The topic progress to characterise LZR MDR *S. capitis*. The chapter ends with a thorough look at antimicrobial resistance, infection prevention and detection of LZR MDR *S. capitis* isolates.

Chapter 3: Genotypic profiles of emerging multidrug-resistant *Staphylococcus capitis* isolates from an ongoing outbreak in critically ill patients

This MSc study is encapsulated in this chapter by using the editorial style of the *Journal of Clinical Microbiology and Infection* with an impact factor of 13.310. The guidelines for the jornal format are detailed in annexure E.

Chapter 4: Conclusion

This chapter highlights the significant findings of the research and details the strengths and shortcomings of the MSc study. Emphasis is placed on the future research that will follow based on the work done in this study.

CHAPTER 1

INTRODUCTION

1.1   Introduction

Coagulase-negative staphylococci (CONS) have emerged as major causes of healthcare-associated infections (HAIs) (Cui *et al.*, 2013; Tufariello *et al*., 2020). Some infections include bacteraemia, central nervous system shunt infection, endocarditis, urinary tract infection (UTI), surgical site infection, endophthalmitis and foreign body infections (Piette and Verschraegen, 2009; Michalik *et al*., 2020). These opportunistic pathogens infect premature neonates, immunocompromised patients admitted to intensive care units (ICUs), patients requiring foreign body devices during hospital stay and patients with dialysis and prosthetic implants (Becker *et al.*, 2014; Michalik *et al*., 2020). Coagulase-negative staphylococci were officially recognised as one of the etiological agents responsible for HAIs in the 1970s but was long thought as a contaminating agent when isolated from specimens, due to their presence in the normal microbiota of the human skin and mucous membrane (Piette and Verschraegen, 2009).

1.1.1   Species within the coagulase-negative *Staphylococcus* group

The species within the CONS group are delineated from *Staphylococcus aureus* (*S. aureus*, the predominant species grouped in coagulase-positive staphylococci) due to this group's inability to produce coagulase, an enzyme that mediates blood clot formation (Ryan, 2018). Coagulase-negative staphylococci can be further grouped as either novobiocin-resistant (*S. cohnii*, *S. saprophyticus*, *S. sciuri* and *S. xylosis*) or novobiocin-susceptible species (*S. auricularis*, *S. capitis*, *S. caprae*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. pasteuri*, *S. saccharolyticus*, *S. schleiferi*, *S. simulans* and *S. warneri*) (Schleifer and Bell, 2015). Lamers *et al*. (2012) proposed a new classification system to classify staphylococci into clusters based on the 16S rRNA elongation factor Tu (*tuf*), β-subunit of RNA polymerase (*rpoB*) and heat shock protein 40 (*dnaJ*) genes. The *Staphylococcus* genus can be grouped in 15 different cluster groups with *S. capitis*, *S. epidermidis* and *S. caprae* forming part of the Epidermidis Cluster Group (ECG) (Lamers *et al*., 2012).

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1.1.2 Antimicrobial resistance in *Staphylococcus capitis*

Staphylococcus capitis was generally susceptible to antistaphylococcal antibiotics, until the emergence of pathogenic multidrug-resistant (MDR) *S. capitis* strains in neonates and adult patients detected in 2012 in French Hospitals (Butin *et al.*, 2017b). Multidrug-resistant *S. capitis* strains have gained resistance mechanisms that improve their survival in the presence of two or more antimicrobial agents (Ryan, 2018). These MDR *S. capitis* strains are of great clinical importance as it spreads clonally in healthcare settings and are the cause of severe infections such as infective endocarditis and osteomyelitis (Butin *et al*., 2017a). Contact spread through touch is the major mechanism of spreading of this opportunistic pathogen (Schleifer and Bell, 2015; De Groote *et al*., 2022). Global travel of healthcare workers and point-source contamination of equipment and/or products are other important aspects contributing to the spread of these successful clones (Carter *et al.*, 2018; Parsons *et al*., 2019).

1.1.3 Linezolid resistance in *Staphylococcus capitis*

The majority of these nosocomial strains are resistant to methicillin and penicillin with a decreased susceptibility to glycopeptides (Butin *et al.*, 2017b). Linezolid, an oxazolidinone was approved for clinical use as an alternative antimicrobial agent for MDR, complicated skin and soft tissue infections on 18 April 2000 (Etebu and Arikekpar, 2016). In 2005, the first linezolidresistant (LZR) *Streptococcus pneumonia* strains were reported (Wolter *et al.*, 2005). The occurrence of the LZR phenotype can be attributed to three mechanisms: i) a mutation in domain V of the 23S rRNA, ii) a mutation in the 50S rRNA or, iii) the acquisition of chloroform reductive dehalogenase A (*cfrA*), phenicol oxazolidinone resistance *(poxt*A) and/or ATP binding cassette(ABC)-F *(optrA)* genes via horizontal gene transfer (HGT) (Mendes *et al.*, 2014; Butin *et al.*, 2017b; Bamford *et al*., 2021).

Although methicillin-resistant *S. epidermidis* is the most frequently isolated CONS in healthcare-associated environments, studies have shown the emergence of LZR MDR *S. capitis* as an important opportunistic pathogen in ICUs and neonatal-ICUs (NICU) (Cui *et al.*, 2013; Butin *et al.*, 2017b; Tevell *et al.*, 2017; Carter *et al.*, 2018). Studies from France, the United Kingdom, Belgium and Australia have identified a distinct clone named the New Reduced-complexity Sequence Type A clone (NRCS-A) to be the most common cause of NICU-associated bacteraemia by detecting a distinct pattern through pulsed-field gel electrophoresis (PFGE) (Butin *et al.*, 2016; Butin *et al.*, 2017b; Carter *et al.*, 2018; Butin *et al.*,

2019). This clone is associated with all methicillin-resistant *S. capitis* strains found in NICUs in France, which suggests a high dissemination of the same clone (Butin *et al.*, 2016). Some of its characteristics include: i) its ability to persist and become endemic, ii) its increased resistance to antimicrobial agents commonly used in NICUs, iii) harbouring type V staphylococcal chromosomal cassettes (SCC*mec*) and iv) a MDR profile with either resistance or hetero-resistance to vancomycin (Butin *et al.*, 2016; Butin *et al.*, 2017b). However, studies have shown more diverse and unrelated banding patterns in adult patients infected with *S. capitis* differing from the distinct patterns seen in neonatal patients (Butin *et al.*, 2016; Decalonne *et al.*, 2020).

One of the most difficult challenges with CONS is the ability to discern the clinical difference between colonising and invasive microorganisms when isolated from a specimen (Frebourg *et al.*, 2000; Becker *et al.*, 2014). This is a crucial identification to make in order to prescribe the patient with the correct antimicrobial agent which will prevent selection of antimicrobial-resistant bacteria (Asaad *et al.*, 2016; Hebeisen *et al.*, 2019).

1.1.4   Pathogenicity of *Staphylococcus capitis*

The pathogenic significance of each CONS species are promoted by its biofilm forming capabilities (Kim *et al.*, 2018). *Staphylococcus capitis* is known to be susceptible to antimicrobial agents and has low virulence compared to *S. epidermidis* and *S. haemolyticus,* however, it has been reported that *S. capitis* gained antimicrobial resistance (AMR) in several patients with sepsis and endocarditis (Butin *et al.*, 2017a). One important virulence factor of *S. capitis* is its biofilm production (Cui *et al.*, 2013). There are four steps to biofilm formation: i) primary attachment, ii) accumulation, iii) maturation and iv) detachment (Becker *et al.*, 2014; Büttner *et al.*, 2015). Coagulase-negative staphylococci form biofilms by adhering to foreign bodies using hydrophobic interactions mediated by two proteins, the biofilm-associated protein homologue (Bhp) and an adhesin/autolysin E (AtlE) (Salgueiro *et al.*, 2017).

The expression of bacterial cell surface proteins occurs through specific interactions with host extracellular matrix (ECM) components (Büttner *et al.*, 2015; Salgueiro *et al.*, 2017). The ECM interacts with a group of proteins, called microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (Salgueiro *et al.*, 2017). There are several of these MSCRAMMs in CONS, each of which bind to a different protein: i) serine aspartate repeat-containing protein F (SdrF), binds to collagen I (structural protein found in skin), ii) Fg-binding

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protein (Fbe), a LPXTG-motif (name based on the main conserved residues) that contains a protein covalently attached to the bacterial cell surface, binds to fibrinogen (soluble protein found in blood plasma) coated surfaces and iii) Extracellular-matrix binding protein (Embp), binds to fibronectin (glycoprotein of the extracellular matrix) (Büttner *et al.*, 2015; Salgueiro *et al.*, 2017). Other proteins called putative adhesins described in *S. epidermidis*, include extracellular lipase (GehD) (that binds to collagen) and the *S. epidermidis* surface proteins (Ses) such as SesI (Salgueiro *et al.*, 2017).

After adherence have occurred, the biofilm needs to accumulate in multi-layered cell aggregates, this can be mediated by polysaccharide adhesins (PIA) and/or proteinaceous adhesins (Becker *et al.*, 2014). A PIA, also known as poly-N-acetylglucosamine (PNAG) connects bacterial cells in the biofilm by electrostatic attraction to the exposed teichoic acids (Widerström *et al.*, 2012; Becker *et al.*, 2014; Büttner *et al.*, 2015) The exopolysaccharide PIA/PNAG is synthesised by intercellular adhesion gene products (icaA, icaB) of the icaAB operon (Widerström *et al.*, 2012; Cui *et al.*, 2013). Other proteinaceous adhesins involved in biofilm formation and accumulation include accumulation-associated protein (Aap), Bap homologue protein (Bhp) and extracellular matrix binding protein (Embp) (Büttner *et al.*, 2015; Salgueiro *et al.*, 2017). The Aap protein can induce the accumulative phase without the presence of PIA/PNAG adhesin proteins (Büttner *et al.*, 2015).

1.1.5 Phenotypic and molecular testing of *Staphylococcus capitis*

The species of CONS can be determined through the use of manual commercial biochemical tests, such as the analytical profile index (API) Staph test (bioMérieux, Marcy l'Etoile, France) and automated systems such as the VITEK® 2 automated system (bioMérieux, Marcy l'Etoile, France) (Becker *et al*., 2014; Kim *et al*., 2018). Laboratories use an incubated purity plate to facilitate identification due to the presence of polymicrobial species (Mahon *et al.*, 2015). Both systems are accurate and rapid, however, the carry-over of samples into separate tests as with the API test, may lead to contamination (Mahon *et al*., 2015). The matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) aids in the identification of bacteria to species level (Singhal *et al.*, 2015). This is achieved by ionising the microogranisms' particles according to their mass-to-charge-ratio (Rychert, 2019). The MALDI-TOF is used in routine diagnostic laboratories for its rapid, sensitive, cost effective and effortless results (Singhal *et al.*, 2015). One major downfall of the MALDI-TOF is that

new isolates can only be detected if the spectral database contains peptide mass fingerprints of the type strains of the specific species (Singhal *et al.*, 2015).

Molecular testing is more sensitive and considered superior to phenotypic testing (Widerström *et al.*, 2012). Molecular testing is mainly used in reference or research laboratories rather than routine diagnostic laboratories due to the high cost (Hirotaki *et al.*, 2011). The most common nucleic-acid based approaches used in the identification of CONS are the polymerase chain reaction (PCR), multiplex (M)-PCR and real-time PCR assays. Other nucleic-acid based approaches that are not widely used in CONS identification are the fluorescent *in situ* hybridisation (FISH), microarrays and high-resolution melting curve analysis (HRMA) (Becker *et al.*, 2014). Some M-PCR applications include pathogen identification, gender screening, linkage analysis and forensic studies (Edwards and Gibbs, 1994). Multiplex-PCR assays are useful because more than one reaction can be run in the same tube but optimisation of the reaction conditions can be tedious and time consuming (Barer *et al.*, 2018; Bender *et al.,* 2019). Real-time PCR is a modification of PCR and can be used to quantitively determine the amount of starting nucleic acid (Varani *et al.*, 2009; Mahon *et al.*, 2015). By using this approach, analysis can be done on mRNA expression, genetic variation and mutations within certain regions (Kralik & Ricchi, 2017).

Genotyping methods are defined as methods comparing genetic material of bacterial strains and have higher discriminatory resolution on closely related strains (Widerström *et al.*, 2012). Pulsed-field gel electrophoresis (PFGE) is based on the digestion of chromosomal DNA with restriction enzymes and separated using pulsating electrophoresis, resulting in strains forming unique banding patterns (Mahon *et al.*, 2015). Although PFGE has the highest discriminatory power of all the genotyping methods, it can be technically demanding and slow (Widerström *et al.*, 2012; Dorneles *et al.*, 2018). Multi-locus sequence typing (MLST) identifies mutations in genes by sequencing allelic variants of the species' housekeeping genes after PCR amplification (Mahon *et al.*, 2015; Salgueiro *et al.*, 2017). Multi-locus sequence typing (MLST) is regularly used because of its excellent intralaboratory and interlaboratory comparisons despite its limited discriminatory power (Widerström *et al.*, 2012; Soroush *et al.*, 2016). Multilocus variable number tandem repeats (VNTR) analysis (MLVA) amplifies variable numbers of repeats in bacterial genomes and subsequently characterises individual strains based on the number of repeats at each investigated locus (Widerström *et al.*, 2012). Like PFGE, MLVA is also highly discriminatory but the variation in different loci may evolve

too quickly to permit reliable data on long term epidemiological relationships and population structures (Dahyot *et al.*, 2018). Conventional MLST can be integrated with next generation sequencing to whole genome MLST (wgMLST). Much more loci (1 500 to 4 000) are considered in wgMLST which will result in a higher typing resolution (Babenko *et al.*, 2016). Whole genome MLST is defined based on allelic variation where both recombination, insertions and deletions count as a single evolutionary event (Babenko *et al*., 2016). One limitation however is that wgMLST requires allele curation (Babenko *et al*., 2016).

Sequencing of bacterial genomes are used to determine the order of the nucleotides in a genome or DNA fragment of a cell at a single time, providing the most comprehensive characterisation (Mahon *et al.*, 2015). The first sequencing procedure, the chain termination method or Sanger sequencing was designed by Frederick Sanger and was thought to be labour intensive and only used for identifying single genes (Watson, 2014). Automated sequencing was developed and applied to large-scale processes such as the Human genome project where the U.S. Department of Energy and National Institute of Health team sequenced a 2.91 billion base pair (bp) consensus sequence of the euchromatic portion of the human genome (Venter *et al.*, 2001). Sequencing is advantageous in that is works with all bacteria (Dylus *et al.*, 2020). Other genotypic methods could be omitted however, it is too expensive to employ in diagnostic or clinical laboratories (Khromykh and Solomon, 2015; Dylus *et al*., 2020).

Little research is available regarding the prevalence and genotypic profiles of the ongoing outbreak of LZR MDR *S. capitis* in the private sector in South Africa. The knowledge of linezolid exposure among patients in this setting is unknown which makes it difficult to apply antimicrobial stewardship and alternative therapies. The purpose of this study was to identify the LZR MDR *S. capitis* isolates from multiple private hospitals in the greater Gauteng area, to compare the genetic relatedness of these LZR MDR *S. capitis* isolates and, to make recommendations for the prevention of its spread.

1.2   Aim

The aim of this study was to investigate the genotypic profiles of emerging multidrug-resistant *Staphylococcus capitis* isolates from an ongoing outbreak in critically ill patients in South Africa's private sectors.

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1.3   Objectives

The objectives of this study were:

- To re-confirm the antimicrobial sensitivities of the *Staphylococcus capitis* isolates with an E-test
- To re-confirm the identification of the *Staphylococcus capitis* isolates using a multiplex-PCR assay
- To detect the presence of the linezolid-resistant plasmid encoded gene, chloramphenicol-florfenicol resistance using a PCR assay
- To compare the genotypic profiles of the multidrug-resistant linezolid resistant *Staphylococcus capitis* isolates using pulsed field gel electrophoresis
- To molecularly characterise selected multidrug-resistant linezolid resistant *Staphylococcus capitis* isolates using whole genome sequencing

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Invasive medical devices (IMDs) are designed for critically ill patients that require either administration of fluids and antimicrobial agents or assistance with drainage of urine in the case of surgery or bedridden patients (Zhang *et al.*, 2016; Bierlaire *et al.*, 2021). The continual use of IMDs however, is not risk free (Cortese *et al.*, 2018). Critical patients using IMDs are at higher risk of healthcare-associated infections (HAIs) (Ory *et al.*, 2019).

A healthcare-associated infection (HAI) is defined as the presence of a nosocomial acquired infection (after 48 hours) that was not present at the time of admission to the hospital (Monegro *et al.*, 2022). The World Health Organization (WHO) estimated that HAIs are the biggest adverse event in healthcare settings regardless of the resources available (WHO, 2011; Steward *et al.*, 2021). Coagulase-negative staphylococci (CONS) have emerged as a major cause of HAIs in neonates and immunocompromised patients (Becker *et al.*, 2014; De Groote *et al.*, 2022). The exact extent of healthcare-associated infections (HAIs) is not known in low- and middle-income countries (LMICs), because of a lack of reported data. Studies conducted in LMICs often only report HAIs from individual hospitals or units, making it difficult to determine the overall burden of these infections (Maki & Zervos, 2021). The ability of CONS to form biofilms on IMDs, such as intravascular catheters (IVCs), are one of the most important virulence factors associated within this group (Becker *et al.*, 2014).

Staphylococcus capitis (*S. capitis*), which was once thought of as part of the normal microflora of the human body, was shown to be one of the etiological agents responsible for biofilm-related infections such as endocarditis and catheter-related bloodstream infections (CRBSI) (Cui *et al.*, 2013). Several studies done in different countries reported an emergence of multidrug-resistant (MDR) *S. capitis* in their healthcare settings, especially in neonatal Intensive Care Units (NICUs) (Gu *et al.*, 2013; Cui *et al.*, 2013; Doern *et al.*, 2016; Butin *et al.*, 2016). The majority of these MDR strains also showed resistance to linezolid, which is known as the standard therapy for MDR infections (Butin *et al.*, 2016).

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Linezolid is an antimicrobial agent that forms part of the oxazolidinone group (Center for Drug Evaluation and Research (CDER), 2004). Linezolid has proven to be active against a broad range of nosocomial Gram-positive bacteria such as vancomycin resistant *Enterococcus faecium* (VRE*fm*), methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant CONS (MR-CONS) (Sadowy, 2018). An international pharmaceutical company (Pfizer) patented linezolid under the brand name Zyvox (MSF, 2014). After August 2014, several affordable linezolid generics came into play once the patent ended (MSF, 2014).

The emergence of LZR MDR *S. capitis* strains have been observed in critically ill patients across the Gauteng province in private hospitals since 2012. This ongoing outbreak is of concern due to the fact that the majority of these LZR MDR *S. capitis* isolates have tested linezolid resistant in routine diagnostics.

2.2 An overview of intravascular catheters and related infections

Intravascular catheters remain an important aspect in improving patient health and management in hospitals (Von Eiff *et al.*, 2005). The majority of IVCs are inserted in the peripheral veins for short-term use, to administer fluids and/or medication while monitoring a postoperative, intensive care unit (ICU) or emergency room (ER) patient (Mahon *et al.*, 2015). The less common, semi-permanent IVCs are inserted in the central veins to perform procedures such as haemodialysis in patients with a renal disease and remain in the central vein from weeks to months (Mahon *et al.*, 2015). More permanent IVCs are implanted in patients that cannot survive without these catheters, an example would be a prosthetic heart valve (Von Eiff *et al.*, 2005). Even though IVCs are useful and efficient, they are prone to be colonised by microorganisms through biofilm formation and subsequent intraluminal migration that results in bloodstream infections (BSI) (Mahon *et al.*, 2015). It has been reported that eight microbial phyla and 136 diverse microbial genera have been detected on the surface of IVCs (He *et al.*, 2019). Gram-positive cocci are reported more frequently on colonised catheters, followed by yeasts and Gram-negative bacilli (Alonso *et al.*, 2019).

2.2.1 Clarification of terminology used in the dissertation

In this study, the term invasive medical device (IMD) will be used to avoid confusion between synonymous terms such as foreign-body devices, implanted medical devices and inserted medical devices. There are a few acronyms that are used to define a catheter related infection. These acronyms are sometimes used interchangeably but does not share the same meaning:

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A central line-associated bloodstream infection (CLABSI) is defined as a laboratory confirmed BSI where an organism is confirmed and a central line is present on the laboratory confirmed BSI (LCBI) day of event (O'Grady *et al.*, 2011; Centers for Disease Control and Prevention (CDC), 2019a). The clinical term, catheter related BSI (CRBSI) is used during patient diagnosis and treatment and never used during surveillance (O'Grady *et al.*, 2011; CDC, 2011). Catheter-associated urinary tract infections (CAUTIs) are defined as a UTI where an indwelling urinary catheter was in place for more than two calendar days on the day of the event (CDC, 2019b).

2.2.2 Types description of intravascular catheters

Invasive medical devices are used in almost every discipline in medical care for diagnostic and therapeutic procedures (Becker *et al.*, 2014). Invasive medical devices are classified based on the branch of medicine where it is most frequently used, as shown in Table 2.1 (Von Eiff *et al.*, 2005).

Table 2.1: Classification of invasive medical devices based on the branch of medicine where these devices are most frequently used (Von Eiff *et al.*, 2005)

Medical branch	Invasive medical devices
Intravascular	Peripheral catheters (venous, arterial), midline catheters, peripherally inserted central venous catheters (PICC), non-tunneled catheters (Cook, Arrow), tunneled catheters (Hickman, Broviac, Groshong), pulmonary artery catheters and totally implanted ports (Port-a-Cath, MediPort, Infusaport)
Cardiovascular	Mechanical heart valves, implantable defibrillators and related devices, vascular grafts, ventricular assist devices, coronary stents and Implantable patient monitors
Neurosurgical	Ventricular shunts, ommaya reservoirs, intracranial pressure devices and implantable neurological stimulators
Orthopaedic	Joint prostheses and other reconstructive orthopaedic implants, spinal implants and Fracture-fixation devices
Urological	Inflatable penile implants
Gynaecological	Breast implants
Otolaryngological	Cochlear implants and middle ear implants
Ophthalmological	Intra-ocular lenses and glaucoma tubes
Dental	Dental implants

Intravascular catheters are grouped into central venous catheters (CVC) and arterial catheters (AC) (Zhang *et al.*, 2016). The following characteristics are used to describe IVCs: i) the type of vessel used (e.g., peripheral, arterial, central), ii) whether it's a temporary, semi-permanent

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or permanent IVC, iii) the insertion site (e.g., subclavian, femoral, internal jugular, peripheral, PICC), iv) the pathway from skin to vessel (e.g., tunnelled, non-tunnelled), v) the length of the IVC, and vi) a special characteristic of the IVC (e.g., cuff present, impregnation with heparin, antibiotics used, antiseptics used, number of lumens) (CDC, 2011). The components of a general IVC are annotated on a tunnelled CVC in Figure 2.1 (University Health Network (UHN), 2017).
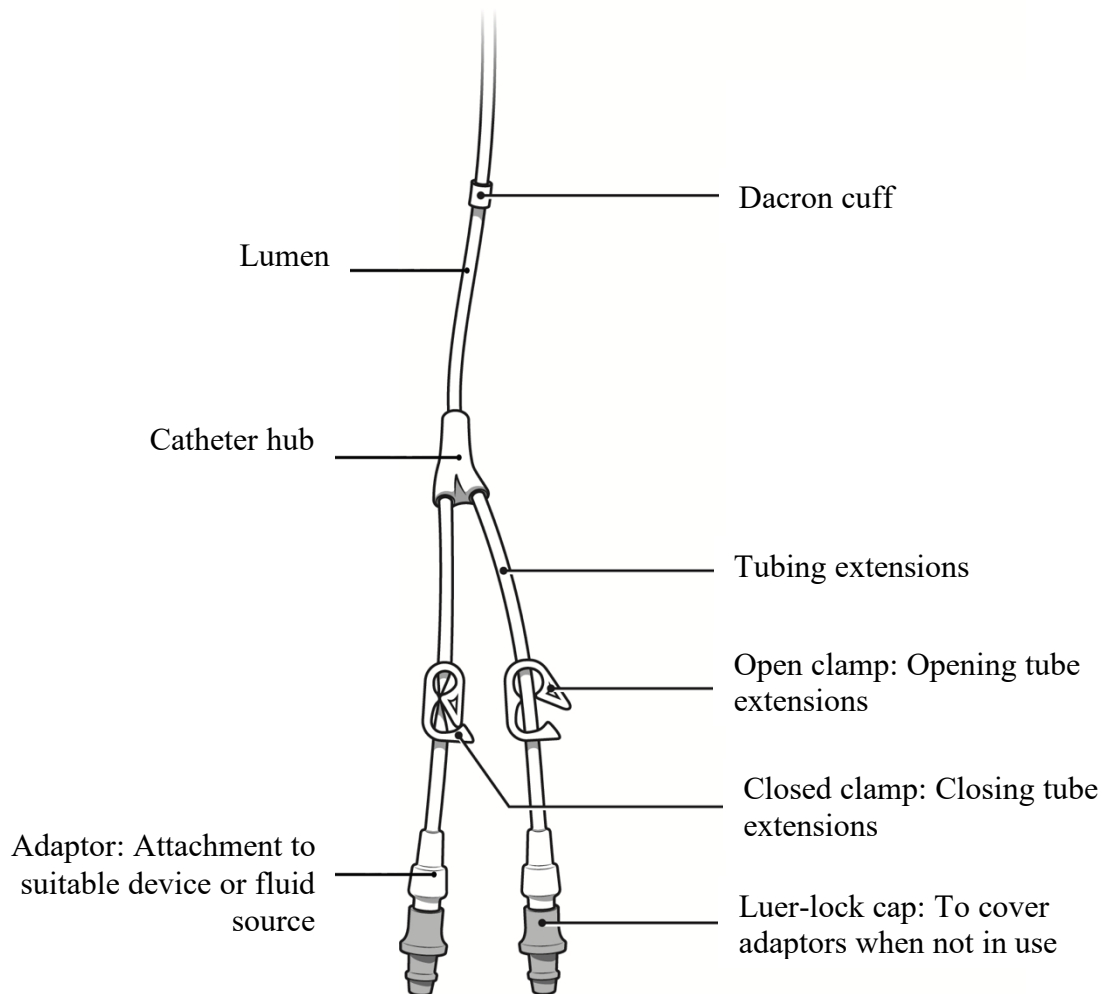
Figure 2.1: **An annotated diagram of a tunnelled central venous catheter (CVC), used for giving treatment, nutrients, IV fluids and taking blood samples (UHN, 2017)**

The Dacron cuff is placed under the skin near the exit site to allow tissue growth to seal the opening and also prevents the catheter from slipping out (UHN, 2017). Each lumen has a clamp (prevent leakage), adaptor and a Luer-lock cap (Figure 2.1) (UHN, 2017). Heparin, an anticoagulant can be given to patients that require prolonged intravenous (IV) therapy to prevent blood clot formation (Shah and Shah, 2007).

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2.2.3   Surveillance of intravascular catheter-related infections

Healthcare associated infections (HAIs) are observed globally, with LMIC at higher risk (Allegranzi *et al.*, 2011). Central line-associated bloodstream infections (CLABSI) are the most common type of HAI reported worldwide (Hebeisen *et al.*, 2019). Rosenthal and colleagues (2008) have shown that the infection rates for HAIs, especially CLABSI, CAUTI and ventilator-associated pneumonia (VAP) are 3 to 5 times higher in LMIC, compared to North America, West Europe and Australian ICUs (Rosenthal *et al.,* 2008).

The first step in reducing CRBSI prior to education and strict adherence to infection prevention and control programmes is to define the problem through surveillance (Strasheim *et al.*, 2015). Surveillance of infections such as CRBSI aid in understanding the natural history of the disease, detecting epidemics, understanding the distribution and spread, evaluating control and prevention measures that are in place, detecting change in healthcare practice and facilitate national and international strategies (Strasheim *et al.*, 2015).

2.2.4   Risk factors and patient populations associated with intravascular catheter-related infections

Patients that are at risk of catheter related infections are those who have had i) a prolonged hospital stay before insertion of a catheter, ii) a catheter for an extended time, iii) microbial colonization at the site of catheter insertion, iv) internal jugular venous catheterization, v) neutropenia, vi) total parenteral nutrition through the catheter and vii) suboptimal care of the catheter (Parsons *et al.*, 2019). Patients at risk include those with prosthetic valves, pacemakers, defibrillators, ventricular assist devices, intravascular catheters, or other IMDs as well as neonates and immunocompromised hosts (Tufariello & Lowy, 2019).

Some risk factors for CRBSI in particular include any underlying disease, the method used for catheter insertion, the site of catheter insertion, the duration (short term vs long term) and the purpose of the catheter (Gahlot *et al*., 2014). Administration of parenteral nutrition through intravascular catheters, increases the risk of CRBSI (Gahlot *et al*., 2014).

2.2.5   Diagnosis of intravascular catheter-related infections

Catheter cultures are usually sent to a diagnostic laboratory if a CRBSI is suspected (Chaves *et al.,* 2018). The methodologies used to diagnose these cultures most often include roll-plate (semiquantitative) and sonification/vortex methods (quantitative) (Chaves *et al.,* 2018).

Qualitative methodologies such as culture-broth immersion is unreliable for distinguishing between a contaminant and an infection (Mer, 2005). When interpreting a semiquantitative culture, the presence of 15 colony forming units (CFUs) is usually indicatory of significant catheter colonisation (Mer, 2005). Quantitative interpretation involves vortexing or flushing the internal surface, when a count of 13 CFU/surface is observed, significant catheter colonisation may be assumed (Mer, 2005). Some additional conservative techniques to diagnose CRBSI include endoluminal brushing, semiquantitative cultures of skin around the catheter site, accompanied with a peripheral blood culture and Gram staining of catheter blood with arcidine orange leukocyte cytospin (AOLC) (Mermel *et al.*, 2009). A molecular technique used for diagnosis of CRBSI is the amplification of the 16S rRNA gene by PCR. Septum sonication is performed along with the PCR assay to confirm ventricular-assist device related bloodstream infection (VAD-RBSI) in patients on antimicrobial therapy (Chaves *et al.,* 2018).

2.2.6 Pathogenesis of intravascular catheter-related infections

The source of CRBSI occurs either through colonisation of the device, or contamination of the fluid administered through the device (infusate-related infection) (Mermel *et al.*, 2009). Infusate contamination is usually regarded an epidemic BSI while CRBSIs are regarded as endemic BSIs (Mermel *et al.*, 2009).

Microorganisms that cause CRBSIs gain access to either the extraluminal or intraluminal surface of the device and adhere to the surface (Haddadin & Regunath, 2019). In order for the microorganisms to have a sustained infection and cause haematogenous spread, a biofilm is formed (Safdar *et al.,* 2004). Microorganisms gain access by one of three routes: i) through the percutaneous tract that is probably facilitated by capillary action, ii) by contaminating the catheter hub or lumen (Figure 2.1) when the catheter is inserted or later manipulated, or iii) by haematogenous transfer from remote sources of local infection (e.g., pneumonia) (Safdar *et al.,* 2004). It is suggested that the origin of CRBSI in short term devices is from the cutaneous site as opposed to luminal colonisation in long term devices (Haddadin & Regunath, 2019).

2.2.7 Pathogens of intravascular catheter-related infections

The majority of microorganisms that cause CRBSI form part of the normal microflora of the skin closest to the insertion site, colonisation of the catheter tip is the major cause of bacteraemia, sepsis and multi-organ failure in ICUs (Mer, 2005). Most CRBSIs occur due to bacterial infection such as *Staphylococcus aureus*, CONS, *Escherichia coli* and

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Klebsiellapneumoniae (Gahlot *et al*., 2014). In the past, Gram-positive bacteria were the most prevalent cause of CRBSI, however, Surapat and colleagues (2020) highlighted the fact that Gram-negative bacteria have gained predominance as the causative pathogen. These Gram-negative pathogens include *Pseudomonas aeruginosa*, *Klebsiella* spp., and *Acinetobacter baumanii* (Suparat *et al.,* 2020).

Various studies reported fungal infections as the causative pathogen in CRBSI (Gahlot *et al*., 2014). Mainly yeasts and non-filamentous fungus have been reported with *Candida* spp. being most prevalent amongst the fungal species (Gahlot *et al*., 2014). Very little is known of viral and parasitic pathogens related to CRBSI (Gahlot *et al*., 2014).

2.3 An overview of clinically relevant infections caused by coagulase-negative *Staphylococcus*

Coagulase-negative staphylococci are regular inhabitants of the human skin and as a result contaminate clinical cultures (Becker *et al.*, 2014). Contradictory to this statement, CONS have been found to be the etiological agent for significant clinical BSIs due to its opportunistic behaviour (Tufariello & Lowy, 2019). Members of the CONS group that most commonly cause infections include: *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. capitis*, *S. lugdunensis*, *S. saphrophytics*, *S. warneri* and *S. caprae* (Asaad *et al.*, 2016).

2.4 Classification of *Staphylococcus capitis*

The *Staphylococcus* genus belong to the *Bacillus-Lactobacillus-Streptococcus* cluster in the *Firmicutes* phylum (Pallen *et al*., 2007; De Vos, 2009). *Staphylococcus capitis* form part of the *Staphylococcaceae* family (Mahon *et al*., 2015). *Staphylococcus capitis* is classified as a CONS due to its inability to produce coagulase (Foster, 1996; Becker *et al*., 2014).

Coagulase-negative *Staphylococcus* were initially classified by delineating the different staphylococcal species from *Staphylococcus aureus,* which were, at the time of introducing this concept, the only known species within this group (Becker *et al.*, 2014)*.* This concept was initially used as a diagnostic procedure-based classification that became a clinical approach to differentiate between pathogenic- *S. aureus* and nonpathogenic- CONS (Becker *et al.*, 2014). An improved understanding of CONS allowed researchers to investigate within the CONS group and found that some, if not all CONS are opportunistic pathogens (Schleifer & Bell, 2015).

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The *Linnaeus* classification of *Staphylococcus capitis*, branched into taxonomic orders, is shown in Table 2.2. *Staphylococcus capitis* can further be divided from species level to subspecies level (Bannerman & Kloos, 1991). Both *S. capitis* subsp. *capitis* and *S. capitis* subsp. *urealyticus* are classified as human pathogens (Tevell *et al*., 2017). Studies have shown that *S. capitis* subsp. *urealyticus* can migrate to habitats other than the human head, during antimicrobial therapy (Tevell *et al*., 2017). Cui and colleagues (2013) conducted a study in Australian NICUs that showed that *S. capitis* subsp. *urealyticus* was not only more predominant in NICUs, it also had a higher probability to express biofilm activity *in vitro* and express more extensive antimicrobial resistance genes (Cui *et al.*, 2013).

Table 2.2: **The *Linnaeus* classification of staphylococci (Schleifer & Bell, 2015)**

Taxonomic order of life	Order in which human-associated staphylococci group
Domain	*Bacteria*
Kingdom	*Eubacteria / Gram-positive bacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Staphylococcaceae*
Genus	*Staphylococcus*
Species	*capitis*
Subspecies	*Staphylococcus capitis subsp. capitis* *Staphylococcus capitis subsp. urealyticus*
Binominal nomenclature	
Staphylococcus capitis	

The intra-genus similarities between the different species within the *Staphylococcus* genus are 96.5%, which is much higher compared to the 93.4% to 95.3% similarity parameter between closely related genera (Schleifer & Bell, 2015). In Figure 2.2, the phylogenetic relationship between *S. capitis* and other *Staphylococcus* spp. are shown. The DNA relatedness between the species was determined based on DNA-DNA hybridisation experiments to differentiate within the genus (Kloos & Schleifer, 1975; Pallen *et al.*, 2007; Schleifer & Bell, 2015).
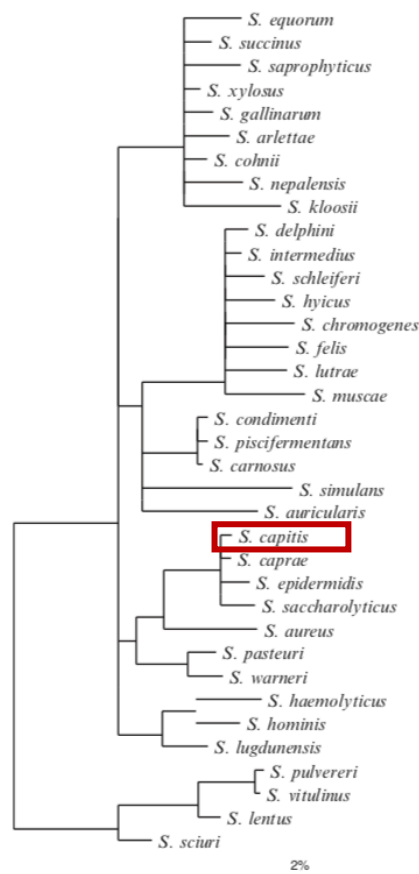
Figure 2.2: **Phylogenetic relationships of staphylococcal species based on 16S rRNA sequences. The length bar indicated 2% sequence divergence (Schleifer & Bell, 2015)**

2.5   General characteristics of *Staphylococcus capitis*

Staphylococcus capitis is commonly found on human skin but more specifically, the scalp (Kumar *et al.*, 2017). *Staphylococcus* spp. such as *S. capitis,* are classified as a CONS if the species does not produce coagulase (Ryan, 2018). This classification is also an important characteristic when diagnosing staphylococcal infections (Mahon *et al.*, 2015). Bacteria that form part of the human microflora can cause infection through catheter insertion, tissue wounds or surgical site if the patient is immunocompromised (Kahn *et al.*, 2017). *Staphylococcus capitis* are opportunistic pathogens, meaning a patient that is immunocompromised can be infected by this bacterium if it invades the body through a wound or site of entry (Ryan, 2018). *Staphylococcus capitis* can transmit from patient to patient in hospitals through direct contact by healthcare workers and environmental sources (Kahn *et al.*, 2017).

Staphylococcus capitis are oxidase-negative, catalase-positive, chemo-organotrophs and facultative anaerobes (Schleifer & Bell, 2015). On a molecular level, *S. capitis* are non-motile, non-spore forming, Gram-positive cocci with an average size of 0.8 μm to 1.2 μm in diameter (Schleifer & Bell, 2015). *Staphylococcus capitis* cells usually occur in pairs and are seen in tetrads due to multiplanar division to form irregular grape-like structures (Schleifer & Bell, 2015). *Staphylococcus capitis* optimally grow on 5% sheeps blood agar at 37°C when culturing but grows on all general media used in diagnostic laboratories (Mahon *et al.*, 2015).

Temperature range for growth is 18°C to 45°C but *S. capitis* grows optimally at 30°C to 40°C (Schleifer & Bell, 2015). The colony characteristics of *S. capitis* include smooth, slightly convex, glistening, opaque white/greyish colonies on blood agar (Mahon *et al.*, 2015). *Staphylococcus capitis* subsp. *capitis* has a colony diameter of 1 mm to 3 mm while *S. capitis* subsp. *urealyticus* produces larger colonies of 4.3 mm to 7.1 mm in size (Mahon *et al.*, 2015). On a biochemical level, *Staphylococcus capitis* subsp. *urealyticus* can be distinguished from *Staphylococcus capitis* subsp. *capitis* by its active urease activity, acid production in anaerobic conditions and its fatty acid profile (Cui *et al.*, 2013). *Staphylococcus capitis* has similar serology compared to other species within the CONS group except for the absence of alkaline phosphatase (Schleifer & Bell, 2015).

2.5.1   Genomic characteristics of *Staphylococcus capitis*

It has been reported that *S. capitis* has the closest evolutionary link to *S. epidermidis* compared to other clinically relevant CONS (Cameron *et al.*, 2015). *Staphylococcus capitis* has a chromosome size of 2.5 Mega base pair (Mbp) with a GC content of 33% (Cameron *et al.*, 2015; Kumar, 2017; Sun *et al.*, 2020). The chromosome of *S. capitis* contains 2 304 protein-coding DNA sequences, 58 tRNA and three rRNA (Cameron *et al.*, 2015).

Watanabe *et al.* (2018) identified the comparative gene orthology of TW2795, AYP1020 and CR01, three well known *S. capitis* strains. These three strains shared 2 064 conserved genes (Watanabe *et al.*, 2018). Sun and colleagues (2020) sequenced 21 *S. capitis* strains and predicted 1 789 core genes, 1 441 accessory genes and 946 unique genes (genes of accessory genome only present in one strain). Hundred and sixty nine (169) different *S. capitis* strains have been uploaded to the National Center for Biotechnology Information's (NCBI) GenBank.

2.5 Epidemiology of *Staphylococcus capitis*

In neonates specifically, Rasigade *et al.* (2012) initially reported the detection of a single MDR clone of *S. capitis*. This clone named the New Reduced-complexity Sequence Type A clone (NRCS-A), was characterised based on the specific PFGE pattern and was detected in NICUs in France, Belgium, the United Kingdom and Australia (Butin *et al.*, 2016). Two years later, the same team observed the NRCS-A PFGE pattern in 154 strains obtained from 17 different countries collected from 1994 to 2015 (Butin *et al.*, 2017a). These countries included Autralia, Belgium, Brazil, Canada, Czech Republic, Denmark, Finland, France, Germany, the Netherlands, New Zealand, Norway, South Korea, Switzerland, Taiwan, the United Kingdom and the United States (Butin *et al.*, 2017). In adult patients however, *S. capitis* isolated from BSIs belonged to distinct and varied pulsotypes (Simões *et al.*, 2013; Butin *et al.*, 2017a).

2.6 Treatment and antimicrobial resistance of *Staphylococcus capitis*

Staphylococcal infections were initially treated with penicillin, a β-lactam antimicrobial agent discovered in 1928 (Lobanovska & Pilla, 2017). Shortly after penicillin was approved for treatment (1945), resistance in staphylococcal strains were reported (Lobanovska *et al.*, 2017). Resistance is mediated through penicillinase, an enzyme that hydrolyses the ß-lactam ring in penicillin, preventing attachment to the penicillin-binding protein (PBP) (Becker *et al.*, 2014; Ryan, 2018). In 1959, a semisynthetic β-lactamase resistant penicillin called methicillin was developed to treat infections caused by β-lactamase producing staphylococci (Ito *et al.*, 2009; Lobanovska *et al.*, 2017). One year after introducing methicillin (1960), the first methicillin-resistant *Staphylococcus aureus* (MRSA) infection was identified in hospital settings in the UK and Denmark (Ito *et al.*, 2009; Becker *et al.*, 2014; Harkins *et al.*, 2017). Methicillin was one of the first penicillinase resistant penicillins used in early therapy, so the term 'methicillin resistance' is still used universally to describe resistance to any β-lactam antimicrobial agent such as nafcillin and oxacillin (John & Harvin, 2007; Mahon *et al.*, 2015).

Since the discovery of penicillin, other antimicrobial agents have been developed and classified based on their chemical structure and proposed mechanism of action (Farrer, 2011). Many of the antimicrobial agents were followed up by the emergence of resistance nosocomial pathogens (Lobanovska *et al.*, 2017; Klemm *et al.*, 2018). Several genes and mutations involved in antimicrobial resistance accelerates the microorganism's evolutionary genetics through selective pressure by means of either intrinsic or acquired resistance (Mulvey *et al.*, 2009; Klemm *et al.*, 2018). Intrinsic resistance is encoded by the innate or inherent genetic

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makeup of the microorganism, meaning the microorganism has always been resistant to a specific antimicrobial group (Mulvey *et al*., 2009). Acquired resistance, however, refers to specific genes that have: i) either undergone mutations to evade the antimicrobial agent or, ii) antimicrobial resistant genes that have been acquired by introduction of mobile genetic elements (MGEs) to the microorganism (Mulvey *et al*., 2009). Mobile genetic elements found in *S. capitis* are further discussed in section 2.7.2.

The ability of microorganisms to acquire antimicrobial resistant genes through MGEs, have posed a great threat to immunocompromised patients in healthcare settings (Gu *et al*., 2013). A multidrug-resistant (MDR) microorganism is defined as a microorganism that is resistant to at least one antimicrobial agent in three or more antimicrobial classes (CDC, 2019c).

Treatment is dependent on the stage of infection and the etiological agent (Mer, 2005). In the case of suspected CRBSIs, the catheter is usually removed and inserted at a different site if necessary (Mer, 2005; National Department of Health (DoH) Standard Treatment Guideline (STG), 2019). Most infectious complications are resolved or self-limited after removal of the catheter, however, some indications will call for antimicrobial therapy (Mer, 2005; Mermel *et al*., 2009). These indications include persistent sepsis, evidence of septic thrombosis of the great veins, clinical or echocardiographic evidence of endocarditis, metastatic foci of infection, underlying valvular heart disease and underlying immunocompromised state (Mer, 2005).

Initial empiric therapy involves the administration of broad-spectrum antimicrobials based on the local epidemiology and clinical presentation (Leekha *et al*., 2011). Empiric antimicrobial therapy should be administered for 48 hours to 72 hours after fever resolution (DoH STG, 2019).

Initial antimicrobial therapy for CONS infections are described below according to the DoH STG of South Africa: i) clindamycin (oral, 450 mg, 8 hourly for 5 days) is prescribed to patients presenting with large areas of erythema around the catheter insertion site (DoH STG, 2019), ii) vancomycin (IV, 30 mg/kg as loading dose, followed by 20 mg/kg/dose 12 hourly) is prescribed when CRBSI is suspected (DoH STG, 2019; Chaves *et al*., 2019), iii) teicoplanin is not recommended to treat CONS infections due to its reduced susceptibility to teicoplanin (DoH STG, 2019), iv) daptomycin may be administered to patients with septic shock, acute kidney injury or recent exposure to vancomycin (DoH STG, 2019), v) Linezolid should only

be administered to patients with contraindications to above mentioned antimicrobial agents (DoH STG, 2019).

If the patient's symptoms persist, treatment is readjusted to direct therapy based on the antibiogram and identification of the microorganism isolated from the catheter tip and the blood culture (Chaves *et al.,* 2019; Sivanandan *et al.*, 2011; Coetzee *et al.*, 2017.). *Staphylococcus* spp. such as *S. capitis* within the CONS group are not identified to species level in routine diagnostics and share the same treatment regimen as CONS (DoH STG, 2019). Some antimicrobial agents that are used as direct therapy for CONS infections are described below: i) cloxacillin and cefazolin are used for CRBSI caused by CONS that are susceptible to methicillin (Chaves *et al.,* 2019), ii) a glycopeptide is prescribed for a methicillin-resistant CONS (MR-CONS) infection (Chaves *et al.,* 2019), iii) teicoplanin may be prescribed if the patient shows serious side effects due to vancomycin (Chaves *et al*., 2019).

Combination therapy is the simultaneous use of two antimicrobial agents to produce a synergistic effect and is especially used to prevent the emergence of antimicrobial resistance (Leekha *et al*., 2011). If the patients antibiogram shows MDR, linezolid is used (Hashemian *et al*., 2018; DoH STG, 2019).

2.6.1 Mechanism of action and resistance of linezolid

Linezolid is a member of the oxazolidanone class of synthetic antimicrobial agents that inhibit bacterial protein synthesis by a unique mechanism (Clemett *et al*., 2000). The morpholino group and the fluoride atom are significant structures that increases the antimicrobial activity of linezolid, as shown in Figure 2.3 (Hashemian *et al*., 2018). Linezolid was approved by the Food and Drug Association (FDA) in 2000 for indications such as complicated and uncomplicated skin and soft tissue infections, community- and healthcare-associated pneumonia (HAP) and MDR Gram-positive infections (Clemett *et al*., 2000; Doern *et al*., 2016).

+
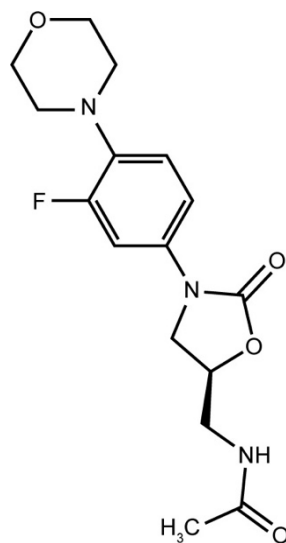
Figure 2.3: **The chemical structure of linezolid indicating the morpholino group op the top and the fluoride atom in the second ring (Hashemian *et al*., 2018)**

By using an *in vivo* cross-linking approach, the mechanism of action of linezolid could be established: linezolid acts by binding with high affinity and great specificity to the catalytic site on the 50S ribosomal subunit, at the ribosomal peptide-transferase centre (PTC), thus, affecting tRNA positioning (Stefani *et al*., 2010). Figure 2.4 provides a schematic representation of where linezolid binds during the translational step in protein synthesis. Linezolid appears to prevent an early step in bacterial protein synthesis by preventing formation of tRNA[fMet]-mRNA-70S or -30S initiation complex (Clemett *et al*., 2000). Linezolid binding to the 50S subunit distorts the binding site for tRNA[fMet](Clemett *et al*., 2000).
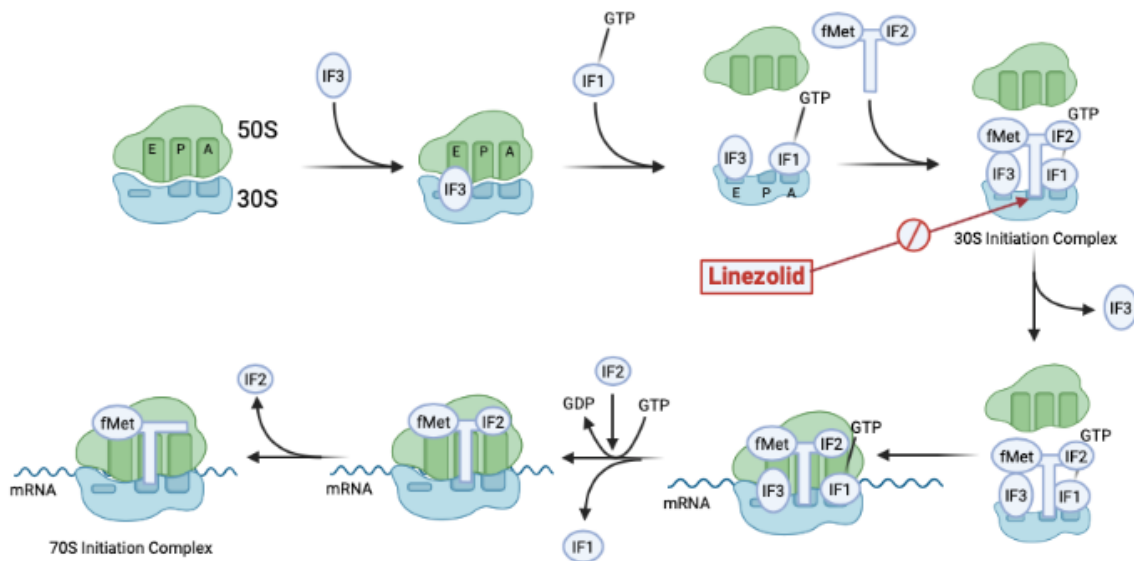
Figure 2.4: **Schematic representation of linezolid's mechanism of action during protein synthesis. The 30S = 30S Ribosomal subunit; 50S = 50S Ribosomal subunit; 70S = 70S Ribosomal complex; IF (1,2,3) = Initiation factors (1,2,3); GTP = Guanosine-5'-triphosphate; GDP = Guanosine diphosphate; mRNA = messenger RNA; Peptide transferase centre (PTC) = [E-site = Exit site; P-site = Peptidyl site; A-site = Aminoacyl site]. This diagram was adapted from Clemett *et al.* 2000 and created using Biorender.com**

The synthetic nature of linezolid is advantageous in that it does not have a natural prototype, therefore it would be expected that there would be no pool of resistance genes that would promote clinical resistance (Stefani *et al.*, 2010). This was true for linezolid until rRNA mutations was observed as a mechanism of resistance (LZR) (Figure 2.5) (Clemett *et al.*, 2000).
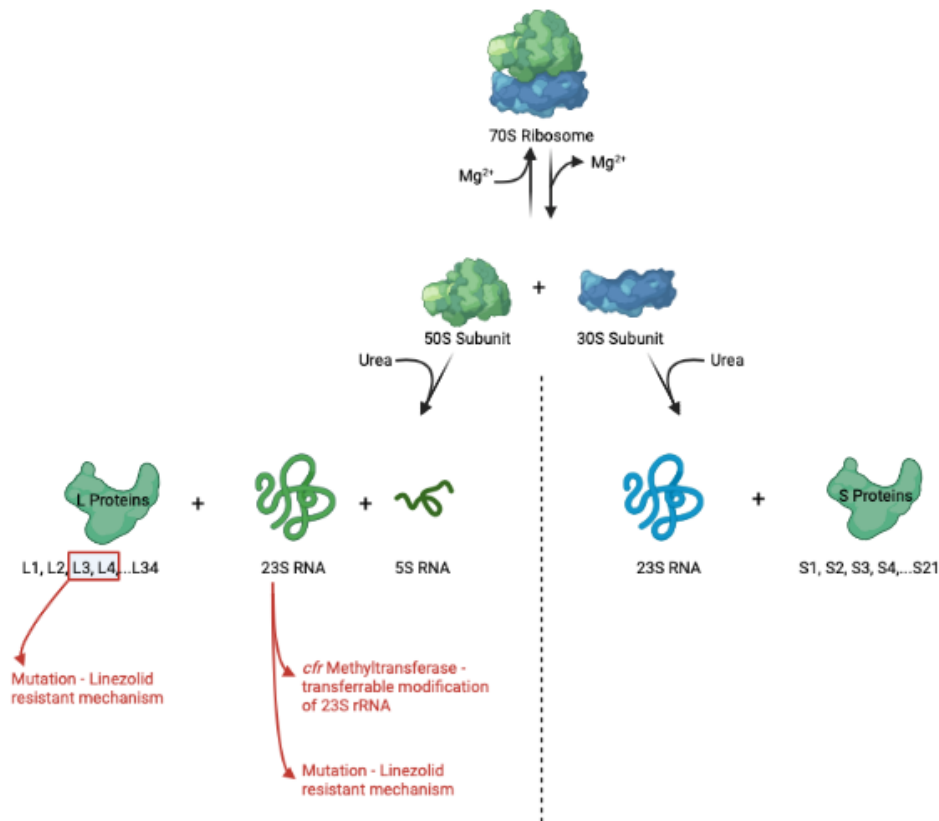
Figure 2.5: Mechanism of resistance illustrated on the 70S ribosomal composition of *Staphylococcus capitis* (Tian *et al*., 2014)

Figure 2.6 indicates the position where the LZR mechanisms occur. The most frequently observed mechanism of resistance is a mutation of domain V of the 23S rRNA gene with a G2576T substitution (Tian *et al*., 2014). The C2104T, G2447T, T2500A, A2503G, T2504A, G2603T and G2631T substitutions have also been observed (Tian *et al*., 2014). The second mechanism of action is through the horisontal transfer of the *cfr* gene, a methyltransferase that modifies adenosine A2503 in the 23S rRNA (Mendes *et al*., 2014). The *cfr* gene is located on a plasmid (pSCFS1) and encodes cross-resistance to phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A or the PhLOPS$_A$ compounds (Tian *et al*., 2014). The *cfr* gene simultaneously methylates the C8 atom (the 8th carbon atom) of the enzyme at the same position which creates a MDR phenotype (Mendes *et al*., 2014). The third known mechanism of resistance is due to alterations in the ribosomal proteins: L3, L4 and L22 and encoded by the ribosomal protein L3 coding gene (*rplC*), ribosomal protein L4 coding gene (*rplD*) and ribosomal protein L22 coding gene (*rplV*) (Locke *et al*., 2020).
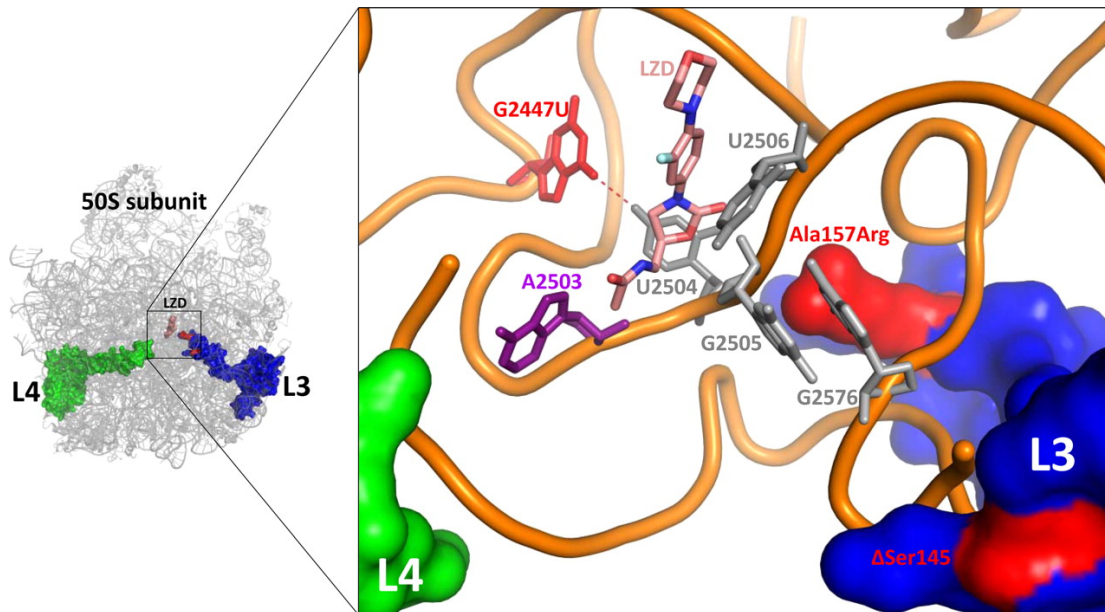
Figure 2.6: **Molecular illustration of the ribosomal mutations in clinical LNR strains: Mutations of ribosomal protein L3 (ΔSer145 and Ala157Arg) and 23S rRNA (G2447U) are shown in red. A PTC-bound LZD molecule is shown in salmon. 23S rRNA bases A2503 (site of methylation by *cfr*), A2504 to 2506 (key residues lining the oxazolidinone binding site in the PTC), and ribosomal protein L4 are shown for reference (Locke *et al.*, 2020)**

2.7 Pathogenesis and virulence of *Staphylococcus capitis*

Staphylococcus capitis can colonise foreign medical devises by producing a polysaccharide slime through the expression of adhesins and as a result, adhere to the medical device (Cui *et al.*, 2013; Osman *et al.*, 2015). Even though CONS are frequently isolated from blood cultures, their isolation does not always reflect a true BSI, but rather a contaminant (Papadimitriou-Olivgeri *et al.*, 2016). Clinically, CONS infections are less severe and lack the virulence determinants responsible for aggression, compared to other BSIs, such as *S. aureus* (Becker *et al.*, 2014; Hitzenbichler *et al.*, 2017). Bacterial biofilms involve a genetically coordinated sequence of events, including initial attachment, microcolony formation and community expansion (Osman *et al.*, 2015).

2.7.1 Virulence factors of *Staphylococcus capitis*

The ability of invasive *S. capitis* to form a biofilm is the primary virulence factor associated with *S. capitis* (Crossley, 2009; Cameron *et al.*, 2015). There are, however three other virulence factors associated with the pathogenicity *of S. capitis* namely: i) phenol-soluble modulins

(PSMs) that modulate an immune response in the host, ii) a δ-toxin with high sequence similarity to the toxin found in *S. aureus* that forms pores in the hosts' red blood cells and iii) the production of poly-γ-glutamic acid (PGA), expressed by the cap operon (Becker *et al*., 2014). The PGA molecule plays a role in both the microorganisms' commensal lifestyle by allowing it to operate in a high salt environment and in development of a biofilm on indwelling devices (Büttner *et al*., 2015). Resistance to antimicrobial peptides produced by the hosts' immune system and evading phagocytosis is also mediated by PGA (Crossley, 2009).

Staphylococcus capitis are common colonisers of the human skin and as a result, contaminate clinical specimens frequently (Becker *et al*., 2014). There are three steps to biofilm formation: i) primary attachment, ii) accumulation, iii) maturation and iv) detachment as seen in Figure 2.7 (Becker *et al*., 2014; Büttner *et al*., 2015).
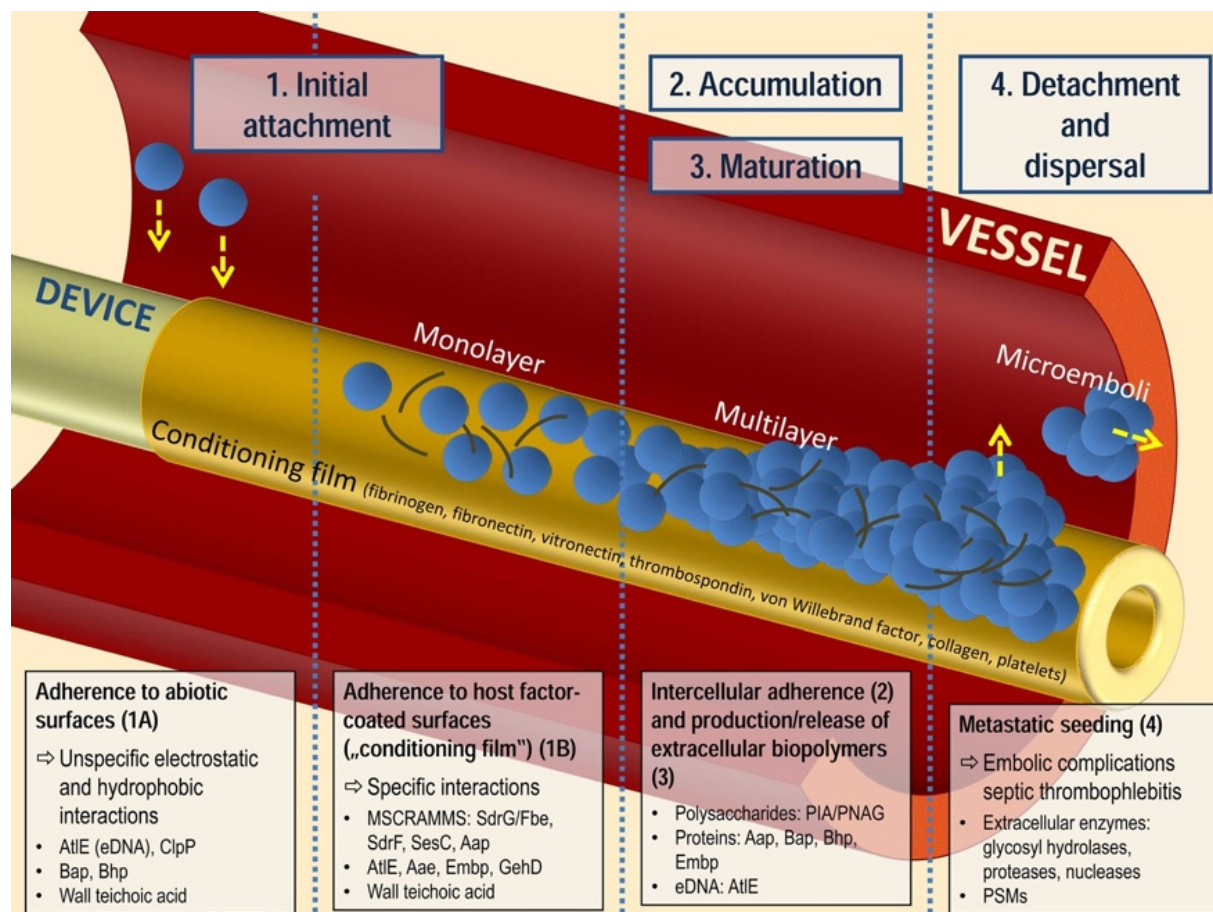
Figure 2.7: Model of biofilm formation on invasive medical devices (Becker *et al.*, 2014)

Staphylococcus capitis form biofilms by adhering to foreign bodies using hydrophobic interactions mediated by two proteins, the biofilm-associated protein homologue (Bhp) and an adhesin/autolysin E (AtlE) (Salgueiro *et al*., 2017). The expression of cell surface proteins

occurs almost simultaneously by mediating specific interactions with host extracellular matrix (ECM) components (Büttner *et al*., 2015; Salgueiro *et al*., 2017). The ECM interacts with a group of proteins, called microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (Salgueiro *et al*., 2017). There are several of these MSCRAMMs in *S. capitis*, of which each binds to a different protein: i) serine aspartate repeat-containing protein F (SdrF), binds to collagen I (structural protein found in skin), ii) Fg-binding protein (SdrG/Fbe), a LPXTG-motif that contains a protein covalently attached to the bacterial cell surface, binds to fibrinogen (soluble protein found in blood plasma) coated surfaces and iii) Extracellelar-matrix binding protein (Embp), binds to fibronectin (glycoprotein of the extracellular matrix) (Büttner *et al*., 2015; Salgueiro *et al*., 2017). Other proteins called putative adhesins described in *S. capitis* include GehD lipase (binds to collagen) and the *S. epidermidis* surface proteins (Ses) such as SesI (Salgueiro *et al*., 2017). After adherence have occurred, the biofilm needs to accumulate in multilayered cell aggregates, this can be mediated by polysaccharide adhesins and/or proteinaceous adhesins (Becker *et al*., 2014)

A polysaccharide intercellular adhesin (PIA), also known as poly-N-acetylglucosamine (PNAG) connects bacterial cells in the biofilm by electrostatic attraction to the exposed teichoic acids (Fey & Olson, 2010; Widerström *et al*., 2012; Becker *et al*., 2014; Büttner *et al*., 2015; Salgueiro *et al*., 2017). The exopolysaccharide PIA/PNAG is synthesised by intercellular adhesion gene products (icaA, icaB) of the icaAB operon (Fey & Olson, 2010; Widerström *et al*., 2012; Büttner *et al*., 2015; Salgueiro *et al*., 2017). Other proteinaceous adhesins involved in biofilm formation and accumulation include accumulation-associated protein (Aap), Bhp and Embp (Fey & Olson, 2010; Widerström *et al*., 2012; Büttner *et al*., 2015; Salgueiro *et al*., 2017). The Aap can induce the accumulative phase without the presence of PIA/PNAG adhesin proteins (Büttner *et al*., 2015).

2.7.2   Mobile genetic elements as virulence factors in *Staphylococcus capitis*

Mobile genetic elements vary in structure to accommodate transfer, based on the environment and specific genetic composition (Ryan, 2018). Common MGEs include insertion sequences, transposons, plasmids, pathogenicity islands, chromosomal cassettes and bacteriophages (Otto, 2009).

2.7.2.1 Staphylococcal cassette chromosome methicillin element as virulence factor of *Staphylococcus capitis*

Methicillin resistance in staphylococci arise when a strain initially susceptible to methicillin acquires a staphylococcal cassette chromosome-methicillin (SCC*mec*) element, harbouring a methicillin-A (*mecA*) gene that integrates into the staphylococcal genome (Otto, 2009; Svensson *et al.*, 2011). The *mecA* gene encodes an altered penicillin-binding protein (PBP2a or PBP2'), preventing the binding (decreased binding affinity) of methicillin to the surface of the bacterial cell wall (John & Harvin, 2007). The complex molecular organisation of staphylococcal β-lactam resistance at gene level allows for diverse *mecA* polymorphisms (Becker *et al.*, 2014). The prevalence of the *mecA* gene was much higher in methicillin resistant CONS (MRCONS) than it was for methicillin resistant *S. aureus* (MRSA) in the 1970s (John & Harvin, 2007). Today almost all clinically relevant CONS possess SCC*mec* elements (Becker *et al.*, 2014).

Several staphylococcal species usually occupy the same niche on a host's epithelial surface, which allow these species to share genetic information in the form of plasmids with each other through horizontal gene transfer (HGT) by conjugation or bacteriophage transduction (Otto, 2009). The SCC*mec* are characterised into different SCC*mec* types based on their composition of gene clusters of the methicillin resistance gene complex (*mec*) and cassette chromosome recombinase gene complex (*ccr*) (Otto, 2009; Svensson *et al.*, 2011). Fifteen SCC*mec* (I to XV) types have been identified in *S. aureus* with novel SCC*mec* types and subtypes also frequently identified (Turlej *et al.*, 2011; Becker *et al.*, 2014; Salgueiro *et al.*, 2017; O'Connor *et al.*, 2018). The same SCC*mec* typing methods (Kondo *et al.*, 2007) used for *S. aureus* can also be used for typing SCC*mec* elements in MR-CONS because of SCC*mec* elements that can be transferred to and from other staphylococcal species, however, the resulting elements only show a prevalence in 13 types (I-XIII), diverse patterns and non-typeable elements (Kaya *et al.*, 2018). The MR-CONS that contained SCC*mec* elements include: *S. epidermidis*, *S. capitis*, *S. cohnii*, *S. chromogens*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, *S. sciuri* and *S. warneri*, where SCC*mec* types III, IV and V were the most prevalent, either alone or in combinations with other subtypes (Harrison *et al.*, 2014; Saber *et al.*, 2017).

The origin of the SCC*mec* is unknown but have been hypothesised to have originated from *S. fleurettii*, a commensal resident on animals that contained a *mecA* gene but was not associated with SCC*mec* (Tsubakishita *et al.*, 2010; Turlej *et al.*, 2011; Becker *et al.*, 2014).

The origin of SCC*mec* could be helpful in effectively controlling MR-CONS and understanding its evolution (Tsubakishita *et al.*, 2010).

The *mec* gene complex includes the *mecA* gene, regulatory genes (*mec*R1 and *mec*I), associated insertion sequences (IS) and hypervariable regions (HVR) (Ito *et al.*, 2009). A *mec* gene is defined as a gene that encodes an alternative PBP with three domains, i) a characteristic N-terminal structure, ii) a transpeptidase domain, and iii) a nonbinding region (Ito *et al.*, 2012). There are five classes of the *mec* gene complex that were identified in *S. aureus*: class A *mec*, class B *mec*, class C1 *mec*, class C2 *mec* and class E *mec* (Ito *et al.,* 2009; Svensson *et al.*, 2011). Table 2.4 shows the difference in composition of the different *mec* classes.

Table 2.4: **The difference in composition of the different *mec* class complexes (IWG-SCC, 2018)**

mec class gene complex	Composition
Class A *mec* (prototype complex)	*mecA* gene; the complete *mecR1* and *mecI* regulatory genes (upstream); HVR and IS431 (downstream)
Class B *mec*	*mecA* gene; a truncated *mecR1* (due to insertion of IS1272 upstream); HVR and IS431 (downstream)
Class C1 *mec*	*mecA* gene; a truncated *mecR1* (due to insertion of IS431 upstream); HVR and IS431 (downstream); IS431 upstream of *mecA* has the same orientation as the IS431 downstream
Class C2 *mec*	*mecA* gene; a truncated *mecR1* (due to insertion of IS431 upstream); HVR and IS431 (downstream); IS431 upstream of *mecA* has the is reversed
Class E *mec*	*mecA* gene; *ΔmecR1* gene; no ISs downstream

The *ccr* gene complex comprise a few genes encoding site-specific recombinases which are mainly responsible for the excision and integration of the SCC*mec* MGE into the chromosome surrounded by open reading frames (ORF) (Salgueiro *et al.*, 2017). The open reading frame, *orfX* is found upstream of the *mec* gene complex which is a conserved sequence among all staphylococcal species and encodes a methyltransferase that methylates 70S ribosomes (Boundy *et al.*, 2013). There are three phylogenetically distinct *ccr* genes with DNA sequence similarities below 50% called *ccrA*, *ccrB* and *ccrC* (Svensson *et al.*, 2011). The *ccrA* and *ccrB* genes are further classified into four allotypes, *ccr*A1-4 and *ccr*B1-4 based on their nucleotide identities that are more than 85%, *ccrC* only has one allotype (Ito *et al.*, 2009).

The joining or junkyard regions (J region) are found between and around the *ccr* and *mec* gene complexes and have essential biological SCC*mec* functions (Svensson *et al.*, 2011). The J region (as seen in Figure 2.8) as the green, orange and pink regions of the gene) consists of three regions (J1 to J3) and are arranged in the same order in all the SCC*mec* types with J1 located on the right side of the cassette, J2 in between the *ccr* and *mec* gene complexes and J3 adjacent to the open reading frame X (*orfX*) (Turlej *et al.*, 2011). The J regions are significant, especially in epidemiological studies, due to their ability to serve as targets for further classification dependent on the presence and absence of plasmids and transposons that carry other antimicrobial and metal resistance determinants, such as: erythromycin, tetracycline, mercury and cadmium (Kondo *et al.*, 2007; Turlej *et al.*, 2011; Becker *et al.*, 2014). Acquiring antimicrobial determinants on a SCC*mec* element may possibly lead to the emergence of MDR staphylococcal strains (Turlej *et al.*, 2011). Figure 2.8 shows the sequence of the SCC*mec*-SCC*cad/asr/cop* element of strain CR01 (neonatal *S. capitis* strain belonging to the NRCS-A pulsotype) and strain SK14 aligned against whole genomes of ST398 *S. aureus* strains S0385 and 08BA02176 (Simões *et al.,* 2013).
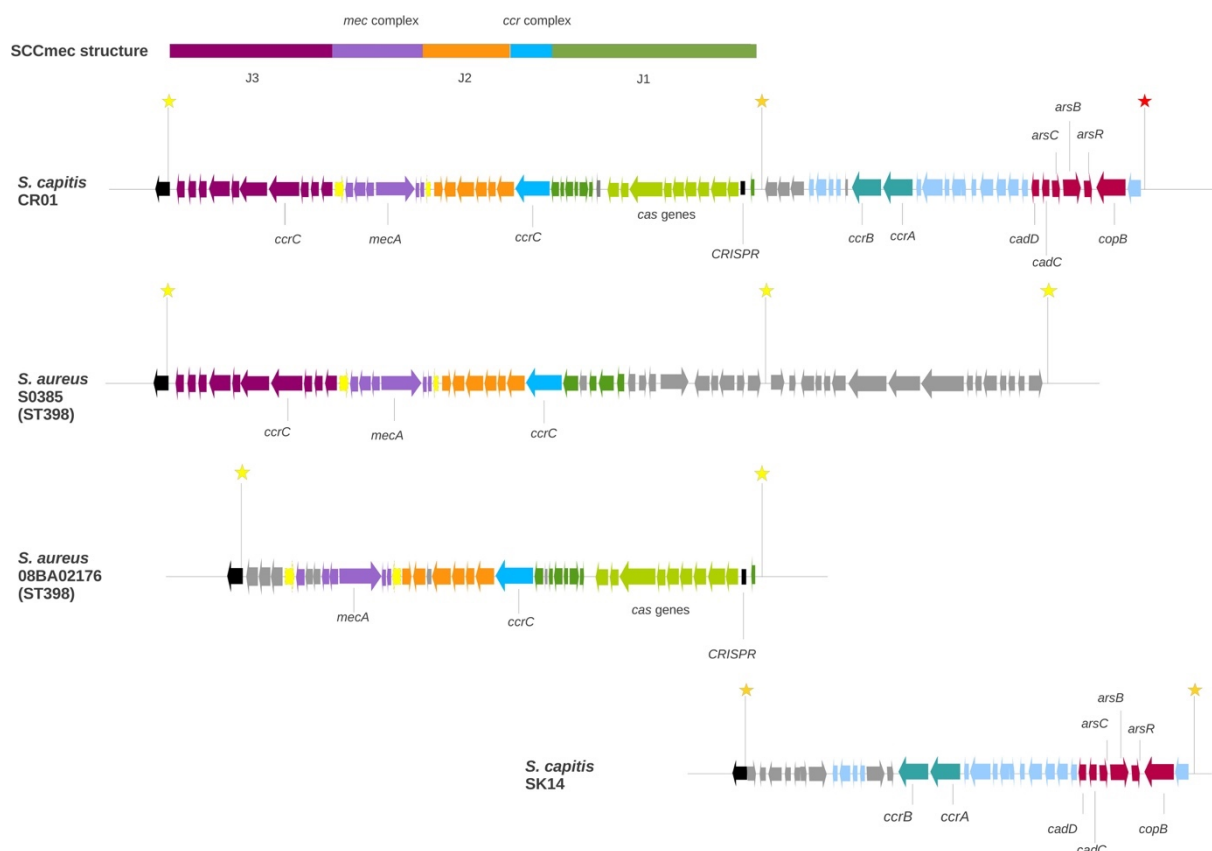
Figure 2.8: Comparative structure analysis of the composite staphylococcal cassette chromosome *mec* SCC*mec*-SCC*cad/asr/cop* element of *S. capitis* strain CR01. Open reading frames (ORFs) are shown as arrows indicating the

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transcription direction and coloured according to the SCC*mec* region to which they belong (J3, *mec* complex, J2, *ccr* complex, or J1). Homologous gene clusters in different strains have similar colours. The chromosomal *orfX* gene and the transposase IS*431* are represented by black and yellow arrows, respectively. Insertion site sequences (ISSs) are indicated by vertical lines and coloured stars as follows: light yellow star, *ccrC* recombinase ISS; dark yellow star, *ccrAB* recombinase ISS with associated direct repeat (DR) sequences; red star, vestigial *ccrAB* ISS and DR sequences. The CRISPR-associated genes (*cas* genes) are indicated in light green within the J3 region, while the *ccr* complex in the SCC*cad/ars/cop* element is coloured in turquoise and the resistance genes in dark red (Simões *et al*., 2013)

2.8   Prevention of a *Staphylococcus capitis* intravascular catheter-related infection

Several prevention techniques are used worldwide and are shown to be effective in lowering the rate of infection in nosocomial settings (O'Grady *et al*., 2011). Continual education of healthcare workers regarding catheter insertion techniques and catheter care remains an important preventative measure (Musco *et al*., 2022). Tunneled CVC is a process by which the lumen of the catheter is inserted through an incision in the chest, tunnelled through the soft tissue under the skin and threaded into the internal jugular or femoral veins to provide direct access to the bloodstream and as a result, reduce the risk of infection (Safdar *et al*., 2004). Cutaneous colonisation or colonisation through the extraluminal route can be prevented by applying chlorhexidine, chlorhexidine-impregnated sponge dressing at the site of insertion or inserting an anti-infective coat catheter (Miller *et al*., 2012).

2.9   Phenotypic and genotypic diagnosis of *Staphylococcus capitis* infections

A large degree of decisions, such as the choice of antimicrobial therapy, hospital stay and patient isolation are made by clinicians based on the results of microbiological, biochemical and molecular diagnosis (Opota *et al.*, 2015). The techniques used to phenotypically differentiate between several isolates are usually based on the status of the metabolic or biological activities of the organism, being either present or absent (Eberle & Kiess, 2012).

2.9.1   Microbiological diagnoses of *Staphylococcus capitis*

Culture-based diagnosis remains the gold standard in identifying the etiologic agent when a bloodstream infection (BSI) is suspected (Opota *et al.*, 2015). Staphylococci grow easily on

any standard routine laboratory culture media, especially sheep blood agar (Mahon *et al.*, 2015). In the case of a contaminated specimen, media such as mannitol salt agar (MSA), Columbia colistin-nalidixic acid agar (CAN), or phenylethyl alcohol (PEA) can be used as selective media for staphylococci due to its high sodium chloride (NaCl) concentration (Mahon *et al.*, 2015). The diagnosis of BSIs entails receiving a blood culture in broth media and incubating the blood culture bottle until it flags positive with bacterial or fungal growth. Once the bottle flags positive, a direct Gram-stain is performed to identify characteristics of the infecting pathogen, for example, whether the organism stains Gram-positive or Gram-negative, as well as cellular morphology and arrangement. The broth is inoculated onto non-selective (5% sheep blood agar and chocolate agar) and selective media (MacConkey agar) and incubated at 35°C in 5% CO_2 for a period of 18 hours to 24 hours. In the case of Gram-positive cocci on the direct Gram stain, a Deoxyribonuclease (DNase) test plate and a Mannitol Salt Agar (MSA) test plate are also inoculated. After the incubation period, the agar plates are analysed by a microbiologist. Gram-positive colonies are subjected to a catalase test to differentiate *Staphylococcus* from *Streptococcus* isolates.

The DNase test is used to differentiate *S. aureus* from other staphylococci that do not produce the enzyme (Mahon *et al.*, 2015). Some *S. aureus* strains however, are slow to react with the hydrochloric acid (HCl) and may appear negative (Koneman *et al.*, 1997). Mannitol-salt agar is used to selectively grow staphylococci in high NaCl concentration (7.5%) and inhibit growth of other bacteria (Chapman, 1945; Ayeni *et al.*, 2017). The MSA test is especially useful if a contaminated isolate is identified but due to nutritional variation, some staphylococcal strains may grow poorly on this medium (Anderson *et al.*, 2005). If the DNase and MSA tests are both positive, a presumptive identification of *S. aureus* is made and goes directly to the MALDI-TOF MS automated system (Bruker, USA). If the DNase and MSA tests are both negative, a presumptive identification of CONS is made and the clinical significance of the isolate is determined by the following criteria: i) the patient presents with infective endocarditis, ii) the patient shows repeated cultures with the same CONS or iii) if the clinician requests the specific identification and susceptibility of the blood culture. If no clinical significance is described, the CONS isolate is deemed to be due to a contamination/colonisation and further processing is terminated. If a discrepancy is observed between the DNase and MSA tests (DNase positive and MSA negative, or vice versa) and no conclusion can be made, a PASTOREX™ STAPH-PLUS (Bio-Rad, USA) test is performed.

2.9.2 Biochemical diagnoses of *Staphylococcus capitis*

Coagulase-negative staphylococci can be speciated through the use of manual commercial biochemical tests and automated systems (Becker *et al.*, 2014; Kim *et al.*, 2018. Manual systems include the analytical profile index (API) Staph test (bioMérieux, Marcy l'Etoile, France), disk diffusion, ETEST® (bioMérieux, France) and broth microdilution while automated systems include VITEK® 2 (bioMérieux, Marcy l'Etoile, France), MALDI-TOF MS automated systems (Bruker, USA), Phoenix (BD, USA) and Microscan (Beckman Coulter, USA) (Yee *et al*., 2021). Laboratories use an incubated purity plate to facilitate identification due to the presence of polymicrobial species (Mahon *et al.*, 2015). Both manual and automated systems are accurate and quite rapid, however, the carry-over of samples into separate tests as with the API test, may lead to contamination (Mahon *et al.*, 2015). The matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker, USA) has many applications but is mainly used in diagnostic laboratories for species identification. Unknown microorganisms are identified to species level by matching the unique proteomic fingerprint of an unknown isolate to an extensive library of reference spectra (Miyoung *et al.*, 2008).

Other phenotypic methods, such as serological typing, phage typing, plasmid profile, slime production detection and protein profile analysis, lack discriminatory power on closely related strains (Widerström *et al.*, 2012). Phenotypic identification is time consuming and labour intensive compared to molecular tests (Miyoung *et al.*, 2008). Phenotypic variation and lack of sufficient biochemical markers are two variables that prevents one hundred percent accuracy when differentiating between CONS species (Hirotaki *et al.*, 2011; Mahon *et al.*, 2015). Automated systems such as the VITEK® 2 system (bioMérieux, Marcy l'Etoile, France), can misidentify the bacteria at genus and species level, therefore results from these automated tests should be used as preliminary results or as results that accompany other tests (Miyoung *et al.*, 2008; Crowley *et al.*, 2012).

2.9.3 Molecular methods for the identification and characterisation of *Staphylococcus capitis*

There are several life-threatening pathogens that can't be readily detected with culture alone, especially in resource limited settings (Okeke & Ihekweazu 2021). Molecular testing is more sensitive and considered superior to phenotypic testing (Widerström *et al.*, 2012). The most common nucleic-acid based approaches used in the identification of *S. capitis* are the

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polymerase chain reaction (PCR), multiplex-PCR and real-time PCR assays. Other nucleic acid based approaches are fluorescent *in situ* hybridisation (FISH), microarrays and high-resolution melting curve analysis (HRMA) (Becker *et al.*, 2014).

A PCR assay can be used in the diagnostic setting to detect valuable markers to confirm the genus, species, as well as the presence of virulence and antimicrobial resistance genes (Ryan, 2018). The 16S rRNA gene is a highly conserved region that has been used to study the evolution and taxonomy of *Staphylococcus* Genus (Ghebremedhin *et al.*, 2008). As reported by Ghebremedhin *et al.,* (2008) the 16S rRNA gene detection is unable to discriminate between phylogenetically close species so the superoxide dismutase (*sodA)* gene is used in conjunction to detect *S. capitis* isolates (Kim *et al.*, 2018). Multiplex PCR assays follow the same principle as PCR assays but determines more than one locus simultaneously (Henegariu *et al.*, 1997). Studies have identified genes for biofilm associated virulence and antimicrobial resistance (Kondo *et* al., 2007; Salgueiro *et al.*, 2017; Bender *et al.*, 2019).

Real-time PCR is an adaption of the standard PCR assay and can be used to quantitively determine the amount of starting nucleic acid. A real-time PCR assay reports the products as they accumulate after each cycle, with the help of either fluorescent dyes, dual-probe FRET, molecular beacons or scorpion primers that bind specifically to the gene of interest and fluoresces in the presence of the gene (Varani *et al.*, 2009; Mahon *et al.*, 2015).

2.9.3.1   Molecular typing of *Staphylococcus capitis*

Genotyping methods are defined as methods comparing genetic material of bacterial strains and have higher discriminatory resolution on closely related strains (Widerström *et al.*, 2012). Pulsed-field gel electrophoresis (PFGE) is based on the digestion of chromosomal DNA with restriction enzymes and separated using pulsating electrophoresis, resulting in strains having unique banding patterns (Mahon *et al.*, 2015). Although PFGE is the gold standard, the method has the highest discriminatory power of all the genotyping methods but it can be technically demanding and laborious (Widerström *et al.*, 2012; Dorneles *et al.*, 2018). By using the *Sma*I restriction endonuclease in PFGE, the clonal diversity and the molecular epidemiology can be further investigated (Rasigade 2012; Wang *et al.*, 2022). Butin *et* al., (2016) reported an alternative restriction endonuclease with increased discriminatory power, which is *SacII*. A dendrogram is constructed from the banding patterns captured on a gel and clustered based on their banding size and pattern (Neoh *et al.*, 2019). A similarity cut-off value of ≥80% allows to group closely related isolates and as a result, will form a pulsotype (Butin *et al.*, 2017a). A

major pulsotype is defined as a cluster consisting of more than five isolates whereas a minor pulsotype is defined as a cluster containing less than five isolates (Tenover *et al*., 1995).

Multi-locus sequence typing (MLST) identifies mutations in genes by sequencing allelic variants of housekeeping genes after PCR amplification (Mahon *et al.*, 2015; Salgueiro *et al.*, 2017). The MLST is regularly used because of its excellent intralaboratory and interlaboratory comparisons despite its limited discriminatory power (Widerström *et al.*, 2012; Soroush *et al.*, 2016). Multilocus variable number tandem repeats (VNTR) analysis (MLVA) amplifies variable numbers of repeats in bacterial genomes and subsequently characterises individual strains based on the number of repeats at each investigated locus (Widerström *et al.*, 2012). Like PFGE, MLVA is also highly discriminatory but the variation in different loci may evolve too quickly to permit reliable data on long term epidemiological relationships and population structures (Dahyot *et al.*, 2018).

2.9.3.2   Whole genome sequencing

Sequencing of bacterial genomes have revolutionised how bacterial pathogens associated with infectious diseases are studied (Kozińska *et al*., 2019). The founding methods introduced the first generation of sequencing, namely: i) Sanger dideoxy synthesis, created in 1977 by Sanger, Nicklen and Coulson and ii) Maxam Gilbert chemical cleavage, created in 1980 by Maxam and Gilbert (Slatko *et al*., 2018). Sanger sequencing was later improved by automation and commercialisation, which led to the sequencing of the human genome (Venter *et al.*, 2001). Although still used today, where high throughput is not required, Sanger sequencing remains costly, laborious and time-consuming (Vincent *et al*., 2017).

A major technological advance was the birth of the second generation of sequencing called next-generation sequencing (NGS) ~~or whole genome sequencing (WGS)~~ (Slatko *et al*., 2018). There are several different NGS platforms that use unique sequencing technologies to sequence small fragments of DNA in parallel and map these sequences according to a reference genome (Behjati *et al*., 2013). Some of the well-known NGS platforms are 454 Pyrosequencing (Qiagen, Germany), Ion Torrent (Thermo Fisher, United States) and Illumina (Illumina, United States), with Illumina being the most popular platform (Slatko *et al*., 2018). Different types of Illumina platforms provide different levels of throughput: i) the MiniSeq provides 7.5 Gigabases (Gb) with 25 million reads/ run at 2X 150 basepair (bp) reads, ii) the MiSeq provides 15 Gb with 25 million reads at 2X 300 bp reads and, iii) the NextSeq provides 120 Gb with 400 million reads at 2X 150 bp reads (Slatko *et al*., 2018). Illumina (Illumina, United States)

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can be used to apply a variety of protocols such as genome sequencing, metagenomics, exome and targeted sequencing, RNA sequencing and CHIP-seq (Slatko *et al.*, 2018).

The third generation of sequencing was developed to sequence long DNA and RNA molecules of up to 30 to 50 kilobases (kb) (Slatko *et al.*, 2018). Pacific Biosciences (PacBio, US), also known as Single Molecule Real Time (SMRT) sequencing is the frontrunner platform with two commercialised sequencing systems, the original RSII model and the Sequal™ (Slatko *et al.*, 2018). Sequencing is advantageous in that is works with all bacteria and all other genotypic methods can be omitted but, it is too expensive to employ in diagnostic or clinical laboratories (Khromykh & Solomon, 2015).

Next generation sequencing can be used to investigate bacterial pathogens on a molecular level (Dylus *et al.*, 2020). Detection and monitoring of outbreaks as well as bacterial pathogen transmission can be determined for epidemiological studies (Dylus *et al.*, 2020). Other tools such as subtyping, resistome and virulome mapping, phenotypic inference and detection of new variants can be used to provide crucial bacterial characteristics (Motro & Moran-Gilad, 2017).

2.10   Summary

Staphylococcus capitis is isolated from the human skin, specifically the scalp and forms part of the human's normal microflora (Becker *et al.*, 2014). *Staphylococcus capitis* are opportunistic pathogens, meaning a patient that is immunocompromised can be infected by *S. capitis* if it invades the body through a wound or site of entry (Ryan, 2018). *Staphylococcus capitis* is one of the etiological agents responsible for biofilm-related infections such as endocarditis and catheter-related bloodstream infections (CRBSI) (Cui *et al.*, 2013).

Several studies done in different countries have reported an emergence of MDR *S. capitis* in healthcare settings (Cui *et al.*, 2013; Gu *et al.*, 2013; Butin *et al.*, 2016; Doern *et al.*, 2016). The majority of these MDR strains show LZR (Butin *et al.*, 2016). The use of linezolid increased as more MDR pathogens emerged which lead to LZR (Butin *et al.*, 2017a).

The ability of invasive coagulase-negative staphylococci to form a biofilm is the primary virulence factor in *S. capitis* (Crossley, 2009). There are, however three other virulence factors associated with the pathogenicity of CONS: phenol-soluble modulins (PSMs) that modulate an

immune response in the host, a δ-toxin with high sequence similarity to the toxin found in *S. aureus* that forms pores in the hosts' red blood cells and the production of poly-γ-glutamic acid (PGA), expressed by the cap operon (Becker *et al.*, 2014).

A large degree of decisions, such as the choice of antimicrobial therapy, hospital stay and patient isolation are made by clinicians based on the results of microbiological, biochemical and molecular diagnosis (Opota *et al.*, 2015). The emergence of LZR MDR *S. capitis* strains have been observed in critically ill patients across the Gauteng province in private hospitals from September 2014. The aim of this study was to identify LZR MDR *S. capitis* isolates; compare the genetic relatedness of these LZR MDR *S. capitis* isolates and find mutations responsible for linezolid resistance. The genetic relatedness between isolates can be used to find possible correlations between patients from the same age or same hospital and it can show the circulation of certain clones. Investigating this ongoing outbreak also provided information that can be compared to other international results.

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CHAPTER 3

Genotypic profiles of emerging linezolid-resistant multidrug-resistant *Staphylococcus capitis* isolates from an ongoing outbreak in critically ill patients

The editorial style of the Journal: Clinical Microbiology and Infection was followed in this chapter

Abstract

An increasing number of linezolid resistant (LZR), multidrug-resistant (MDR) *Staphylococcus capitis* infections have been observed in private hospitals in the greater Gauteng area since 2014. The aim of this study was to investigate the genotypic profiles of emerging LZR MDR *S. capitis* isolates from an ongoing outbreak in critically ill patients in South Africa's private sectors. A total of 119 *S. capitis* isolates from 29 private hospitals were identified and reported as linezolid resistant. The antimicrobial resistance patterns of the LZR MDR *S. capitis* isolates were: erythromycin 99.2% (118/119), amoxycillin/clavulanate 98.3% (117/119), cloxacillin 98.3% (117/119), clindamycin 97.5% (116/119), fucidic acid 84% (100/119), gentamycin 74.8% (89/119), cotrimoxazole 27.2% (33/119), rifampicin 16.8% (20/119), daptomycin 2.5% (3/119), vancomycin 1.7% (2/119) and teicoplanin 0.8% (1/119). The *cfr* gene was found in one isolate, while the *optr*A and *poxt*A genes were not detected with multiplex (M)-PCR. The pulsed-field gel electrophoresis (PFGE) dendrogram showed 1 major pulsotype consisting of 76 isolates, 3 minor pulsotypes with nine, five and three isolates respectively and 10 singletons. Fifteen isolates were classified as untypeable. Whole genome sequencing analysis of five representative *S. capitis* isolates showed a less known point mutation at G2604T on the rRNA gene conferring resistance to linezolid. Antimicrobial resistant genes identified included: *tetK, aac(6')-le-aph(2")-la, fusB, sepA, sdrM, mupA, mdeA, mecA, blaZ, ermC, dfrC*. Phenotypic antibiotic susceptibility did not show expression of all the genotypic genes detected. The results showed that highly resistant LZR MDR *S. capitis* isolates are circulating in these private hospitals among adult patients in ICUs. This emphasizes the importance of continious surveillance (with the inclusion of molecular epidemiological investigations) to monitor the transmission and spread of these circulating LZR MDR *S. capitis* strains in clinical settings in South Africa.

Keywords: *Staphylococcus capitis*, linezolid resistance (LZR), multidrug-resistance (MDR)

3.1 Introduction

Coagulase-negative staphylococci (CONS) have emerged as a major cause of healthcare associated infections (HAI) in neonates and immunocompromised patients (Sun *et al*., 2020). The ability of CONS to form biofilms on indwelling foreign devices such as catheters is the most important virulence factor (Becker *et al*., 2014). *Staphylococcus capitis* (*S. capitis*) is one of the etiological agents in biofilm-related infections such as endocarditis, urinary tract infections (UTI) and catheter-related bacteraemia (Stenmark *et al*., 2019). The literature suggests that *S. capitis* is an emerging opportunistic pathogen in premature neonates and renal dialysis patients (Butin *et al*., 2016; Carter *et al*., 2018; Michalik *et al*., 2021). In France, Butin and colleagues (2016) reported that the majority of neonatal strains are resistant to methicillin and penicillin with a decreased susceptibility to glycopeptides.

The emergence of LZR MDR *S. capitis* in South African ICUs and renal dialysis units have been observed in the private sector since September 2014 in patients of varying age groups. Even though all patients from this study were admitted to either an ICU or a renal dialysis unit, the exact number remained undisclosed. Therefore, the patients in this study were rather referred to as critically ill. This observance is of major concern seeing that all the isolates were MDR and LZR, of which linezolid is the last resort antimicrobial agent used for MDR and severe Gram-positive infections. Linezolid-resistant *S. capitis* strains were reported shortly after the introduction of the oxazolidinone as treatment option for nosocomial infections (Etebu and Arikekpar, 2016). Linezolid resistance can occur either by acquisition of plasmid-mediated genes such as *cfr, optrA* and *poxtA* or through a point mutation in either the 23S rRNA or the 50S rRNA (Michalik *et al*., 2021). Linezolid-resistant strains are being frequently reported since affordable generic antimicrobial agents are available to administer both oral and intravenously (IV), which leads to more patients being prescribed linezolid (Médecins Sans Frontières (MSF), 2014). A French study conducted by Rasigade *et al*. (2012) found a unique clone circulating among neonates with late-onset sepsis (LOS), in seven distant NICUs in France designated New Reduced-complexity Sequence Type A clone (NRCS-A) which suggested a high potential for dissemination of some clones. Contradicting to these findings, it has been reported that *S. capitis* isolates in adult patients belong to many distinct clones (Butin *et al.,* 2016).

No research is available regarding the prevalence, genotypic profiles and molecular epidemiology of the ongoing outbreak of LZR MDR *S. capitis* noted in the private sector in South Africa among diverse patient age groups. The purpose of this study was to characterise these LZR MDR *S. capitis* isolates using PCR assays, followed by PFGE to determine the genetic relatedness and WGS performed on five representative isolates to determine the resitome, virulome, mobilome and genomic characteristics.

3.2   Materials and Methods

The outline of the Methods and Materials section was based on a study done by Butin *et al.* (2017) who investigated the emergence and dissemination of a LZR *S. capitis* clone in Europe. In addition, a study by Bender *et al.* (2019) later identified genes apart from the *cfr* gene to detect the plasmid-mediated LZR mechanism that were also utilised in this study.

3.2.1   Study design, setting and sampling

This was a retrospective study conducted in the Department of Medical Microbiology, Faculty of Health Sciences, University of Pretoria. The patient population of this study included immunocompromised patients as reported by the laboratory pathologist from four different age groups: i) the paediatric group (0 days to 14 years), ii) the young group (15 to 47 years), iii) the middle age group (48 to 63 years) and iv) the elderly group (≥64 years). Isolates were recovered from clinical blood specimens collected from patients in various private hospitals in the greater Gauteng province from September 2014 to September 2019 as part of routine diagnostig procedures. Table 3.1 presents the distribution of 119 isolates arranged by the year of collection, along with age groups, gender and region of the 29 hospitals. Ethical approval was obtained from the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (Ethics number 115/2019). Five representative isolates were selected for whole genome sequencing and was outsourced to the Sequencing Core Facility at the National Institute of Communicable Diseases (NICD). The selection of these isolates was based on their clustering on the dendrogram, as well as the patient demographics and the irregular antimicrobial susceptibility profiles. The samples included in this study were determined by the following inclusion and exclusion criteria:

Inclusion criteria	Exclusion criteria
- Age 0 to 99 years - Routine diagnostics showed blood culture was positive for multidrug-resistant *S. capitis* - Multidrug-resistant *S. capitis* was specifically resistant to linezolid	- Routine diagnostics showed blood culture was negative for multidrug-resistant *S. capitis* - Multidrug-resistant *S. capitis* was susceptible to linezolid

3.2.2 Bacterial isolate collection and storage

According to the routine analysis of the private diagnostic laboratory the following procedures were conducted: i) the blood specimens were cultured ii) identified with MALDI-TOF (Bruker, USA) iii) antimicrobial sensitivity testing was performed with the VITEK® 2 automated system (bioMérieux, France) and iv) LZR profiles were tested with an ETEST® (bioMérieux, France) to determine the minimum inhibitory concentration (MIC) according to the Clinical and Laboratory Standards Institute (CLSI) staphylococcal breakpoint guidelines (CLSI, 2019). Demographical data and an antibiogram were obtained from the private diagnostic laboratory in Pretoria. Clinical data such as current and previous antimicrobial treatment including linezolid, complications, diagnosis and morbidity/mortality status were unfortunately not available.

The LZR MDR *S. capitis* isolates were transferred in cryotubes (Merck, Germany) from a private diagnostic laboratory in Pretoria to the Department of Medical Microbiology laboratory, streaked out on 5% sheeps blood agar (Thermo Fisher, USA) and incubated at 37°C (Vacutec, South Africa) for 24 hours. The isolates were Gram-stained (Bartholomew & Mittwer, 1952) to ensure that the cultures were pure, free of contamination and to establish an initial broad identification. In addition to distinguishing between Gram-positive and Gram-negative bacteria, Gram staining also provided information about the morphology (shape) of bacterial cells (Ryan *et al.,* 2018). The Gram staining procedure is detailed in Annexure B. A single colony of each of the isolates were inoculated into 5 mL sterile Brain Heart Infusion (BHI) broth (LabM Limited, UK) and placed in a shaking incubator (Stuart Orbital Incubator, UK) at 37°C for 24 hours. The overnight broth (LabM Limited, UK) was used for DNA extraction (section 3.2.4) and isolate storage in triplicate. Seven-hundred and fifty microlitres (750 µL) of the overnight broth (LabM Limited, UK) were added to sterile cryotubes (Merck, Germany) along with 750 µL of 50% sterile glycerol solution (Merck, Germany). One set of

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the cryotubes (Merck, Germany) were stored at -20°C (Samsung, Korea) and a duplicate set of the cryotubes (Merck, Germany) were stored at -80°C (New Brunswick, USA) for future analysis.

3.2.3 Total genomic DNA extraction of presumptive multidrug-resistant *Staphylococcus capitis* isolates

One and a half millilitres (1.5 mL) of the overnight BHI broth (LabM Limited, UK) was centrifuged (Spectrafuge 24D, Labnet International, Inc., USA) at 5 000 x *g* for 5 minutes at room temperature (25°C ±5°C). The supernatant was discarded and the previous step was repeated in the same microcentrifuge tube to obtain a visible pellet to provide sufficient genomic DNA (Corning Life Science, Axygen, USA). The cell pellet was resuspended in 1 mL of 1 x phosphate buffered saline (PBS) [pH 7.2] (GIBCO®, Invitrogen, Nieu- Zealand) and used for DNA extraction using the boiling method (Queipo-Ortuño *et al*., 2018). The purity and concentration of the extracted DNA were determined using the Nanodrop 1 000 (Thermo Scientific NanoDrop Spectrophotometer, USA). The extracted DNA were stored in 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA) at -20°C (Samsung, Korea) until required for further analysis.

3.2.4 Molecular identification of the *Staphylococcus capitis* isolates

The first Multiplex-Polymerase Chain Reaction (M-PCR) assay was performed to simultaneously identify: i) the genus *Staphylococcus* by amplifying a conserved fragment of the 16S rRNA gene (Yousun Chung *et al*., 2016) and ii) the superoxide dismutase (*sodA*) gene that encodes a metalloprotein and inactivates harmful superoxide radicals to confirm the species namely *S. capitis* (Kim *et al*., 2018). The primer sequences can be viewed in Table 3.2.

All primers used in this research study were synthesised by Inqaba Biotechnical Industries (Pty) Ltd (Inqaba, South Africa). The M-PCR assay was prepared and performed using the Bioline M-PCR kit (Bioline, South Africa), followed by the Bioline protocol (Bioline, South Africa). The master mix (Bioline, UK) that contains a HotStart *Taq* DNA polymerase, a Bioline M-PCR buffer, a dNTP mix and 3.0 mM $MgCl_2$ (pH 8.7) were prepared according to the manufacturer's instructions (Bioline, South Africa). The M-PCR reaction composition is summarised in Table 3.3. The PCR assay was performed using a thermocycler (T100 Thermal Cycler, Bio-Rad) and included an initial activation step of 95°C for 15 minutes, to activate the HotStart *Taq* DNA polymerase. A gradient PCR was performed to establish the optimal

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annealing temperature of the primers between 44.6°C and 58°C for sufficient amplification. A *S. capitis* reference strain (ATCC 35661) was used as a positive control while Nuclease-Free water (QIAGEN, Germany) was used as a negative control.

3.2.5 Antimicrobial sensitivity testing of the *Staphylococcus capitis* isolates

All MDR *S. capitis* isolates were LZR and were re-tested for antimicrobial susceptibility using the ETEST® (bioMérieux, France) method to confirm resistance and MIC values. A single colony from the sheeps blood agar plates (Thermo Fisher, USA) was used to make a saline cell suspension with a turbidity equal to a 0.5 McFarland standard, measured with a Densicheck (bioMérieux, France). The surface of a Mueller-Hinton agar plate (Oxoid Ltd, UK) was evenly covered by 30 μL of the cell suspension using an inoculation loop after which a linezolid ETEST® (bioMérieux, France) strip was placed aseptically on the surface in the centre of the plate and incubated (Vacutec, South Africa) at 37°C for 18 hours.

The MIC value was read from the gradient where the ellipse edge intersects the ETEST® strip (bioMérieux, France) and reported. The MIC scores were interpreted as susceptible (≤ 4 μg/mL), intermediate, or resistant (≥ 8 μg/mL) by comparing the breakpoint values of linezolid with the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) staphylococcal breakpoint guidelines (CLSI, 2020).

3.2.6 Molecular detection of the plasmid-mediated linezolid resistant mechanisms in *Staphylococcus capitis*

The identified *S. capitis* isolates were screened for the acquired linezolid-resistance mechanisms: i) *cfr* gene, ii) *optr*A gene and iii) *poxt*A gene. These genes are found on plasmids, insertion sequences and transposons and facilitates interspecies spread (Bender *et al*., 2019). This screening was done by using a Multiplex-PCR assay (Bender *et al*., 2019). The primers used for the detection of the plasmid-mediated resistance genes is shown in Table 3.2. The same master mix (Bioline, UK) as explained in 3.2.5 was used as well as the same M-PCR reaction composition as described in Table 3.3. The PCR assay was performed using a thermocycler (T100 Thermal Cycler, Bio-Rad) and included an initial activation step of 96°C for two minutes to activate the HotStart *Taq* DNA polymerase. Positive controls for all the genes were not available but the published size of the relevant genes were used to compare the

amplification product sizes (Bender *et al.,* 2019). Nuclease-Free water (QIAGEN, Germany) was used as a negative control.

3.2.7 Detection of Polymerase Chain Reaction amplification products of the multidrug-resistant *Staphylococcus capitis* strains

The DNA amplicons were detected using a 1% SeaKem® LE Agarose (Lonza, USA) gel in 1× Tris-Borate-EDTA (1× TBE) (Merck, Germany) buffer, stained with 5 µL of ethidium bromide (10 µg.mL^{-1}) (Sigma-Aldrich, USA). A 100 plus base pair (bp) DNA Gene Ruler (Thermo Scientific, USA) was included as a molecular weight marker for each gel. The amplicons were visualised under ultraviolet (UV) light (Transilluminator, Ultra-violet Products Incorporated, USA) and all visible bands were manually compared relative to the 100 bp DNA Gene Ruler (Thermo Scientific, USA).

3.2.8 Pulsed-field gel electrophoresis of multidrug-resistant *Staphylococcus capitis* strains

The genetic relatedness of 119 *S. capitis* isolates was determined using pulsed-field gel electrophoresis (PFGE). The PFGE was done using the Bio Rad CHEFDR II/III electrophoresis system (Bio Rad, United States) using *SmaI* as the restriction enzyme (Thermo Scientific, USA). The unified PFGE protocol for Gram-positive bacteria was used with the slight modification of an overnight lysis step of the plugs at 51°C (Stuart, UK) to ensure complete lysis (CDC, 2019). A cell suspension was made at an optical density of 1.2 at 630 nm (PerkinElmer Lambda 25, UV/UIS spectrometer, USA). The cell suspension was added to sterile microcentrifuge tubes (Scientific Specialities Inc., USA) and 20 mg/mL lysozyme stock solution (Sigma-Aldrich, USA) was added and incubated in a heating block (LabNet, USA) after which 20 mg/mL proteinase K (Sigma-Aldrich, USA) and 1 mg/mL Lysostaphin (Sigma-Aldrich, USA) were added. Four hundred microliters of 1.2% agarose (Seakem LE agarose, USA) was prepared using Tris-EDTA buffer (pH 8.0) (Sigma-Aldrich, USA) and added to the suspension after which it was dispensed into wells of the casting mould. The plugs were removed and placed into conical tubes (Bio One, Germany) filled with a lysis mixture containing cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0) and proteinase K (Sigma-Aldrich, USA) and incubated (Vacutec, South Africa) at 51°C for 18 hours with constant shaking at 170 revolutions per minute (rpm) (Hardy diagnostics, USA).

The plugs were washed using 10 mL ultrapure water (Purite Select HP, UK) and 10 mL Tris-EDTA buffer (pH 8.0) and either stored at 4°C or used for restriction enzyme digestion. The digestion of the plugs consisted of a 1:10 dilution of *SmaI* (Thermo Scientific, USA) and nuclease-free water (QIAGEN, Netherlands). The plugs were cut into thin slices using a sterile scalpel and transferred to sterile microcentrifuge tubes (Scientific Specialities Inc., USA) containing Tris-EDTA buffer (pH 8.0) (Sigma-Aldrich, USA). The cut slices were incubated at 37°C (AccuBlock Digital Dry Bath, LabNet, USA) for 10 minutes. The buffer was discarded and a restriction enzyme mastermix consisting of nuclease-free water (QIAGEN, Netherlands) and CutSmart™ restriction buffer (New England Biolabs, UK) was added to the conical tubes (Bio One, Germany) containing the plug and incubated at 37°C (AccuBlock Digital Dry Bath, LabNet, USA) for 3 hours.

A 1.2% agarose gel (Seakem LE agarose, USA) was prepared and cast on to the CHEFDR II/III casting tray (Bio Rad, United States). The plug slices were loaded into 15 respective wells with three molecular ladders (ATCC 12600) added in the first, middle and last well in the 1.2% agarose gel (Seakem LE agarose, USA) after it had solidified. The wells were sealed with the remaining molten 1.2% agarose (Seakem LE agarose, USA). The gel was placed in the PFGE chamber filled with 0.25x Tris-EDTA buffer (pH 8.0) (Sigma-Aldrich, USA). The configuration parameters were set to run for 23 hours at a chamber temperature of 14°C and a voltage of 220 V linear to 200 V. The interval switch time was set to 5 seconds linear to 40 seconds. After the PFGE run, the gel was submerged in an ethidium bromide staining solution (0.25 µg/mL) (Sigma-Aldrich, USA) and stained in darkness for 30 minutes. The ethidium bromide staining solution was decanted and sterile ultrapure water (Purite Select HP, UK) was used to destain the gel for a further 30 minutes. The gel was viewed under UV light with a Gel Doc XR+ System (BioRad, UK). The banding patterns obtained from the gel was photographed and imported to the BioNumerics Seven (Applied Maths, Belgium) software for analysis. A major pulsotype was defined if the cluster consisted of more than five isolates whereas a minor pulsotype was defined if the cluster contained less than five isolates (Tenover *et al*., 1995). The PFGE dendrogram was constructed with a similarity cut-off value of ≥80%. The Dice Coefficient (0.5% tolerance and 0.5% optimisation) was used to estimate the band-based similarity coefficient (Tenover *et al*., 1995).

3.2.9   Whole genome sequencing of selected *Staphylococcus capitis* isolates

Whole genome sequencing was performed on five LZR MDR *S. capitis* isolates that were chosen based on various criteria, including patient demographics and irregular antimicrobial susceptibility profiles. Isolate 30 was selected because the patient was the youngest (9 months old) and showed resistance to seven antimicrobial agents. Isolates 66 and 103 were chosen due to their high linezolid MIC value (256 μg/mL) and the patients were 72 and 70 years old, respectively. Isolate 116, from a patient aged 26 years, was selected because it showed resistance to all 12 antimicrobial agents tested. Isolate 145, aged one year and seven months, was chosen because it showed resistance to vancomycin. Isolates 30, 66, 103 and 116 were selected from pulsotype A, while isolate 145 was from pulsotype B. Based on the dendrogram analysis, these five isolates were deemed representative of the LZR MDR *S. capitis* isolates studied.

The DNA extraction of the representative five LZR MDR *S. capitis* isolates were performed using a commercial DNA extraction kit, *Quick*-DNATM Fungal/Bacterial Miniprep according to the manufacturer's (Zymo Research, USA) instructions with a slight modification of adding 500 μL of 0.5% (v/v) β-mercaptoethanol (Merck, Germany) to the Genomic Lysis Buffer for optimal performance. The purity and concentration of the extracted DNA was determined using the Nanodrop 1 000 (Thermo Scientific NanoDrop Spectrophotometer, USA). All extracted DNA were stored in 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA) at -20°C (Samsung, Korea) until required for further analysis.

The Nextera DNA sample preparation kit (New England Biolabs, UK) was used according to the manufacturer's instructions to prepare all samples by simultaneously fragmenting, tagging and amplifying the input DNA. Sequencing of all samples were done using the Hiseq Illumina sequencer (Illumina Inc., USA) and performed by the NICD, Johannesburg, South Africa. Processing and analysis of the sequence reads generated were done using Price of MetaVelvet (BioTools, USA) software for sequence assembly and CARMA for taxonomic binning. The sequence assemblies were run against the following database pipelines: i) Kmer Species for species confirmation, ii) Kmer Resistance, Comprehensive Antibiotic Resistance Database (CARD) and ResFinder to identify resistance genes and mutations within the domain V of the 23S rRNA, iii) VirulenceFinder to determine the virulence genes, iv) PlasmidFinder to identify any plasmids present and v) SCCmecFinder to identify the SCC*mec* type.

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3.3 Results

The aim of this study was to investigate the genotypic profiles of emerging multidrug-resistant *Staphylococcus capitis* isolates from an ongoing outbreak in critically ill patients in South Africa's private sectors. The available demographic data was used to compare the results with the year the isolates were collected.

3.3.1 Prevalence of multidrug-resistant and linezolid resistant *Staphylococcus capitis* in private hospitals in the greater Gauteng area

A total of 119 isolates from 167 [71% (119/167)] collected isolates were used in this study. The 35 [21% (35/167)] isolates that underwent initial testing but were not included in this study could not be revived for further analysis due to inappropriate storage conditions (e.g., faulty freezer). Out of the 35 isolates that could not be revived, eight were collected in 2015, two were collected in 2016, 15 were collected in 2017, one was collected in 2018, and nine had unknown collection data. However, for 13 isolates [8% (13/167)], no growth was observed, and therefore no demographic data could be requested.

A total of 119 isolates from 29 different private hospitals in the greater Gauteng area were identified as LZR MDR *S. capitis*. All isolates were taken from positive blood culture specimens isolated from patients with catherer-related bloodstream infections (CRBSI) in critically ill patients. Linezolid resistant MDR *S. capitis* infections were more prevalent in the Johannesburg region (region D) representing 17 hospitals 58% (69/119) followed by 37% (44/119) from eight hospitals representing the Pretoria region (region A) and 4.2% (5/119) from outlier regions within the greater Gauteng area. The location of one in-patient was unknown 0.8% (1/119). The patients' distribution map was drawn up using hospital coordinates and number of isolates collected from a specific hospital from September 2014 to September 2019 by using Tableau (Salesforce Inc, USA). The resulting map is presented in Figure 3.1. The regions were denoted by alphabetical letters A to G for ease of reference and the hospitals in specific regions were denoted by a numerical value to secure anonymity of the hospitals (e.g., B2).

The demographic data showed that the patients with LZR MDR *S. capitis* infections ranged from nine months to 92 years old with a mean age of 54. The demographic data also indicated that the infection was more prevalent in males, accounting for 60.5% (72/119) of cases, primarily between the ages of 15 and 47 [21.8% (26/119)]. In females, LZR MDR *S. capitis*

infections were less common, accounting for 32.8% (39/119) of cases and was more prevalent in patients aged 64 years or above [16.8% (20/119)]. The gender of eight (6.7%) in-patients and the age of five (4.2%) were unknown. Clinical data such as current and previous antimicrobial treatment, complications, diagnosis and morbidity/mortality status were unfortunately not available.

The majority of the isolates were collected from Gauteng with two isolates collected from North West province, two from Mpumalanga and one from Polokwane, therefore the greater Gauteng region was used as a collective. When examining the distribution of isolates over time, demographic data was recorded as follows (Table 3.1): In 2014, only six isolates were collected, consisting of one female and five males with an age range of 31 to 77 and a mean age of 62. All the isolates collected in 2014 were from region D (Johannesburg), with two isolates collected at one hospital (D3), two more isolates collected at a second hospital (D15) and the remaining two isolates collected at two distinct hospitals (D1 and D11).

A total of 17 isolates were collected in 2015, including six females, 10 males and one of unknown gender. The age range of isolates collected in 2015 was 24 to 84, with a mean age of 58. The isolates collected in 2015 were, i) 53% from region D (Johannesburg), with three collected from one hospital (D3), two from a second hospital (D1) and four from four distinct hospitals (D2, D4, D13 and D16), ii) 41% were from region A (Pretoria), with five collected from one hospital (A5) and two collected from a second hospital (A4) and iii) 6% were collected from region C (Midrand), with one isolate collected from a distinct hospital (C2).

A total of 24 isolates were collected in 2016, including 11 females, 11 males and two of unknown gender. The age range of these patients was from one year and seven months to 92 years, with a mean age of 52. The isolates collected in 2016 were: i) 71% from region D (Johannesburg), with five isolates collected at one hospital (D1), four isolates collected at a second hospital (D11), two isolates collected from a third hospital (D5) and two isolates collected from a fourth hospital (D17). Four isolates were collected from four distinct hospitals (D3, D6, D7 and D10), ii) 29% were from region A (Pretoria), with four collected at one hospital (A5), two collected at a second hospital (A4) and one collected at a distinct hospital (A2).

In 2017, a total of 12 isolates were collected, consisting of three females, seven males and two of unknown gender. The age range of the patients from whom the isolates were collected was

from nine months to 74 years old, with a mean age of 51. Nine of the 12 isolates were, collected from region D (Johannesburg). These nine isolates were collected from six different hospitals in the region. Two isolates were collected from each of the first three hospitals (D3, D8 and D10), and the remaining three isolates were collected from the three distinct hospitals (D1, D6 and D16). The remaining 25% (3 isolates) were collected from region A (Pretoria). Two of these isolates were collected from one hospital (A5) and the third isolate was collected from a different hospital (A4).

In 2018, a total of five isolates were collected, consisting of one female, three males and one of unknown gender. The age range of individuals from whom the isolates were collected was from 26 to 57 years, with a mean age of 43. From the five isolates collected in 2018, one isolate (20%) was collected at distinct hospitals from each of the following regions: A (Pretoria, A5), B (Centurion, B1), C (Midrand, C1), D (Johannesburg, D7) and E (Limpopo, Polokwane).

A total of 33 isolates were collected in 2019, including 13 females, 19 males and one of unknown gender. The age range of these patients was from one year and seven months to 71 years, with a mean age of 48. From the isolates collected in 2019: i) 39% were from region A (Pretoria), with five isolates collected at one hospital (A5), four isolates collected at a second hospital (A4), two isolates collected from a third hospital (A2) and two isolates collected from two distinct hospitals (A1 and A3), ii) 39% were from region D (Johannesburg), with three collected at one hospital (D1), two collected at a second hospital (D10), two collected at a third hospital (D11) and six collected from six distinct hospitals (D2, D3, D9, D13, D14 and D17), iii) 12% were from region B (Centurion), with four isolates collected from the same hospital (B1), iv) 6% were from region C (Midrand) with two isolates collected from two distinct hospitals (C1 and C2) and v) one (3%) isolate collected from region F (Rustenburg, North West).

The collection date of 22 isolates could not be retrieved and therefore, were classified as unknown. These isolates included 4 females, 17 males and one of unknown gender. The age range of these patients was from 19 to 83 years, with a mean age of 58. From the isolates collected with unknown collection dates: i) 64% were from region D (Johannesburg) with 11 isolates collected at one hospital (D2), two isolates collected at a second hospital (D10) and one isolate collected from a third hospital (D12), ii) 18% were from region A (Pretoria), with three collected at one hospital (A5) and one collected at a second hospital (A2), iii) 9% were from region G (Trichardt, Mpumalanga), with two isolates collected from the same hospital

(G), iv) one (5%) were from region F (Rustenburg, North West) from a distinct hospital (F2) and v) one (5%) from an unknown location (U).

3.3.2   Identification of the *Staphylococcus capitis* isolates

The multiplex-PCR assay confirmed the identification of the 119 LZR MDR *S. capitis* isolates included in the study. The identification of these isolates were in agreement with the MALDI-TOF (Bruker, USA) results performed at the private diagnostic laboratory. The amplicons were visualised using gel electrophoresis as shown in Figure S1.

3.3.3   Antimicrobial susceptibility profiles of the *Staphylococcus capitis* isolates

All the LZR MDR *S. capitis* isolates were confirmed as LZR with the ETEST® strip (bioMérieux, France) [100% (119/119)] and agreed with the initial MIC values obtained from the private diagnostic laboratory. The distribution of the LZR MIC among patients ranged from 8 μg/mL to >256 μg/mL according to the 2020 CLSI guideline. The results were interpreted as follows: i) susceptible with a concentration ≤ 4 μg/mL, ii) intermediate with a concentration between ≥5 μg/mL and ≤7 μg/mL or iii) resistant with a concentration of ≥ 8 μg/mL by comparing the breakpoint values of linezolid. The MIC values for LZD isolated from *S. capitis* were found to be widely distributed across a range of values: i) 9.2% (11/119) of the isolates with a MIC of 8 μg/mL, ii) 33.6% (40/119) with a MIC of 16 μg/mL, iii) 0.8% (1/119) with a MIC of 24 μg/mL, iv) 34.5% (41/119) with a MIC of 32 μg/mL, v) 0.8% (1/119) with a MIC of 34 μg/mL, vi) 13.4% (16/119) with a MIC of 64 μg/mL, vii) 0.8% (1/119) with a MIC of 96 μg/mL, viii) 3.4% (4/119) with a MIC of 128 μg/mL and ix) 3.4% (4/119) with a MIC of >256 μg/mL. These values have been tabulated in Table 3.4.

The private diagnostic laboratory's routine diagnostics determined the phenotypic antimicrobial resistance patterns of the LZR MDR *S. capitis* isolates according to the VITEK® 2 automated system (bioMérieux, France) were as follow: erythromycin 99.2% (118/119), amoxycillin/clavulanate 98.3% (117/119), cloxacillin 98.3% (117/119), clindamycin 97.5% (116/119), fucidic acid 84% (100/119), gentamycin 74.8% (89/119), cotrimoxazole 27.2% (33/119), rifampicin 16.8% (20/119), daptomycin 2.5% (3/119), vancomycin 1.7% (2/119) and teicoplanin 0.8% (1/119).

Out of the 119 isolates included in the panel, only isolate 116 [1/119 (0.8%)] demonstrated resistance to all 12 antimicrobial agents. Six isolates [5% (6/119)] showed resistance to nine

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different antimicrobial agents, while 23 isolates [19% (23/119)] demonstrated resistance to eight antimicrobial agents. Additionally, 63 isolates [53% (63/119)] were resistant to seven antimicrobial agents, 18 isolates [15% (18/119)] were resistant to six antimicrobial agents, seven isolates [6% (7/119)] were resistant to five antimicrobial agents and one isolate [6% (7/119)] showed resistance to linezolid with other antimicrobial agents not reported. The distribution of isolates resistant to different numbers of antimicrobial agents is presented in Table 3.4.

3.3.4 Screening of the plasmid-mediated linezolid resistance genes among the *Staphylococcus capitis* isolates

The *cfr* gene was found in one [0.8% (1/119)] isolate, while the *optr*A and *poxt*A genes were not detected with the M-PCR assay. The linezolid MIC value for the *cfr* positive isolate was 8 µg/mL indicating that there was no correlation between a high MIC value and the presence of the *cfr* gene in this study. The amplicons were visualised using gel electrophoresis as shown in Figure S2.

3.3.5 Genetic relatedness of the *Staphylococcus capitis* isolates using pulsed-field gel electrophoresis

A hundred and four [87.4% (104/119)] LZR MDR *S. capitis* isolates were successfully digested by *SmaI*, the remaining 12.6% (15/119) could not be digested and were classified as untypeable. The PFGE dendrogram was constructed using a Dice Coefficient (0.5% tolerance and 0.5% optimisation) to estimate the band-based similarity coefficient and an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) for cluster analysis. At the similarity cut-off value of ≥80%, the dendrogram showed one major pulsotype with 76 isolates and three minor pulsotypes with nine, five and three isolates respectively and 10 singletons, as shown in Figure 3.2. The 76 isolates from pulsotype A represented 22 of the 29 hospitals with 14 of the isolates originating from the same hospital (A5). The remaining isolates from pulsotype A were collected from the 21 remaining hospitals: nine from D1, eight from D2, five from B1, D3, and D11 each, four from A4 and D10 each, two from A2, C1, D7, D8, D13, D15, and D16 each, and one from A3, C2, D5, D9, D12, D14, D17, and G, respectively. The majority of isolates that clustered in pulsotype A were from male patients [(57.9%) 44/76] compared to the percentage of female patients [(36.8%) 28/76]. Isolates in pulsotype A showed the highest prevalence of 36.8% (28/76) from the elderly age group (≥ 64 years).

The nine isolates that clustred in pulsotype B represented eight of the 29 hospitals with two of the isolates originating from the same hospital (D3). The other seven isolates from pulsotype B were collected from the seven remaining hospitals: A4, A5, D1, D5, D10, D17 and F1. The majority of isolates that formed in pulsotype B were also from male patients [(55.6%) 5/9] compared to the percentage from female patients [(33.3%) 3/9]. Patients isolates that formed in pulsotype B [44.4% (4/9)] also belonged to the elderly age group (≥ 64 years).

The five isolates that clustered in pulsotype C represented five of the 29 hospitals, with one isolate from a hospital in the Pretoria region (A5), three isolates from three different hospitals in the Johannesburg region (D1, D6 and D11) and one isolate with an unknown location. The majority of isolates in pulsotype C were from male patients [(60%) 3/5] while the gender of the other two patients were unknown. The patient isolates in pulsotype C were found equally in the young (15 years to 47 years), middle aged (48 years to 63 years) and elderly (≥ 64 years) age groups.

The three isolates that formed in pulsotype D represented three of the 29 hospitals with one isolate from the Pretoria region (A5) and two isolates from the Johannesburg region (D3 and D6). Two of the three patient isolates in pulsotype D were from male patients [(67%) 2/3] and two of the three patients were in the young (15 years to 47 years) age group [(67%) 2/3].

3.3.6   Whole genome sequencing of the *Staphylococcus capitis* isolates

Whole genome sequencing was performed on five representative LZR MDR *S. capitis* isolates based on the dendrogram, the patient demographics and patients with irregular antimicrobial susceptibility profiles. Isolates 30, 66, 103 and 116 was selected from pulsotype A and isolate 145 from pulsotype B. All five isolates were re-confirmed as *S. capitis* with the Kmer Species program, which was also in agreement with the MALDI-TOF (Bruker, USA) and M-PCR assay results.

All the *S. capitis* isolates were predicted as human pathogens by PathogenFinder (Center of Genomic Epidemiology (CGE), Denmark). The antimicrobial-resistant genes detected among the five *S. capitis* isolates through the Comprehensive Antibiotic Resistance Database (CARD) and the Kmer Resistance database showed the following AMR genes: tetracycline K (*tetK*), aminoglycoside resistance (*aac*(6')-le-*aph*(2")-la), fusidic acid resistance (*fusB*), staphylococcal efflux pump (*sepA*), multidrug efflux pump (*sdrM*), mupirocin resistance (*mupA*), multidrug efflux pump (*mdeA*), methicillin A (*mecA*), beta-lactamase (*blaZ*), rRNA

adenine N-6-methyltransferase (*ermC*), dihydrofolate reductase (*dfrC*), gyrase subunit B (*gyrB*) and antiseptic resistance protein A (*qacA*). Isolates 30, 66, 116 and 145 harboured the antiseptic resistance protein D (*qacD*) gene while isolate 103 harboured the chloramphenicol acetyltransferase (*cat)* gene. All the isolates showed a point mutation at G2604T and T173A in the 23S rRNA gene conferring resistance to linezolid and aminocoumarin, respectively. No virulence genes were detected using the VirulenceFinder (CGE, Denmark). All *S. capitis* isolates carried the following plasmids as detected with PlasmidFinder: repL (pDLK1), repA (pLW043), repA (SAP016A), ORF (pKH1). Isolates 66, 103 and 145 also carried a rep (pSSAP1) plasmid. Isolate 103 carried an additional repI (pGB354) plasmid. Accroding to SCCmecFinder, all five isolates carried SCC*mec* type Vb(5C2&5). The *S. capitis* isolates could not be assigned a sequence type (ST) as there is no MLST scheme for *S. capitis* to date.

3.4 Discussion

This study reported findings on the genetic relatedness and AST of LZR MDR *S. capitis* found in private hospitals in the greater Gauteng area. Linezolid is used as a last resort antimicrobial agent to treat MDR infections as well as other severe Gram-positive infections (Sadowy, 2018). Although LZR has been extensively reported in other countries, limited data regarding this resistance is available from South Africa. The results of this ongoing outbreak provide valuable insights into the prevalence and characteristics of LZR MDR *S. capitis* in the greater Gauteng area in South Africa.

The results of this study indicated that LZR MDR *S. capitis* is distributed across various age group, but the majority of cases were found in young adults (aged 15 to 47 years) and the elderly (over 64 years). The age of patients in the study ranged from nine months to 92 years, which contrasts with some international studies that identified LZR *S. capitis* primarily in neonatal wards (Tevell *et al*., 2020). This implies that LZR MDR *S. capitis* is not confined to a particular age group, infections occurred at a higher prevalence in males [60.5% (72/119)] compared to females [32.8% (39/119)]. The gender of eight (6.7%) in-patients and the age of five (4.2%) patients were unknown. This information is important for understanding the epidemiology and transmission of LZR MDR *S. capitis* and can inform infection control and prevention strategies. It is also important for clinicians to be aware of the potential for LZR MDR *S. capitis* infections in patients of all ages, especially those who are immunocompromised or have other risk factors for infection.

Blood samples were collected from September 2014 to September 2019. In 2014, only six cases of LZR MDR *S. capitis* infections were reported, while in 2019, the prevalence of LZR MDR *S. capitis* increased to 33 cases. Although there was an overall increase in infections observed between 2014 and 2019, the number of cases fluctuated in the intervening years (Table 3.1). When including the 35 isolates that were not able to be revived, the data suggests that the infection rate remained relatively stable between 2015 and 2017. However, in 2018, there was a decrease in the infection rate, followed by a rapid increase in 2019.

All six LZR MDR *S. capitis* isolates that were collected in 2014 originated from four distinct hospitals in the Johannesburg area. This observation may indicate that the source of the outbreak could be traced back to private hospitals in Johannesburg. The first cases of LZR MDR *S. capitis* in Pretoria were only reported during late 2015 in seven distinct private hospitals. A South African publication by Bamford *et al*. (2021) reported on LZR *S. capitis* infections in private healthcare settings in the Western Cape and showed an increase in infection from 5.9% in 2019 to 22.5% in 2021. The study by Bamford *et al*. (2021) did not specify the patient demographics.

A multiplex-PCR (M-PCR) assay was employed to detect *S. capitis* using the 16s rRNA and *sodA* genes. The method for detection was developed by Al-Talib *et al*. (2009) and Kim *et al*. (2018) respectively. The findings obtained from the M-PCR assay were consistent with the outcomes obtained from MALDI-TOF (Bruker, USA).

The ETEST® strip (bioMérieux, France) used for linezolid susceptibility testing showed a wide range of MIC values from 8 μg/mL to >256 μg/mL. The majority of the isolates had an MIC of either 16 μg/mL [33.6% (40/119)] or 32 μg/mL [34.5% (41/119)] which is considered high and indicates reduced susceptibility to linezolid. In such cases, alternative treatment decisions should be made by a licensed medical professional based on the individual patient's medical history, the severity of the infection and the potential risks and benefits of alternative treatment options, such as: daptomycin, tedizolid quinupristin-dalfopristin, tigecycline and ceftaroline (Cappelletti *et al*., 2016). However, the selection of alternative antimicrobial agents should be based on AST results (Cappelletti *et al*., 2016). Four isolates [3.4% (4.119)] that had an MIC of >256 μg/mL were collected in Johannesburg between 2014 and 2015. When comparing each year from 2014 to 2019, there was no discernible pattern observed in the LZR MIC and antibiogram of LZR MDR *S. capitis*. The VITEK® 2 (bioMérieux, France) results and results from the ETEST® strip (bioMérieux, France) were in agreement.

All LZR *S. capitis* isolates were MDR according to the VITEK® 2 (bioMérieux, France) analysis. Multidrug-resistance is defined as a microorganism that is resistant to two or more antimicrobial agents (Ryan, 2018). One isolate, isolated from a 26 year old female patient in the Pretoria region showed resistance to all 12 [1/119 (0.8%)] different antimicrobial agents included in the panel while the majority 52% (62/119) of the isolates showed resistance to seven different antimicrobial agents throughout the collection period (September 2014 to September 2019) in varying ages and hospital distribution. In 2015, 5% (6/119) of the isolates were resistant to only five antimicrobial agents, compared to 2.5% (3/119) that were reported as resistant. The resistance of the remaining 2.5% was unknown. Table C3 in Annexure C shows the complete antibiogram for the LZR MDR *S. capitis* isolates. Using AST such as VITEK® 2 (bioMérieux, France) and ETEST® strip (bioMérieux, France) is important to guide antibiotic treatment for antimicrobial stewardship. In the case of isolate 116 where the LZR MDR *S. capitis* isolate were resistant to all 12 antimicrobial agents tested, the patient's care team may need to explore alternative treatments such as experimental antimicrobial agents, immunotherapy, or phage therapy. Additionally, infection control measures, such as isolating the patient, using strict hand hygiene and disinfection protocols as well as minimizing the use of invasive procedures, may be necessary to prevent further spread of the resistant bacterial pathogen. It is important to involve infectious disease specialists and microbiologists in the management of such cases to develop a personalized treatment plan and minimize the risk of complications (Kollef & Bassetti, 2021).

The second M-PCR assay only detected the presence of the *cfr* gene in isolate 143 [0.8% (1/119)], a 75 year old male from the Pretoria region collected in 2019. Although Figure S2 displays two *cfr* positive isolates, it is important to clarify that isolate 146 was not included in this study as it was identified as *Staphylococcus epidermidis*. The *cfr* positive isolate had a low MIC value of 8 μg/mL which contradicts other studies that reported MIC values four to 16 fold higher in isolates exhibiting the *cfr* gene (Wali *et al*., 2022). The reason for the low MIC value in the *cfr* isolate might be due to a point mutation in the open reading frame (ORF) of the *cfr* gene or a frameshift mutation in the chromosomal *cfr* (*cfr*$_{Q148K}$) as reported by Lee and Yang (2023). The low prevalence of the *cfr* gene found in this study contradicts the presence of the *cfr* gene with other studies that reported the presence of *cfr* in all their isolates from the Harbin Medical University in China (Han *et al*., 2022). The *cfr* gene is not widely distributed yet in these setings but may change in future. None of the isolates in this study were positive for the *poxt*A or the *optr*A genes which mediates resistance to linezolid. The findings in this study

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showed that the *poxtA* and *optrA* genes did not contribute to LZR and are consistent with a study conducted at Huashan Hospital in Shanghai that investigated various mechanisms of LZR (Ding *et al*., 2020).

The genetic relatedness of the LZR MDR *S. capitis* isolates were determined using PFGE. Only 104 [87.4% (104/119)] LZR MDR *S. capitis* isolates were successfully digested by *SmaI* while 12.6% (15/119) could not be digested and were classified as untypeable. In a study by Butin *et al*. (2016) *S. capitis* isolates were restricted with both *SmaI* and *SacII* restriction endonucleases (RE) and it was found that *SacII* has a higher discriminatory power. In future studies, the *SacII* RE can be used to digest all isolates that were untypeble by *SmaI* restriction. The PFGE dendrogram showed the genetic relatedness of the LZR MDR *S. capitis* isolates collected from private hospitals in the greater Gauteng area. The isolates in each pulsotype shares >80% similarity cut-off in their *Sma*I restriction and therefore can be inferred to belonging to the same clone as reported by Butin *et al*. (2017).

The LZR MDR *S. capitis* isolates were characterized by a major pulsotype (pulsotype A), consisting of 76 isolates. Among these, five isolates were collected in 2014, 10 in 2015, 17 in 2016, eight in 2017, three in 2018, 22 in 2019, and 11 had unknown collection dates. The age of the patients ranged from nine months to 86 years, with a mean age of 53 years. Forty four of the patients were male, 28 were female and four gender classifications were unknown. The majority of the isolates in pulsotype A were collected in region D (Johannesburg), nine from one hospital (D1), eight from a second hospital (D2), five from a third hospital (D3), five from a fourth hospital (D11), four from a fith hospital (D10), two each from five distinct hospitals (D7, D8, D13, D15 and D16) and 1 each from five distinct hospitals (D5, D9, D12, D14 and D17). Twenty one isolates were collected in region A (Pretoria), 14 from one hospital (A5), four from a second hospital, two from a third hospital (A2) and one from a fourth hospital (A3). Three isolates in pulsotype A were collected in region C (Midrand), two from one hospital (C1) and one from a second hospital (C2). Five isolates were collected from a hospital (B1) in region B (Centurion) and another was collected from a hospital in region G (Trichardt, Mpumalanga). The information provided indicated that the isolates in pulsotype A were collected over a period of several years, from different hospitals and regions and from patients of different ages and genders. An increase in infections is seen from 2014 to 2019 with the majority of isolates collected from the same region (region D, Johannesburg) which might be a clonal dissemination of the LZR MDR *S. capitis* strain.

Pulsotypes B, C and D were classified as minor pulsotypes. Pulsotype B consisted of nine isolates. Among these, one isolate was collected in 2014, two in 2015, two in 2016, one in 2017, two in 2019 and one isolate had an unknown collection date. The age of the patients ranged from one year, seven months to 83 years, with a mean age of 63 years. Five of the patients were male, three were female and one gender classification was unknown. The majority of the isolates in pulsotype B were collected in region D (Johannesburg), with two isolates from one hospital and four isolates collected from four distinct hospitals. Two isolates were collected in region A (Pretoria), both from distinct hospitals. One isolate was collected in region F (Rustenburg, North West). The information provided indicated that the isolates in pulsotype B were collected over a period of several years, from different hospitals and regions, and from patients of different ages and genders.

Pulsotype C consisted of five isolates. Among these, one isolate was collected in 2015, one in 2016, one in 2017, one in 2019 and one isolate had an unknown collection date. The age of the patients ranged from 40 years to 76 years, with a mean age of 59 years. Three of the patients were male and the remaining three genders classifications were unknown. The majority of the isolates in pulsotype B were collected in region D (Johannesburg), with three isolates from three distinct hospitals. One isolate was collected in region A (Pretoria) and one isolate had an unknown location. The information provided indicates that the isolates in pulsotype C were collected over a period of several years, from different hospitals and regions and from patients of different ages and genders.

Pulsotype D consisted of three isolates. Among these, one isolate was collected in 2015, one in 2016 and one in 2018. The age of the patinets ranged from 32 years to 61 years, with a mean age of 46 years. Two of the patients were male and the remaining one was female. Two of the isolates in pulsotype B were collected in region D (Johannesburg) from two distinct hospitals while one was collected from a hospital in region A (Pretoria). The information provided indicated that the isolates in pulsotype D were collected over a period of several years, from different hospitals and regions and from patients of different ages and genders.

Isolates from each pulsotype came from different hospitals in Gauteng suggesting clonal dissemination through different routes of transmission such as contact between staff and patients, staff transferring between hospitals and staff transferring to different wards (Sikora & Zahra, 2022). Another source of transmission is through the community, particularly through direct contact with an infected person or contaminated surfaces, or through contact with items

such as towels or clothing that have been in contact with an infected person (Sikora & Zahra, 2022).

Whole genome sequencing performed on the five representative isolates showed the presence of the following AMR genes that was also observed in the phenotypic testing: *aac*(6')-le-*aph*(2")-la conferring resistance to aminoclycosides (such as gentamycin), *blaZ* conferring resistance to amoxacilin/clavulanic acid and cloxacillin, *ermC* conferring resistance to lincosamides (clindamycin) and macrolides (erythromycin) and *fusB* conferring resistance to fusidic acid. Only isolate 103 and 116 harboured the *dfrC* gene conferring resistance to sulfonamides (cotrimoxazole). Only isolate 103 harboured the chloramphenicol acetyltransferase (*cat*) gene which has been reported to confer resistance to chloramphenicol and is also known as a multidrug transporter (Schwarz *et al*., 2004). It is important to note that molecular genes detected aren't necessarily expressed, that is why phenotypic testing in diagnostic laboratories remain important (Yee *et al*., 2021). The gene for vancomycin resistance was not detected with whole genome sequencing analysis even though isolate 145 and 116 were resistant to vancomycin according to the VITEK® 2 (bioMérieux, France) results. Possible explanations for not detecting the vancomycin resistance genes include genetic resistance mechanisms such as changes in cell wall thickness, undetected genetic mutations in analysis using underdeveloped pipelines, acquisition of resistance through other mechanisms, or phenotypic resistance without genetic alterations (Ahmed & Babtiste, 2018). Additional investigations using complementary sequencing techniques may be needed to uncover the underlying resistance mechanisms that were not captured by the sequencing methods employed.

According to Michalik *et al*. (2021) the most common LZR mechanism is a 23S rRNA mutation G2576U, but this mutation was not detected in any of the five isolates analysed after WGS. However, all five LZR *S. capitis* isolates carried a previously identified mutation of the 23S rRNA gene, G2604T (Nguyen *et al*., 2020). Similar findings were reported by a study conducted in Vietnam by Nguyen *et al.* (2020), identifying the same mutation in clinically relevant CONS at the University Medical Centre. The single point mutation caused a basepair change from guanine to thymine at position 2604 in the sequence of the central loop of domain V in the 23S rRNA and as a result changed the amino acid sequence. This change in the amino acid sequence can alter the structure of the protein, potentially reducing the binding affinity of linezolid to the ribosome and leading to resistance to the antibiotic (Tewhey *et al*., 2014). While

the identified point mutation is capable of conferring resistance to linezolid, it is important to acknowledge that the development of resistance could involve multiple mechanisms working in tandem, as reported by Han *et al*. (2022). Furthermore, research has revealed a correlation between the frequency of linezolid resistance and the presence of multiple mutations in the gene encoding 23S rRNA (Meka *et al*., 2004). Future investigations will need to include the analysis of potential point mutations that may have been missed by the CARD database used in this study.

Several plasmids were identified with WGS among the five isolates: i) Plasmid repL (pDLK1) with a size of 2.4 kilo bases (Kb) was detected in all isolates which encodes only the *ermC* gene and a replication gene (repL) (Ullah *et al*., 2022). This plasmid was only previously reported in MRSA isolates form Pakistan, ii) Conjugative plasmid repA (pLW043) with a size of 59.7 Kb was detected in all isolates and encodes the *vanA* operon in the Tn1546 transposon, a beta-lactamase operon, the *aac*(6')-le-*aph*(2")-la gene and the *dfr* gene (Mlynarczyk-Bonikowska *et al*., 2022). This plasmid was reported in isolates that were vancomycin-resistant *S. aureus* (VRSA) in the USA (Weigel *et al*., 2003), iii) a mobilizable plasmid, repA (SAP016A) was also found in all isolates however limited information is available on this plasmid (Lebard *et al.,* 2008). This plasmid was previously reported in *S. epidermidis* from the USA, iv) The rep (pSSAP1) plasmid was detected in three of the isolates namely isolates 66, 103 and 145 and contained uro-adherence factor B (*uafB*) which encodes an adhesive-encoding gene that mediates adhesion to fibrinogen and fibronectin (Magnan *et al.,* 2022). This plasmid was previously reported in *Staphylococcus pettenkoferi* in France (Magnan *et al.,* 2022). According to VirulenceFinder no virulence genes were detected in the five representative isolates, however the presence of the rep (pSSAP1) plasmid, which encodes a virulence factor gene, *uafB* contradicts the results. The repI (pGB354) plasmid was only found in isolate 103 and is associated with chloramphenicol resistance which is in agreement with the CARD database (Freitas *et al*., 2020). This plasmid was previously reported in *Streptococcus agalactiae* in Portugal (Freitas *et al*., 2020)*.*

The SCC*mec* types identified through SCCmecFinder in all five representative isolates harbored a type Vb (5C2&5) SCC*mec*. This finding is in agreement with Simões *et al*. (2013) who identified the type V (5C2&5) SCC*mec* element in the NRCS-A *S. capitis* strain, CR01. Due to the lack of a MLST typing scheme for *S. capitis*, a ST could not be assigned to the

isolates, however Wang *et al.* (2022) reported that researchers are in the early processes of developing a core genome MLST (cgMLST) scheme for future research.

Studies conducted in France, the United Kingdom, Belgium, Australia and Sweden have reported the presence of neonatal LZR MDR *S. capitis* in their NICUs which all belong to a specific clone, NRCS-A (Gu *et al.*, 2013; Butin *et al.*, 2017; Decalonne *et al.*, 2020; Bamford *et al.*, 2021). In the current study, a high prevalence of LZR MDR *S. capitis* was observed from private healthcare settings mostly among young patients (15 to 47 years) and elderly patients (≥ 64 years). While the previous studies focused on the NRCS-A clone in NICUs, the current study suggests that this potential clone is also present in other healthcare settings in varying patient populations.

One major limitation of this study is that clinical information was not available, which could have helped to establish further correlations between isolates in the same clusters. Another limitation of this study is that the pipelines for WGS analysis may not be specifically designed to analyze CONS such as *S. capitis*. The findings of this study are significant as they represent one of the first reports in South Africa on the transmission of a possible established clone between private hospitals. No reports have indicated linezolid resistance in critically ill patients in public hospitals but a rise in linezolid resistance in tuberculosis patients have been observed. Surveillance of linezolid resistance in the public settings will therefore be beneficial.

There are several future aspects that could be considered for further investigation in this study, such as: i) Collecting more detailed clinical information on the patients infected with *S. capitis* could provide further insights into the epidemiology and transmission dynamics of this pathogen, as well as the potential risk factors associated with infection, ii) Genomic analysis of additional isolates, expanding the sample size by analyzing more isolates from different hospitals and regions in South Africa could help to provide a more comprehensive picture of the spread and diversity of *S. capitis* clones in the country, iii) Further investigating the molecular mechanisms underlying the antimicrobial resistance observed in *S. capitis* isolates, including the presence of plasmids or other mobile genetic elements, could help to identify potential targets for future therapies and iv) Conducting studies on the effectiveness of infection prevention and control measures in reducing the spread of *S. capitis*, especially in private healthcare settings where the prevalence is high, could be useful in developing more effective strategies to control outbreaks and prevent future infections. This could be achieved by continuing collection from Sep 2019 and reporting on new findings.

3.5 Conclusion

The majority of the LZR MDR *S. capitis* isolates were closely related and harboured similar ARGs, plasmids and *SCCmec* elements which suggests the establishment and spread of a successful clone since September 2014. The results indicated no significant correlation between the age and gender of patients in the cluster. However, the findings suggest that the ongoing outbreak may have originated in region D (Johannesburg) and subsequently spread to other regions over the years. Many of the ARGs identified in this study are carried on plasmids, which may promote the fitness and dissemination of this clone. These results showed the importance of effective surveillance to monitor the spread of LZR MDR *S. capitis* in hospitals in South Africa. The detection of LZR MDR *S. capitis* is of great concern as these strains are associated with high morbidity, mortality and increased healthcare costs. The majority of the isolates remained susceptible to daptomycin, teicoplanin and vancomycin, indicating that these antibiotics could be used as last resort treatment options for LZR MDR *S. capitis* infections. Alternative therapies such as immunotherapy and phage therapy are in the pipeline for Gram-positive infections and will be beneficial to treat patients that are resistant to most of the antimicrobial agents.

Ethics Statement

Ethical approval was obtained for the study from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (Ethics reference number: 115/2019). Individual patient consent was waivered since the study was retrospective and patient care was not influenced at any stage.

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Table 3.1: **The distribution of the 119 linezolid resistant multidrug-resistant** *Staphylococcus capitis* **isolates arranged by the year of collection, along with age groups, gender and region of the hospitals. Female = F; Male = M; Unknown = U; A = Pretoria; B = Centurion; C = Midrand; D = Johannesburg; E = Limpopo; F = Rustenburg; G = Trichardt**

Year collected	Prevalence (n)	Gender (n)	Age range (years old)	Median age	Hospital region
2014	6	1 F	31 to 77	62	100% D
		5 M			
2015	17	6 F	24 to 84	58	53% D
		10 M			41% A
		1 U			6% C
2016	24	11 F	1 year, 7 months to 92	52	71% D
		11 M			29% A
		2 U			
2017	12	3 F	9 months to 74	51	75% D
		7 M			25% A
		2 U			
2018	5	1 F	26 to 57	43	20% A
		3 M			20% B
		1 U			20% C
					20% D
					20% E
2019	33	13 F	1 year, 7 months to 71	48	39% A
		19 M			39% D
		1 U			12% B
					6% C
					3% F
U	22	4 F	19 to 83	58	64% D
		17 M			18% A
		1 U			9% G
					5% F
					5% U

Table 3.2: **Genes, primers and PCR conditions that were used for the amplification of fragments to identify *Staphylococcus capitis* and detect plasmid-mediated linezolid resistant mechanisms in *S. capitis* isolates**

Gene	Primers	Primer sequence (5'-3')	PCR conditions	Amplicon size (bp)
*sodA**	*CT103-F*	TCAGATATTCAAACTGCAGTACG	95°C, 15 min; 30 cycles of: 95°C, 30s; 58°C, 30s; 77°C, 70s; final extension 72°C, 2 min.	103
	CT103-R	CTACTTCACCTTTTTCTTCAGA		
16S rRNA†	*rRNA*-F	GCAAGCGTTATCCGGATTT		597
	rRNA-R	CTTAATGATGGCAACTAAGC		
cfr‡	*cfr*-fw	TGAAGTATAAAGCAGGTTGGGAGTCA	96°C, 2 min; 30 cycles of: 96°C, 30s; 50°C, 30s; 72°C, 30s; final extension 72°C, 5 min. (hold: 8°C)	746
	cfr-rv	ACCATATAATTGACCACAAGCAGC		
optrA§	*optrA*-fw	TACTTGATGAACCTACTAACCA		422
	optrA-rv	CCTTGAACTACTGATTCTCGG		
poxtA¶	*poxtA*-fw	AAAGCTACCCATAAAATATC		533
	poxtA-rv	TCATCAAGCTGTTCGAGTTC		

*(Kim *et al.*, 2018)

†(Al-Talib *et al.*, 2009)

‡(Kehrenberg and Schwarz, 2006)

§(Brenciani *et al.*, 2016)

¶(Bender *et al.*, 2019)

Table 3.3: **The Master mix reaction composition for the M-PCR assay to identify the *Staphylococcus capitis* isolates and for the detection of the *cfr* gene in *S. capitis* isolates**

Content	Volume/tube (μL)	Final concentration
2 x Bioline PCR Master mix	6.25	1 x (3 mM MgCl₂)
10 X Primer mix	1.25	2 μM
Nuclease-free water	4.00	-
DNA	1.00	< 1 μg DNA / 25 μL
Total	**12.5**	-

Table 3.4: **Distribution of 119 linezolid resistant, multidrug-resistant *Staphylococcus capitis* isolates by year of collection, linezolid resistant minimum inhibitory concentration, and number of antimicrobial agents resistant (MDR). n = number of isolates; U = unknown**

	n	LZR MIC									Number of isolates that are resistant to a certain number of antimicrobial agents (MDR)								
		8	16	24	32	34	64	96	128	>256	1	5	6	7	8	9	10	11	12
2014	**6**	-	-	-	-	-	2	-	1	3		-	-	5	1	-	-	-	-
2015	**17**	1	4	-	6	1	2	1	1	1		3	1	6	7	-	-	-	-
2016	**24**	-	9	-	8	-	6	-	1	-		-	1	21	2	-	-	-	-
2017	**12**	1	5	-	5	-	-	-	1	-		-	2	8	2	-	-	-	-
2018	**5**	-	1	-	2	-	2	-	-	-		-	-	4	-	1	-	-	-
2019	**33**	5	12	1	13	-	2	-	-	-		-	9	13	7	4	-	-	-
U	**22**	4	9	-	7	-	2	-	-	-	1	3	5	7	4	1	-	-	1
Total	**119**	11	40	1	41	1	16	1	4	4	1	7	18	63	23	6	-	-	1
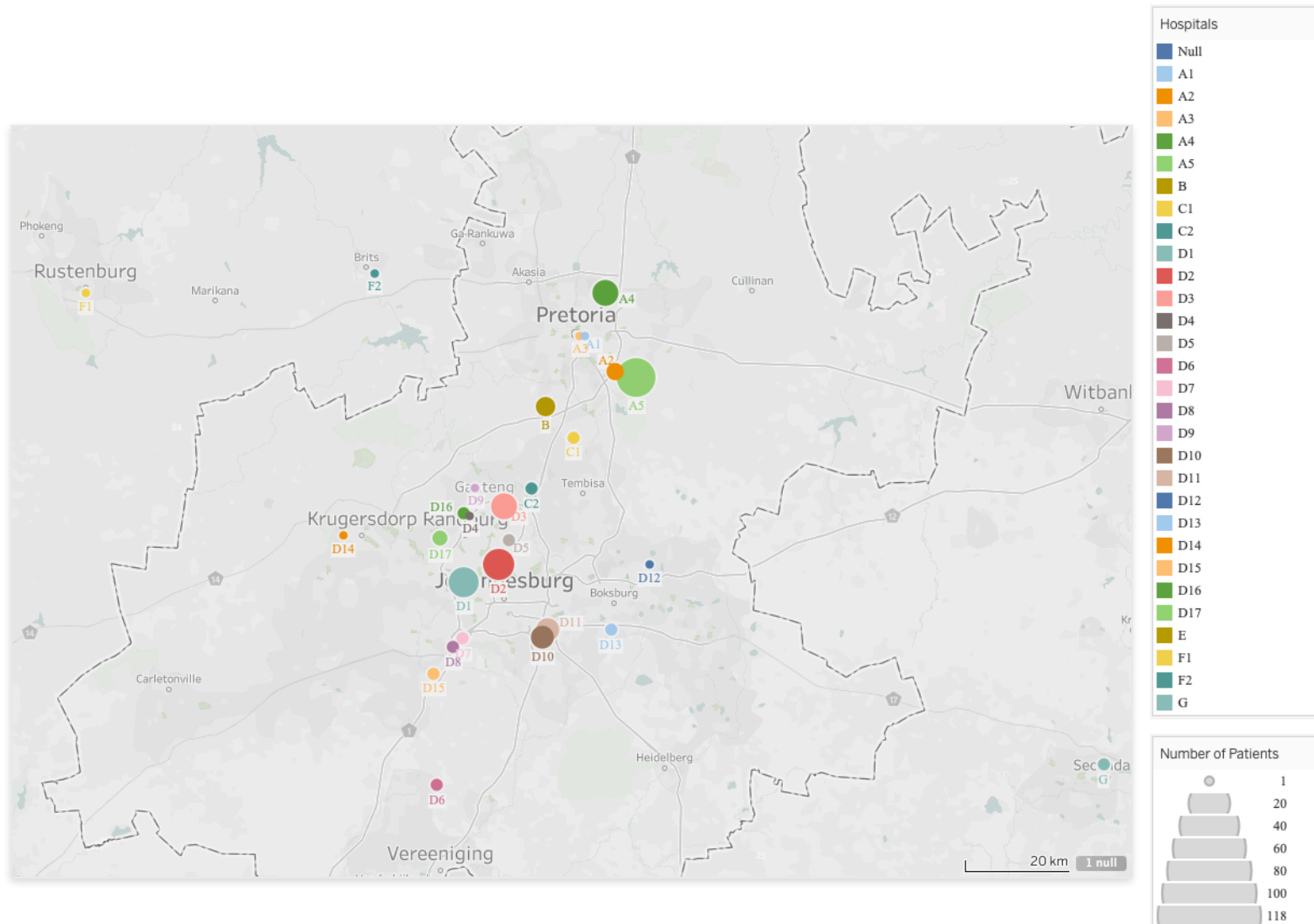
Figure 3.1: The map displays the locations of hospitals in the greater Gauteng area where isolates were collected. Regions are labelled A to G, hospitals are sub-labelled by numbers and differentiated by color. The size of each label corresponds to the number of isolates collected from that hospital. The map was created using Tableau (Salesforce Inc, USA).

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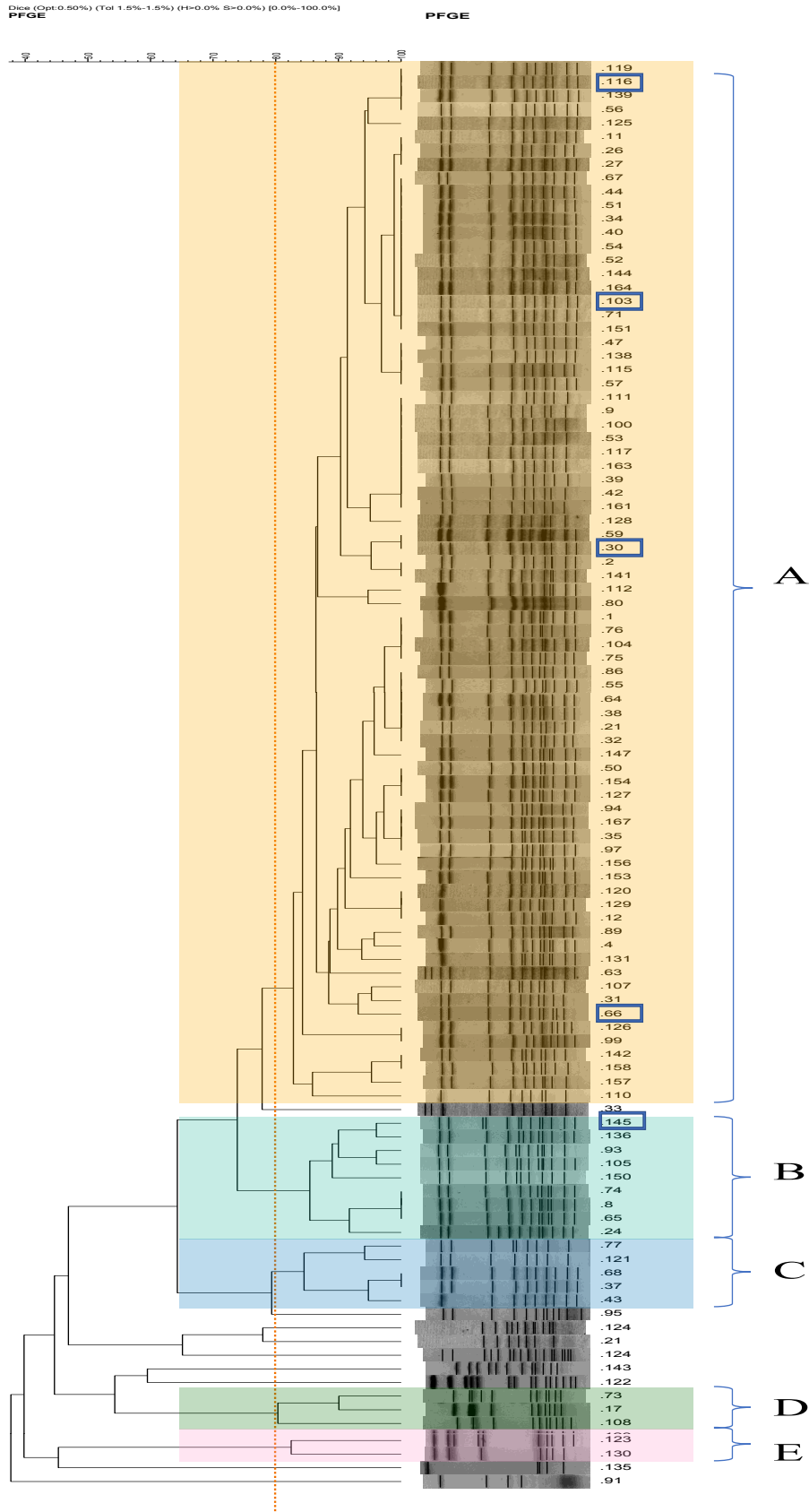
FIGURE 3.2: Dendrogram showing the genetic relatedness of the *Staphylococcus capitis* isolates in the study. The various pulsotypes are labelled A to E. The blue box outline indicates the isolates that were chosen for WGS.

CHAPTER 4

CONCLUSION

4.1   Concluding remarks

Antimicrobial resistance (AMR) poses a great threat against modern medicine in that it prevents recovery from life-threatening diseases (WHO, 2021). The World Health Organization has listed AMR as one of the top ten public health threats facing humanity and set out a global action plan to improve awareness, strengthen surveillance, reduce incidence of infection and to optimise use of antimicrobial therapy (WHO, 2021). Since 2014, a rise in linezolid-resistant multidrug-resistant (LZR MDR) *Staphylococcus capitis* (*S. capitis*) infections were observed in private hospitals in the greater Gauteng area. These infections were mainly observed in critically ill patients in intensive care units (ICUs). The clinical data such as previous antimicrobial treatment including linezolid, complications, diagnosis and morbidity/mortality status were unfortunately not available. This information could aid in identifying the cause of the outbreak, determining its spread and informing public health response efforts (Houlihan & Whitworth, 2019). This study presents one of the first investigations into the mechanism of LZR and molecular epidemiology of this ongoing *S. capitis* outbreak in the greater Gauteng area. This outbreak of LZR MDR *S. capitis* is of particular concern as linezolid is seen as one of the last resort antimicrobial agents used to treat MDR infections as well as other severe Gram-positive infections (Sadowy, 2018).

Several phenotypic and genotypic techniques were used to characterise the staphylococci obtained from the private diagnostic laboratory. The identification of *S. capitis* and the characterisation of plasmid-mediated LZR was performed using conventional multiplex PCR (M-PCR) assays. The M-PCR assays were successfully optimised according to the published articles (Kehrenberg and Schwarz, 2006; Al-Talib *et al.*, 2009; Kim *et al.*, 2018a). Even though the M-PCR assays were very time consuming, this technique remains cost-effective compared to WGS (Mahon *et al.*, 2015). All isolates in this study were successfully confirmed as *S. capitis* with the M-PCR assay and was in agreement with the MALDI-TOF (Bruker, USA) results.

An ETEST® (bioMérieux, France) was performed on all *S. capitis* isolates to re-confirm their minimum inhibitory concentration (MIC) to linezolid after the initial antimicrobial susceptibility testing (AST) with the VITEK® 2 (bioMérieux, France). The linezolid ETEST® (bioMérieux, France) showed a wide range of MICs that ranged from 8 µg/mL to >256 µg/mL. All the *S. capitis* isolates included in this study were reported as MDR with the majority [54% (64/119)] showing resistance to seven antimicrobial agents. These antimicrobial agents include linezolid, amoxacilin/clavulanic acid, cloxacillin, gentamicin, clindamycin, erythromycin and fucidic acid. These LZR MDR *S. capitis* isolates have gained resistance mechanisms that improve their survival in the presence of two or more antimicrobial agents (Ryan, 2018). Using AST such as VITEK® 2 (bioMérieux, France) and ETEST® strip (bioMérieux, France) is important to guide antibiotic treatment for antimicrobial stewardship.

The *cfr* gene confers cross-resistance to five classes of 50S ribosomal subunit-targeted antibiotics (phenotype PhLOPS$_A$) which includes phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (Tian *et al*., 2014). The *cfr* gene was only detected in isolate 143, a 75 year old male in the Pretoria region. The presence of the *crf* gene enables a higher rate of dissemination through horizontal gene transfer of a plasmid or a composite transposon to closely related species (Han *et al*., 2022). Data regarding the prevalance of the *cfr* gene among the *Staphylococcus* genus remains limited in South Africa. Therefore, continuous surveillance should be set in place to detect possible *cfr* genes in future collections. The *poxtA* and *optrA* genes are also acquired through plasmids or composite transposons and have been reported to mediate resistance to linezolid (Bender *et al*., 2019). However, these genes were not detected in any of the isolates.

To determine the genetic relatedness, pulsed-field gel electrophoresis (PFGE) was performed and the isolates were digested with a *Sma*I restriction endonuclease (RE). The isolates were restricted with a restriction endonuclease, *Sma*I which successfully restricted 87.4% (104/119) of the isolates,12.6% (15/119), however, could not be digested with *Sma*I and were classified as untypeable. In future studies, the untypeable isolates can be further analyzed using the *Sac*II restriction enzyme. According to a previous study (Butin et al., 2016), this restriction endonuclease has been reported to have a higher discriminatory power, as it recognizes specific genomic sequences. It has been reported that *S. capitis* isolates in adult patients usually belong to many distinct banding patterns (Butin *et al.,* 2016), however this contradicts with the findings in this study that showed a high similarity in the banding patterns of the patients from

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varying age groups. The PFGE dendrogram constructed at the similarity value of ≥80% showed one major pulsotype, three minor pulsotypes and ten singletons. The banding patterns observed in the pulsotypes were not consistent with those of any known clonal groups in *S. capitis* such as the NRCS-A clone. As a result, it can be inferred that a different clone has been successfully identified and established in these clinical settings. This conclusion was drawn through visual interpretation of the banding patterns which showed high similarity. This increased specificity can be attributed to variations in the genome sequences, which may be associated with factors such as geographic location, environmental pressures such as genetic drift and independent evolution (Bengtsson-Palme *et al*., 2018).

Five representative LZR MDR *S. capitis* isolates, selected based on their pulsotype, patient demographics, low antimicrobial susceptibility profiles and amount of anticimrobial agents the isolates were resistant to, were subjected to WGS. Whole genome sequencing showed the presence of AMR genes that was also observed in the phenotypic testing. Antimicrobial resistant genes genes detected with WGS aren't necessarily expressed in phenotypic testing, that is why phenotypic testing remain an important test in diagnostic laboratories (Yee *et al*., 2021). An additional gene that were detected with WGS and not phenotypic testing were chloramphenicol acetyltransferase (*cat*) gene which has been reported to confer resistance to chloramphenicol and is also known as a multidrug transporter (Schwarz *et al*., 2004).

According to Michalik *et al*. (2021) the most common LZR mechanism is a 23S rRNA mutation G2576U, however this mutation was not detected in any of the five isolates analysed after WGS. All five LZR MDR *S. capitis* isolates however, carried a previously identified mutation of the 23S rRNA gene, G2604T which is it associated with LZD. This mutation has only been reported in one paper from Vietnam in 2020 and was detected in methicillin-resistant CONS (Nguyen *et al*., 2020).

Several plasmids were identified among the five isolates with WGS: i) Plasmid repL (pDLK1), ii) Conjugative plasmid repA (pLW043), iii) A mobilizable plasmid, repA (SAP016A) and iv) The rep (pSSAP1) plasmid was detected in three of the isolates. According to VirulenceFinder no virulence genes were detected in the five representative isolates, however this contradicts with the presence of the uro-adherence factor B (*uafB*) gene located on the rep (pSSAP1) plasmid, which encodes a virulence factor gene assocated with biofilm formation. The repI (pGB354) plasmid was only found in isolate 103 and is associated with chloramphenicol resistance which was also in agreement with the CARD database (Freitas *et al*., 2020). This

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plasmid was previously reported in *Streptococcus agalactiae* in Portugal (Freitas *et al*., 2020). This finding indicates horizontal gene transfer (HGT) between different genera.

The SCC*mec* types identified through SCCmecFinder after WGS in all five representatives was type Vb (5C2&5) SCC*mec*. Notably, the findings of the current study were consistent with those reported by Simões *et al.* (2013), who identified a novel type V (5C2&5) SCC*mec* element in the NRCS-A *S. capitis* strain, CR01. This observation represents the only correlation between the results of this study and the characteristics of the NRCS-A clone. Due to the lack of a MLST typing scheme for *S. capitis*, a ST could not be assigned to the isolates, however Wang *et al*. (2022) reported that the researchers are in the early processes of developing a core genome MLST (cgMLST) scheme for future research.

Several surveillance programs exist to control the spread of nosocomial infections but usually doesn't include South Africa due to poorly functioning health systems, lack of skilled personnel and financial constraints. In South Africa, existing surveillance programs in healthcare settings do not include monitoring of CONS species. This may be attributed to the fact that staphylococci obtained from blood cultures are often considered to be contaminants and are typically discarded during routine diagnostics. However, the current study's results underscore the importance of processing staphylococcal isolates in routine diagnostics and initiating surveillance programs to better understand the prevalence and distribution of CONS in healthcare settings. Although *S. capitis* might not be as virulent as *S. aureus*, its spread through the private hospitals as well as the detection of a circulating LZR MDR clone is alarming. One major limitation of this study is the lack of available clinical information, which could have facilitated the establishment of possible correlations between isolates in the same clusters, patient co-morbidities and previous linezolid treatment. Another limitation of this study is that the pipelines for WGS analysis may not be specifically optimized to analyze CONS species, such as *S. capitis*. Despite these limitations, the findings of this study are significant and represent one of the first reports in South Africa on the transmission and spread of a possible LZR MDR *S. capitis* clone between private hospitals from September 2014 to September 2019.

4.2   Future research

The findings in this study highlight the urgent need for authorities to recognize LZR MDR *S. capitis* as an important pathogen that is spreading in private hospitals in South Africa and to implement measures to control its spread and prevent further infections. A better understanding

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of LZR epidemiology among CONS in South Africa is required to effectively apply improved infection control in healthcare settings. The point mutation responsible for LZR resistance, G2604T, was previously reported in only one other study (Nguyen et al., 2020) Genomic analysis of additional isolates could identify G2604T mutations as well as other emerging mutations in this circulating clone to help to provide a more comprehensive understanding of the spread and diversity of *S. capitis* clones in the country. Further investigation of the G2604T mutation is required to determine its location within the *S. capitis* genome and understand the factors contributing to its prevalence in South Africa. The location of the mutation can be useful as a target for alternative treatment. This can be done with transcriptomics and metabolomics to further investigate the mutation.

The knowledge of linezolid exposure among patients in this setting is unknown which make it difficult to apply antimicrobial stewardship and alternative therapies. Other clinical data such as, complications, diagnosis and morbidity/mortality were also unknown which could help understand the transmittability LZR MDR *S. capitis*. Possible routes of transmission of LZR MDR *S. capitis* include transmission between staff and patients, staff transferring between hospitals and staff transferring to different wards. In addition, transmission may also occur through the community, particularly through direct contact with an infected person or contaminated surfaces.

The pipelines for whole genome sequencing analysis used in this study is not optimised for all microorganisms but rather focuses on pathogens causing serious infections. Discrepancies found in this study could be resolved with a more established network of pipelines that would include the analysis of species within the *Staphylococcus* genus. Finally, once the development of the cgMLST scheme for *S. capitis* is completed, the isolates used in this study can be subjected to the scheme to establish their sequence types (ST) and compare them to publicly available online libraries. This ongoing outbreak should be carefully surveilled by continuing isolate collection, determining the genetic relatedness of these isolates and monitoring the antibiogram profiles and detecting possible plasmid-mediated linezolid resistance genes. It is important to continue investigating and reporting any additional linezolid resistant mechanisms and and other last resort antimicrobial resistance to mitigate the spread in critically ill patients.

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ANNEXURE A

List of reagents and buffers used in experimental procedures

1. **Culturing and storage of bacteria implicated in microbiologically confirmed bloodstream infections (BSI)**

a) **Brain Heart Infusion (BHI) Broth (pH 7.4 ± 0.2 at 25°C)**   **(500 mL)**

    BHI powder (LabM Limited, UK)   18.5 g*

    Distilled water (dH$_2$O)   500 mL

    $*\frac{500\ mL}{1000} \times 37 = 18.5$ g in 500 mL

    Dissolve 18.5 g of BHI broth powder in 400 mL of dH$_2$O, bring the volume to 500 mL with the remaining dH$_2$O and heat slightly. Sterilise the solution by autoclaving (121°C for 15 min at 15 Barr).

b) **50% Glycerol (1:1) solution**   **(500 mL)**

    Glycerol (Merck, Germany)   250 mL

    dH$_2$O   250 mL

    Add 250 mL of glycerol to 150 mL of dH$_2$O and bring the volume to 500 mL with the remaining dH$_2$O. Sterilise the solution by autoclaving (121°C for 15 min at 15 Barr).

2. **Gram-staining of bacteria implicated in microbiologically confirmed bloodstream infections (BSI) (Claus, 1992)**

a) Crystal violet (Diagnostic Media Products, NHLS, South Africa), primary stain

b) Sterile dH$_2$O, used for fixation of a bacterial colony to a microscope slide

c) Gram's Iodine (Diagnostic Media Products, NHLS, South Africa), mordant

d) Ethyl-alcohol (95%), decolouriser

e) Safranin (Diagnostic Media Products, NHLS, South Africa), counterstain

3. **Reagents required for the total DNA extraction of CoNS isolates (Zymo Research *Quick*-DNA™ Fungal/Bacterial Miniprep Instruction Manual v2.1.0, 2018)**

a) **Reagents included in the *Quick*-DNA™ Fungal/Bacterial Miniprep (D6005) kit (Zymo Research, USA)**

    i)   BashingBead™ Buffer   40 mL

ii)	Genomic Lysis Buffer	100 mL
iii)	DNA Pre-Wash Buffer	15 mL
iv)	g-DNA Wash Buffer	50 mL
v)	DNA Elution Buffer	10 mL

b) **Reagents not included in the *Quick*-DNA™ Fungal/Bacterial Miniprep (D6005) kit (Zymo Research, USA)**

i) 1 X Phosphate buffered saline (PBS) [pH 7.2] (GIBCO®, Invitrogen, Nieu-Zealand)

ii) Add 500 µL of 0.5% (v/v) β-mercapto-ethanol (Merck, Germany) to the Genomic Lysis Buffer for optimal performance.

4. **Reagents required for gel electrophoresis (Green & Sambrook, 2012)**

a) **Stock solution of 0.5 M EDTA (pH 8.0)** **(1 000 mL)**

0.5 M of EDTA (Sigma-Aldrich, USA) (E5134 – 500 g)	186.1 g*
Sodium hydroxide (NaOH) pellets (Merck, Germany)	20 g
dH$_2$O	800 mL

*$0.5\,\text{M} \times 1\,\text{L} \times 372.24\frac{\text{g}}{\text{mol}} = 186.1\,\text{g}$

Weigh the components and add to dH$_2$O. Bring to a total volume of 1 000 mL. Sterilise by autoclaving (121°C for 15 min at 15 Barr).

b) **Stock solution of 5X Tris Boric EDTA (TBE) buffer (pH 8.3)** **(1 000 mL)**

0.45 M Trizma base (Sigma-Aldrich, USA) (T1503 – 1 kg)	54 g
0.44 M Boric acid (Sigma-Aldrich, USA) (B7901)	27.5 g
0.5 M EDTA (pH 8) (prepared in 4a)	20 mL
dH$_2$O	700 mL

Weigh the components and add to dH$_2$O. Bring to a total volume of 1 000 mL. Sterilise by autoclaving (121°C for 15 min at 15 Barr).

c) **Working solution of 1X TBE buffer (pH 8.0)** **(1 000 mL)**

5X TBE buffer (pH 8.0) (prepared in 4b)	200 mL*

dH$_2$O   700 mL

$$*\frac{1X \times 1\,000\text{ mL}}{5X} = 200\text{ mL}$$

Measure the components and add to dH$_2$O. Bring to a total volume of 1 000 mL.

d)   SeaKem® LE agarose gel (1%)   **(100 mL)**

SeaKem® LE agarose gel powder (Lonza, USA)   1 g

1X TBE buffer (pH 8.0) (prepared in 4c)   100 mL

Ethidium bromide (10 µg.mL^{-1}) (Sigma-Aldrich, USA)   5 µL

Weigh the agarose and add to 100 mL of 1X TBE buffer. Let the agarose soak in the 1X TBE for 15 min to prevent excessive foaming. Weigh the beaker and the solution before microwaving. Microwave until the agarose is completely dissolved and allow boiling for 1 min. Add dH$_2$O to re-establish the initial weight of the beaker and solution. Prior to casting the gel, add 5 µL of Ethidium bromide to allow UV-light visualisation.

2.2.3   Buffers required for pulsed-field gel electrophoresis (PFGE):

a) Stock solution of 0.5 M EDTA (pH 8.0) (500 mL)

0.5 M of EDTA   93.05 g
Sodium hydroxide (NaOH) pellets   10 g
Sterile ultrapure water   500 mL

Weigh the components and add to ultrapure water. Bring to a total volume of 500 mL. Autoclave.

b) Stock solution of 1 M Tris (pH 8.0) (500 mL)

Trizma base (Tris)   60.55 g
Concentrated hydrochloric acid (HCl)   21 mL
Ultrapure water   500 mL

Dissolve 60.57 g of Trizma base in 300 mL of ultrapure water. Adjust the pH to 8.0 with HCl. Bring to a total volume of 500 mL. Autoclave.

e) Sodium acetate (NaOAc) (pH 4.5) (10 mL)

20 mM Sodium acetate   164.01 mg
Ultrapure water   10 mL

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Glacial acetic acid

Weigh NaOAc out and dissolve in 6 mL of ultrapure water. Adjust the pH to 4.5 using glacial acetic acid and bring to a total volume of 10 mL. Autoclave.

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

1 M Tris (pH 8.0)	10 mL
0.5 M EDTA (pH 8.0)	2 mL
Sterile ultrapure water	1 000 mL

Measure the components and add to ultrapure water. Bring to a total volume of 1 000 mL. Autoclave.

Use in the plug preparation of Gram-positive bacteria, the washing and storage of all plugs and acts as the casting agarose during plug preparation.

e) Cell suspension buffer (CSB) (100 mM Tris: 100 mM EDTA) (pH 8.0) (500 mL)

1 M Tris (pH 8.0)	50 mL
0.5 M EDTA (pH 8.0)	100 mL
Sterile ultrapure water	500 mL

Measure the components and add to 300 mL ultrapure water. Bring to a total volume of 500 mL. Autoclave.

f) Cell lysis buffer (50 mM Tris: 50 mM EDTA + 1% sarcosine + 0.1 mg/mL Proteinase K) (pH 8.0) (1 000 mL)

1 M Tris (pH 8.0)	50 mL
0.5 M EDTA (pH 8.0)	100 mL
N-lauroylsarcosine sodium salt (sarcosyl)	10 g OR
10% N-lauroylsarcosine sodium salt (sarcosyl)	100 mL
Sterile ultrapure water	1 000 mL
Proteinase K (20 mg/mL) (per 5 mL)	25 µL*

Add 50 mL of Tris and 100 mL of EDTA to sterile ultrapure water. Dissolve 10 g of sarcosyl in the solution and top up to 1 000 mL. Ensure the sarcosyl dissolves by warming it to approximately 50°C for approximately 60 min or leave at room temperature for about 2 hours to completely dissolve the sarcosyl. The solution should not be autoclaved and can be stored for up to a year.

*Add Proteinase K to the cell lysis buffer only when lysing the cells and not prior

g) Stock solution of 5X TBE buffer (pH 8.3) (1 000 mL)

0.45 M Trizma base	54 g
0.44 M Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 mL
Ultrapure water	1 000 mL

Weigh the components and add to ultrapure water. Bring to a total volume of 1 000 mL. Autoclave.

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

5X TBE buffer	136.5 mL
Ultrapure water	2 500 mL

Measure the components and add to 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 gel, 5 mL during the restriction enzyme digest inactivation and 25 mL in the sealing of the plug slices.

i) SeaKem® LE agarose gel (1.2%) (325 mL)

SeaKem® LE agarose gel powder	3.9 g
0.25X TBE buffer (pH 8.0)	325 mL

Weigh the agarose and add to 325 mL of 0.25X TBE buffer. Weigh the beaker and the solution before microwaving. Microwave until the agarose is completely dissolved. Add dH2O to re-establish the initial weight of the beaker and solution. No ethidium bromide is added to the molten agarose for PFGE.

j) Ethidium bromide staining solution (0.25 µg/mL) (1 000 mL)

Ethidium bromide solution (10 mg/mL)	250 µL
Sterile ultrapure water	1 000 mL

Add 250 µL of ethidium bromide 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 5 times.

2.2.4 Enzymes required for pulsed-field gel electrophoresis:

a) Proteinase K (20 mg/mL) (12.5 mL)*

Proteinase K	250 mg
Nuclease-free water	12.5 mL

Add 12.5 mL of nuclease-free water to the re-packaged 250 mg Proteinase K. Allow it to dissolve completely. Divide the stock solution into small aliquots (765 µL) and store at -20°C. Only thaw the number of phials required and discard any unused Proteinase K. Do not allow more than one freeze-thaw cycle after the initial dissolving. Require 45 µL per isolate per PFGE run.

*Rest before aliquoting the solution. Mix by gently inverting the microcentrifuge tube.

b) Lysozyme (20 mg/mL) (5 mL)

Lysozyme	100 mg
TE buffer (pH 8)	5 mL

Dissolve 100 mg of lysozyme 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 (150 µL) and store at -20°C. Only thaw the number of phials required and discard any unused lysozyme. Do not allow more than one freeze-thaw cycle after the initial dissolving. Require 20 µL per isolate per PFGE run.

c) Lysostaphin (1 mg/mL) (5 mL)

Lysostaphin	5 mg
20 mM NaOAc (pH 4.5)	5 mL

Add 5 mL of NaOAc to the pre-packaged 5 mg lysostaphin. Allow it to dissolve completely. Divide the stock solution into small aliquots (85 µL) and store at -20°C. Only thaw the number of phials required and discard any unused lysostaphin. Do not allow more than one freeze-thaw cycle after the initial dissolving. Require 5 µL per isolate per PFGE run.

d) SmaI restriction enzyme digestion per plug slice (50 U/plug slice)

Nuclease-free water	173 µL
10X Tango buffer	22 µL
10 U/µL SmaI	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, followed by 22 μL of 10X Tango buffer (with bovine serum albumin) and 5 μL of the SmaI enzyme, to prevent adherence of the enzyme to the microcentrifuge tube. If more than one plug slice is to be digested, use 200 μL of restriction enzyme master mix per plug slice.

e) XbaI restriction enzyme digestion per plug slice (50 U/plug slice)

Nuclease-free water	175.5 μL
10× NEBuffer	22 μL
20U/μL XbaI	2.5 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 175.5 μL of nuclease-free water, followed by 22 μL of 10X NEBuffer and 2.5 μL of the XbaI enzyme, to prevent adherence of the enzyme to the microcentrifuge tube. If more than one plug slice is to be digested, use 200 μL of restriction enzyme master mix per plug slice.

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Zymo Research Quick-DNA™ Fungal/Bacterial Miniprep Instruction Manual v2.1.0, 2018

Detailed description of experimental procedures

a) Gram-staining protocol (Bartholomew & Mittwer, 1952)

1. Prepare a clean microscope slide and label it with the name of the sample to be stained.

2. Using an inoculating loop or sterile swab, transfer a small amount of the bacterial culture to the slide and spread it out to form a thin, even film. Allow the slide to air dry completely.

3. Heat-fix the bacterial cells by passing the slide through the flame of a Bunsen burner or alcohol lamp a few times. This will help to attach the cells firmly to the slide and denature their proteins.

4. Flood the slide with crystal violet stain (Diagnostic Media Products, NHLS, South Africa) and let it sit for 1 minute. Rinse the slide gently with distilled water to remove any excess stain.

5. Flood the slide with Gram's iodine (Diagnostic Media Products, NHLS, South Africa) and let it sit for 1 minute. This will help to form a complex between the crystal violet and iodine, which will enhance the retention of the stain within the bacterial cells.

6. Decolorize the slide by gently rinsing it with 95% ethanol (Diagnostic Media Products, NHLS, South Africa) for about 10-20 seconds, or until the runoff appears clear. It is important to decolorize the slide properly as over-decolorization can cause Gram-positive cells to appear Gram-negative, and under-decolorization can cause Gram-negative cells to appear Gram-positive.

7. Counterstain the slide with safranin stain for 1 minute, which will stain the decolorized Gram-negative cells pink, and leave the Gram-positive cells purple. Rinse the slide with distilled water to remove any excess stain.

8. Gently blot the slide dry with a paper towel or air dry it.

9. Observe the slide under a microscope (Nikon Eclipse, Tokyo) at 1000x magnification, using immersion oil if necessary. Gram-positive cells will appear purple, while Gram-negative cells will appear pink.

b) DNA extraction protocol according to the Boiling method for multiplex polymerase chain reaction (PCR) assays (Green and Sambrook, 2012)

1. Inoculate a single colony into fresh BHI broth (LabM Limited, UK) and incubate at 37°C for 24 to 48 hours

2. Add 1 000 μL of overnight broth to a sterile 1.5 mL microcentrifuge tube (Scientific Specialities Inc., USA)

3. Centrifuge the microcentrifuge tube (Scientific Specialities Inc., USA) at 5 000 x g for 5 min

4. Discard the supernatant, without disturbing the pellet

5. Resuspend the pellet in 1 000 μL of PBS (GIBCO®, Invitrogen, Nieu-Zealand)

6. Centrifuge the tube at 5 000 x g for 5 min

7. Discard the supernatant, without disturbing the pellet

8. Resuspend the pellet in 50 μL of PBS (GIBCO®, Invitrogen, Nieu-Zealand)

9. Incubate the tube in a heating block at 95°C for 15 min

10. Incubate the tube in a ultrasonic bath for 15 min

11. Centrifuge the tube at max speed (13 300 x g) for 5 min

12. Transfer the supernatant into a new microcentrifuge tube

13. Store at -20°C until required for PCR amplification

c) **DNA extraction protocol according to the Zymo Research *Quick*-DNA™ Fungal/Bacterial Miniprep kit (Modified) for whole genome sequencing (WGS)**

1. Add 50 – 100 mg (wet weight) bacterial cells that have been resuspended in to 200 μl phosphate buffered saline (PBS) [pH 7.2] (GIBCO®, Invitrogen, Nieu-Zealand) to a ZR BashingBead™ Lysis Tube (0.1 mm & 0.5 mm). Add 600 μl BashingBead™ buffer to the tube.

2. Secure in a bead beater (Disruptor Genie™, Zymo Research, USA) fitted with a 2 ml tube holder assembly and process at maximum speed for 20 minutes.

3. Centrifuge the ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) in a microcentrifuge (Spectrafuge 24D, Labnet international Inc., USA) at 10,000 x g for 1 minute.

4. Transfer 400 μl supernatant to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuge (Spectrafuge 24D, Labnet international Inc., USA) at 8,000 x g for 1 minute.

5. Add 500 μL of 0.5% (v/v) β-mercapto-ethanol (Merck, Germany) to the Genomic Lysis Buffer

6. Add 1,200 μl of Genomic Lysis Buffer to the filtrate in the Collection Tube from Step 4.

7. Transfer 800 μl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge (Spectrafuge 24D, Labnet international Inc., USA) at 10,000 x g for 1 minute.

8. Discard the flow through from the Collection Tube and repeat Step 6.

9. Add 200 μl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge (Spectrafuge 24D, Labnet international Inc., USA) at 10,000 x g for 1 minute.

10. Add 500 μl g-DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge (Spectrafuge 24D, Labnet international Inc., USA) at 10,000 x g for 1 minute.

11. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml microcentrifuge tube and add 100 μl (35 μl minimum) DNA Elution Buffer directly to the column matrix. Centrifuge (Spectrafuge 24D, Labnet international Inc., USA) at 10,000 x g for 30 seconds to elute the DNA.

d) Pulsed field gel electrophoresis (PFGE) protocol according to the CHEF-DR II System (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.

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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 EL×800 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 (20mg/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) 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 minutes.

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 ML 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)   The plugs were cut for the appropriate reference size standard and a single plug was out 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), CutsmartM restriction buffer (22 μL per plug slice) and *Smal* 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 L 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), the18-well gel comb (Biometra, Germany) was positioned and the gel was poured and allowed to solidify for 45 min at room temperature ($\pm$ 23°C). Enough molten agarose ($\pm$ 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 0.25 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 B1 below.

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

Table B1: The PFGE controller configuration parameters used for staphylococci and enterococci

Parameter	Staphylococci	Enterococci
Duration	21 hours	25 hours
Temperature	14°C	13°C
Interval (Switch time)	5 s linear to 40 s	3.5 s linear to 23.5 s
Interval inverse	OFF	OFF
Angle	120°C	120°C
Voltage	220 V linear to 200 V or 6 V/cm	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 detained 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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Captured results

Table C1:   **Available demographics of critically ill patients, specimen type and when the specimen was collected**

Isolate	Patient Age	Gender	Hospital	Collection Date	Specimen type
1	58	F	A5	2019/03/29	BC
2	23	M	D1	2016/01/01	BC
4	86	F	A4	2016/06/25	BC
8	58	M	D3	2014/09/21	BC
9	31	M	D11	2014/09/22	BC
11	65	M	D15	2014/11/01	BC
12	61	M	A5	2015/11/23	BC
17	61	M	D3	2015/12/10	BC
21	60	F	D11	2019/03/29	BC
24	83	F	A5	2015/08/05	BC
26	24	M	D2	2015/05/04	BC
27	50	M	D14	2019/03/13	BC
30	9 month	NA*	D10	2017/04/05	BC
31	28	M	D10	2017/06/21	BC
32	55	M	D10	2016/04/25	BC
33	51	F	A4	2016/05/14	BC
34	62	M	A5	2016/06/02	BC
35	74	M	A5	2017/03/16	BC
37	59	M	D6	2017/04/04	BC
38	69	F	D16	2017/06/21	BC
39	59	M	D8	2017/05/07	BC
40	83	F	A2	2016/08/08	BC
42	77	M	D3	2014/09/22	BC
43	NA*	NA*	A5	2015/08/09	BC
44	70	F	D1	2014/12/31	BC
47	84	F	D1	2015/10/30	BC
48	54	M	D4	2015/11/06	BC
50	70	F	D1	2017/05/30	BC
51	1 Year 7 months	NA*	D1	2016/04/04	BC
52	23	M	D11	2016/03/29	BC
53	28	M	A5	2016/08/08	BC
54	35	M	D8	2017/03/15	BC
55	72	F	D5	2016/06/02	BC

*NA – not available

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Table C1: **Available demographics of critically ill patients, specimen type and when the specimen was collected (continued)**

Isolate	Patient Age	Gender	Hospital	Collection Date	Specimen type
56	NA*	NA*	A5	2016/06/08	BC
57	25	F	A5	2015/12/14	BC
58	41	M	C2	2015/11/30	BC
59	92	M	D3	2016/02/06	BC
63	61	M	D17	2016/01/14	BC
64	48	F	D3	2017/05/04	BC
65	62	M	A4	2017/06/18	BC
66	72	M	D15	2014/12/08	BC
67	70	F	D16	2015/08/06	BC
68	NA*	NA*	NA*	NA*	NA*
69	58	M	D3	2017/04/14	BC
70	NA*	NA*	A5	2017/04/12	BC
71	36	M	D11	2016/03/24	BC
72	62	F	A5	2016/03/01	BC
73	46	F	D6	2016/06/29	BC
74	83	F	D5	2016/06/29	BC
75	23	M	D11	2016/03/29	BC
76	77	F	D1	2016/06/16	BC
77	76	M	D11	2016/04/28	BC
80	73	M	A5	2015/12/12	BC
86	77	F	D1	2016/05/26	BC
89	36	M	D3	2015/11/29	BC
91	46	M	A4	2015/08/08	BC
93	38	F	D17	2016/02/05	BC
94	48	F	D7	2016/02/20	CVP
95	3	M	D1	2016/01/11	BC
97	30	F	A5	2019/03/08	BC
98	57	F	E	2018/09/29	BC
99	64	M	A4	2019/01/02	BC
100	NA*	NA*	D7	2018/12/24	BC
103	70	F	D1	2015/11/17	BC
104	77	F	D13	2015/10/30	BC
105	76	M	D3	2015/10/25	BC
107	68	F	B	2019/03/09	BC
108	32	M	A5	2018/12/05	CVP
110	26	M	B	2018/12/20	BC
111	56	M	C1	2018/10/20	CVP

*NA – not available

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Table C1: **Available demographics of critically ill patients, specimen type and when the specimen was collected (continued)**

Isolate	Patient Age	Gender	Hospital	Collection Date	Specimen type
112	46	M	A4	2015/08/08	BC
115	26	F	B	2019/03/09	BC
116	26	F	A5	NA*	BC
117	73	F	A2	2019/03/21	BC
119	43	F	B	2019/05/14	BC
120	34	M	A5	2019/05/08	BC
121	40	M	D1	2019/05/07	BC
122	20	M	D17	2019/05/04	BC
123	61	F	D2	2019/05/03	BC
124	33	M	A4	2019/04/20	BC
125	23	F	D1	2019/07/10	BC
126	1	F	A4	2019/07/12	BC
127	56	M	C2	2019/07/15	BC
128	44	M	B	2019/05/14	BC
129	31	M	A5	2019/06/17	BC
130	58	M	D10	2019/06/22	BC
131	39	M	A5	2019/06/25	BC
132	69	F	A2	2019/05/23	BC
133	69	F	A2	NA*	BC
134	50	M	D11	2019/05/27	BC
135	55	M	A4	2019/01/26	BC
136	71	M	D1	2019/02/16	BC
138	67	M	D13	2019/02/22	BC
139	71	F	D10	2019/02/09	BC
140	83	M	D2	NA*	BC
141	7	M	A3	2019/04/14	BC
142	78	M	D3	2019/04/14	BC
143	75	M	A1	2019/04/15	BC
144	69	M	D9	2019/04/20	BC
145	1 year 7month	NA*	F1	2019/09/01	BC
147	71	F	C1	2019/09/05	BC
150	32	M	D10	NA*	BC
151	79	M	D12	NA*	BC
152	83	M	G	NA*	BC
153	59	M	G	NA*	BC
154	37	F	D2	NA*	BC
155	54	F	D10	NA*	BC

*NA – not available

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Table C1: **Available demographics of critically ill patients, specimen type and when the specimen was collected (continued)**

Isolate	Patient Age	Gender	Hospital	Collection Date	Specimen type
156	56	M	D2	NA*	BC
157	48	M	A5	NA*	BC
158	43	M	D2	NA*	BC
159	74	M	D2	NA*	BC
160	79	M	F2	NA*	BC
161	19	M	D2	NA*	BC
162	75	M	D2	NA*	BC
163	63	M	D2	NA*	BC
164	81	M	D2	NA*	BC
165	72	M	A5	NA*	BC
166	55	M	D2	NA*	BC
167	25	M	D2	NA*	BC

*NA – not available

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Table C2: **Identification and confirmation of *Staphylococcus capitis* using M-PCR assays**

Isolate	Microorganism detected	*sod*A and 16S rRNA genes detected	*cfr* gene detected	*optr*A and *poxt*A genes detected
1	*S. capitis*	Pos	Neg	Neg
2	*S. capitis*	Pos	Neg	Neg
4	*S. capitis*	Pos	Neg	Neg
8	*S. capitis*	Pos	Neg	Neg
9	*S. capitis*	Pos	Neg	Neg
11	*S. capitis*	Pos	Neg	Neg
12	*S. capitis*	Pos	Neg	Neg
17	*S. capitis*	Pos	Neg	Neg
21	*S. capitis*	Pos	Neg	Neg
24	*S. capitis*	Pos	Neg	Neg
26	*S. capitis*	Pos	Neg	Neg
27	*S. capitis*	Pos	Neg	Neg
30	*S. capitis*	Pos	Neg	Neg
31	*S. capitis*	Pos	Neg	Neg
32	*S. capitis*	Pos	Neg	Neg
33	*S. capitis*	Pos	Neg	Neg
34	*S. capitis*	Pos	Neg	Neg
35	*S. capitis*	Pos	Neg	Neg
37	*S. capitis*	Pos	Neg	Neg
38	*S. capitis*	Pos	Neg	Neg
39	*S. capitis*	Pos	Neg	Neg
40	*S. capitis*	Pos	Neg	Neg
42	*S. capitis*	Pos	Neg	Neg
43	*S. capitis*	Pos	Neg	Neg
44	*S. capitis*	Pos	Neg	Neg
47	*S. capitis*	Pos	Neg	Neg
48	*S. capitis*	Pos	Neg	Neg
50	*S. capitis*	Pos	Neg	Neg
51	*S. capitis*	Pos	Neg	Neg
52	*S. capitis*	Pos	Neg	Neg
53	*S. capitis*	Pos	Neg	Neg
54	*S. capitis*	Pos	Neg	Neg
55	*S. capitis*	Pos	Neg	Neg
56	*S. capitis*	Pos	Neg	Neg
57	*S. capitis*	Pos	Neg	Neg
58	*S. capitis*	Pos	Neg	Neg
59	*S. capitis*	Pos	Neg	Neg

Table C2: Identification and confirmation of *Staphylococcus capitis* using M-PCR assays (continued)

Isolate	Microorganism detected	*sod*A and 16S rRNA genes detected	*cfr* gene detected	*optr*A and *poxt*A genes detected
63	*S. capitis*	Pos	Neg	Neg
64	*S. capitis*	Pos	Neg	Neg
65	*S. capitis*	Pos	Neg	Neg
66	*S. capitis*	Pos	Neg	Neg
67	*S. capitis*	Pos	Neg	Neg
68	*S. capitis*	Pos	Neg	Neg
69	*S. capitis*	Pos	Neg	Neg
70	*S. capitis*	Pos	Neg	Neg
71	*S. capitis*	Pos	Neg	Neg
72	*S. capitis*	Pos	Neg	Neg
73	*S. capitis*	Pos	Neg	Neg
74	*S. capitis*	Pos	Neg	Neg
75	*S. capitis*	Pos	Neg	Neg
76	*S. capitis*	Pos	Neg	Neg
77	*S. capitis*	Pos	Neg	Neg
80	*S. capitis*	Pos	Neg	Neg
86	*S. capitis*	Pos	Neg	Neg
89	*S. capitis*	Pos	Neg	Neg
91	*S. capitis*	Pos	Neg	Neg
93	*S. capitis*	Pos	Neg	Neg
94	*S. capitis*	Pos	Neg	Neg
95	*S. capitis*	Pos	Neg	Neg
97	*S. capitis*	Pos	Neg	Neg
98	*S. capitis*	Pos	Neg	Neg
99	*S. capitis*	Pos	Neg	Neg
100	*S. capitis*	Pos	Neg	Neg
103	*S. capitis*	Pos	Neg	Neg
104	*S. capitis*	Pos	Neg	Neg
105	*S. capitis*	Pos	Neg	Neg
107	*S. capitis*	Pos	Neg	Neg
108	*S. capitis*	Pos	Neg	Neg
110	*S. capitis*	Pos	Neg	Neg
111	*S. capitis*	Pos	Neg	Neg
112	*S. capitis*	Pos	Neg	Neg
115	*S. capitis*	Pos	Neg	Neg
116	*S. capitis*	Pos	Neg	Neg

Table C2: Identification and confirmation of *Staphylococcus capitis* using M-PCR assays (continued)

Isolate	Microorganism detected	*sod*A and 16S rRNA genes detected	*cfr* gene detected	*optr*A and *poxt*A genes detected
117	*S. capitis*	Pos	Neg	Neg
119	*S. capitis*	Pos	Neg	Neg
120	*S. capitis*	Pos	Neg	Neg
121	*S. capitis*	Pos	Neg	Neg
122	*S. capitis*	Pos	Neg	Neg
123	*S. capitis*	Pos	Neg	Neg
124	*S. capitis*	Pos	Neg	Neg
125	*S. capitis*	Pos	Neg	Neg
126	*S. capitis*	Pos	Neg	Neg
127	*S. capitis*	Pos	Neg	Neg
128	*S. capitis*	Pos	Neg	Neg
129	*S. capitis*	Pos	Neg	Neg
130	*S. capitis*	Pos	Neg	Neg
131	*S. capitis*	Pos	Neg	Neg
132	*S. capitis*	Pos	Neg	Neg
133	*S. capitis*	Pos	Neg	Neg
134	*S. capitis*	Pos	Neg	Neg
135	*S. capitis*	Pos	Neg	Neg
136	*S. capitis*	Pos	Neg	Neg
138	*S. capitis*	Pos	Neg	Neg
139	*S. capitis*	Pos	Neg	Neg
140	*S. capitis*	Pos	Neg	Neg
141	*S. capitis*	Pos	Neg	Neg
142	*S. capitis*	Pos	Neg	Neg
143	*S. capitis*	Pos	*Pos*	Neg
144	*S. capitis*	Pos	Neg	Neg
145	*S. capitis*	Pos	Neg	Neg
147	*S. capitis*	Pos	Neg	Neg
150	*S. capitis*	Pos	Neg	Neg
151	*S. capitis*	Pos	Neg	Neg
152	*S. capitis*	Pos	Neg	Neg
153	*S. capitis*	Pos	Neg	Neg
154	*S. capitis*	Pos	Neg	Neg
155	*S. capitis*	Pos	Neg	Neg
156	*S. capitis*	Pos	Neg	Neg
157	*S. capitis*	Pos	Neg	Neg

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Table C2: **Identification and confirmation of *Staphylococcus capitis* using M-PCR assays (continued)**

Isolate	Microorganism detected	*sod*A and 16S rRNA genes detected	*cfr* gene detected	*optr*A and *poxt*A genes detected
158	*S. capitis*	Pos	Neg	Neg
159	*S. capitis*	Pos	Neg	Neg
160	*S. capitis*	Pos	Neg	Neg
161	*S. capitis*	Pos	Neg	Neg
162	*S. capitis*	Pos	Neg	Neg
163	*S. capitis*	Pos	Neg	Neg
164	*S. capitis*	Pos	Neg	Neg
165	*S. capitis*	Pos	Neg	Neg
166	*S. capitis*	Pos	Neg	Neg
167	*S. capitis*	Pos	Neg	Neg

Table C3: Antimicrobial susceptibility testing (AST) and the linezolid E-test MIC (µg/mL) represented in a gradient table. Dark grey indicates resistance. Light grey indicates susceptibility. White indicates no results.

Isolate	LZR	AMOX/CLAV	CLX	GEN	CLI	ERY	CMZ	FUS	RIF	TCP	VAN	DAPT	MDR
1	32												
2	32												
4	16												
8	64												
9	256												
11	128												
12	32												
17	16												
21	32												
24	16												
26	96												
27	16												
30	16												
31	16												
32	32												
33	32												
34	16												
35	32												
37	8												
38	32												
39	16												
40	64												
42	256												
43	32												
44	64												
47	34												
50	32												
51	32												
52	16												
53	128												
54	32												
55	16												
56	16												

*LZR, Linezolid; AMOX/CLAV, Amoxicillin/Clavulanate; CLX, Cloxacillin; GEN, Gentamicin; CLI, Clindamycin; ERY, Erythromycin; CMZ, Cotrimoxazole; FUS, Fusidic acid; RIF, Rifampicin; TCP, Teicoplanin; VAN, Vancomycin; DAPT, Daptomycin; MDR, Multidrug-resistant

Table C3: Antimicrobial susceptibility testing (AST) and the linezolid E-test MIC (µg/mL) represented in a gradient table. Dark grey indicates resistance. Light grey indicates susceptibility. White indicates no results (continued)

Isolate	LZR	AMOX/CLAV	CLX	GEN	CLI	ERY	CMZ	FUS	RIF	TCP	VAN	DAPT	MDR
57	32												
58	64												
59	32												
63	32												
64	16												
65	32												
66	>256												
67	32												
68	16												
69	>128												
70	16												
71	64												
72	64												
73	16												
74	16												
75	64												
76	32												
77	16												
80	128												
86	64												
89	16												
91	32												
93	32												
94	16												
95	64												
97	64												
98	64												
99	16												
100	16												
103	>256												
104	32												
105	8												
107	32												

*LZR, Linezolid; AMOX/CLAV, Amoxicillin/Clavulanate; CLX, Cloxacillin; GEN, Gentamicin; CLI, Clindamycin; ERY, Erythromycin; CMZ, Cotrimoxazole; FUS, Fusidic acid; RIF, Rifampicin; TCP, Teicoplanin; VAN, Vancomycin; DAPT, Daptomycin; MDR, Multidrug-resistant

**Table C3:** Antimicrobial susceptibility testing (AST) and the linezolid E-test MIC (µg/mL) represented in a gradient table. Dark grey indicates resistance. Light grey indicates susceptibility. White indicates no results (continued)

Isolate	LZR	AMOX/CLAV	CLX	GEN	CLI	ERY	CMZ	FUS	RIF	TCP	VAN	DAPT	MDR
108	32												
110	64												
111	32												
112	64												
115	32												
116	8												
117	32												
119	16												
120	64												
121	16												
122	8												
123	32												
124	32												
125	8												
126	24												
127	32												
128	32												
129	16												
130	32												
131	32												
135	16												
136	16												
138	8												
139	16												
141	16												
142	16												
143	8												
144	8												
145	32												
147	16												
150	64												

*LZR, Linezolid; AMOX/CLAV, Amoxicillin/Clavulanate; CLX, Cloxacillin; GEN, Gentamicin; CLI, Clindamycin; ERY, Erythromycin; CMZ, Cotrimoxazole; FUS, Fusidic acid; RIF, Rifampicin; TCP, Teicoplanin; VAN, Vancomycin; DAPT, Daptomycin; MDR, Multidrug-resistant

Table C3: **Antimicrobial susceptibility testing (AST) and the linezolid E-test MIC (μg/mL) represented in a gradient table. Dark grey indicates resistance. Light grey indicates susceptibility. White indicates no results (continued)**

Isolate	LZR	AMOX/CLAV	CLX	GEN	CLI	ERY	CMZ	FUS	RIF	TCP	VAN	DAPT	MDR
151	8												
153	8												
154	32												
156	32												
157	16												
158	32												
161	32												
163	64												
164	16												
167	32												

*LZR, Linezolid; AMOX/CLAV, Amoxicillin/Clavulanate; CLX, Cloxacillin; GEN, Gentamicin; CLI, Clindamycin; ERY, Erythromycin; CMZ, Cotrimoxazole; FUS, Fusidic acid; RIF, Rifampicin; TCP, Teicoplanin; VAN, Vancomycin; DAPT, Daptomycin; MDR, Multidrug-resistant

Supplementary Figures
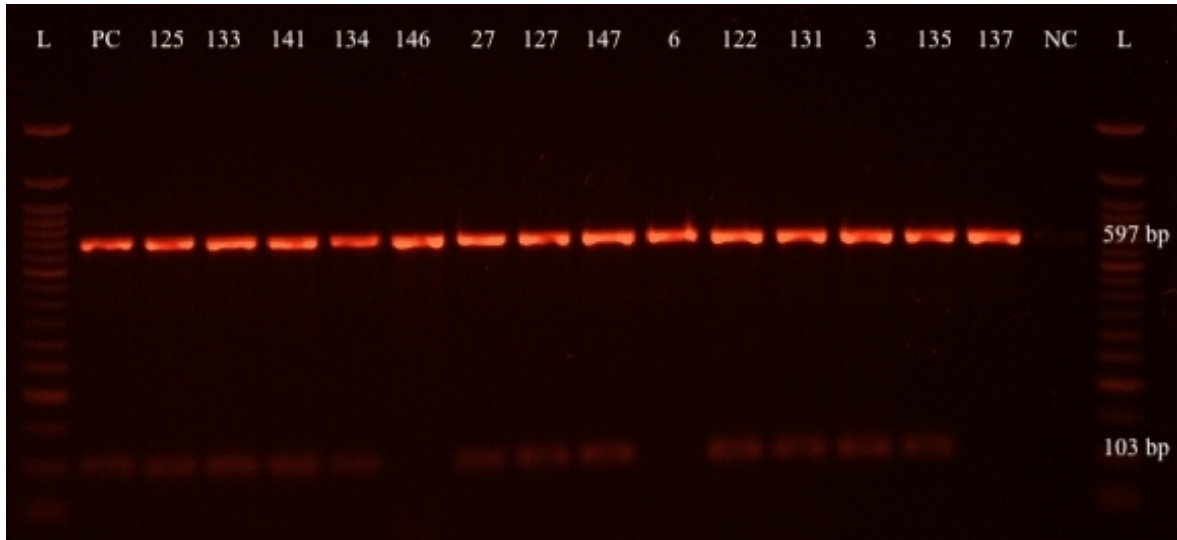
FIGURE S1: Multiplex PCR assay for the identification of *Staphylococcus capitis* isolates. PC = ATCC 35661 *S. capitis* positive control; NC = negative control
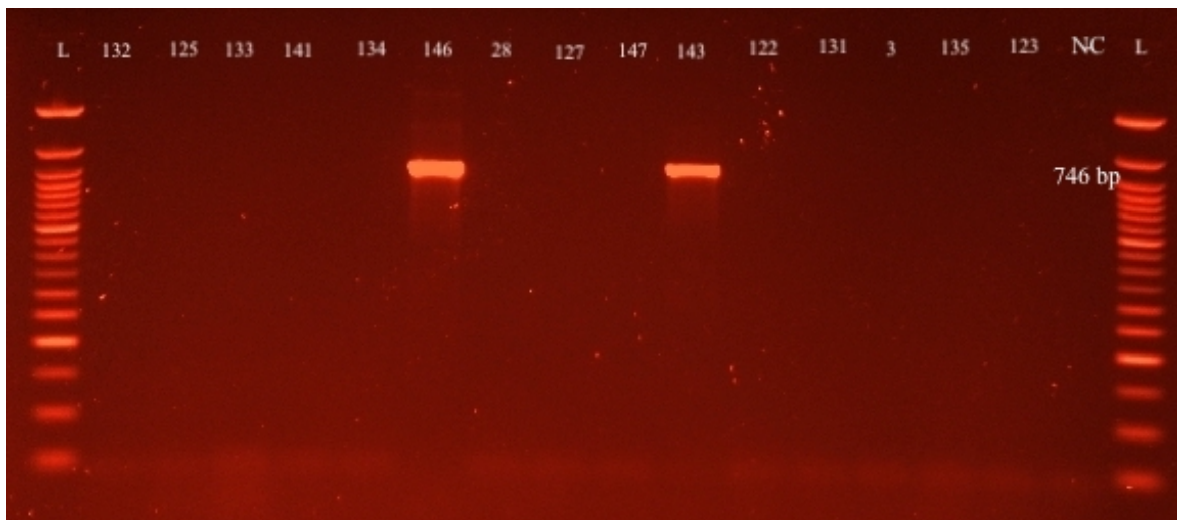
FIGURE S2: Multiplex PCR assay for the detection of the *cfr* (746 bp), *optrA* and *poxtA* genes in *Staphylococcus capitis* isolates. NC = negative control

Journal Guidelines

CLINICAL MICROBIOLOGY AND INFECTION

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AUTHOR INFORMATION PACK

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DESCRIPTION

Clinical Microbiology and Infection (CMI) is a monthly publication in English of the European Society of Clinical Microbiology and Infectious Diseases and publishes peer-reviewed papers that present basic and applied research relevant to therapy and diagnostics in the fields of microbiology, infectious diseases, virology, parasitology, immunology and epidemiology as related to these fields.

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https://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(17)30636-5/fulltext

For our full list of policies, please see the Website Collection 'Editorial Policies':
https://www.clinicalmicrobiologyandinfection.com/content/collection-editorial-policies

Article types
Original Article
We accept the submission of Original Articles in any format. However, we encourage authors to provide a structured abstract (see below) and a text shorter than 2500 words which will be of help to the editors in the decision process before sending the article for peer-review. When accepted, we will ask the article to be formatted according to the CMI Guide for Authors (please see below).

We allow the publishing of preprints before submission.

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The title of Original Articles should be descriptive rather than declarative. The first part of the title should describe the question that was addressed (e.g. Incidence of severe pain after herpes zoster; or Antibiotic A versus antibiotic B for diverticulitis; or In vitro synergy of antibiotic A and antibiotic B against *Escherichia coli*); and the second part the design of the study (e.g., prospective cohort study; or randomized controlled trial; or using the checkerboard technique).

A structured abstract of 250 words maximum should be divided into the following sections: Objectives; Methods; Results; Conclusions. The Abstract should be as informative as possible: e.g. it should include the absolute number of included patients or of strains tested; denominators and numerators rather than just rates or percentages; actual, numerical data and not only *p* values or odds-ratios or relative risks.

Original Articles should not exceed 2500 words. Original Articles should contain a maximum of 4–5 tables and figures (more can be included as supplementary material. Please refer to the section below for further guidelines). Data should be provided in Tables and Figures and not repeated in the text. Text should be used to emphasize important points. The text should be divided into the following sections: Introduction; Methods; Results; Discussion.

We urge authors to structure their Discussion according to the recommendations of Docherty and Smith: BMJ 1999;318:1224-5; namely: summary of the principal findings; findings of the present study in light of what was published before; strengths and limitations of the study; meaning of the study; understanding possible mechanism; implications for practice or policy; implications for future research.

The maximum number of references is 30.

Articles will be published online only or in the print issue as well at the discretion of the Editor. Like all articles published in CMI, online-only Original Articles will be fully citable and indexed. Each Original Article will have the abstract included in the accompanying print issue and will be referenced in the table of contents.

If you consider submitting the results of a survey as an Original Article please read our guidance for authors of surveys http://dx.doi.org/10.1016/j.cmi.2016.08.015

Research Note
Research Notes are restricted to a maximum of 1200 words; 1–2 tables or figures; and 15 references. They should contain a structured abstract and structured text as described above. All Research Notes will be published online only, and their abstracts included in the accompanying print issue and referenced in the table of contents.

Systematic Review
Systematic Reviews address a clear question and use pre-defined methods to identify and include studies, appraise their methodological rigour, and extract data. Meta-analysis is optional. More details on our expectations on Systematic Reviews are given in:http://dx.doi.org/10.1016/j.cmi.2016.04.012; DOI:10.1016/j.cmi.2020.11.006 and DOI: 10.1016/j.cmi.2018.05.022.

Systematic Reviews of interventions should contain a structured abstract of maximum 300 words with the following sections: Background; Objectives; Methods: Data sources, Study eligibility criteria, Participants, Interventions, Assessment of risk of bias, Methods of data synthesis; Results; Conclusions.

Systematic Reviews of diagnostic tests should contain a structured abstract of maximum 300 words with the following sections: Background; Objectives; Methods: Data sources, Study eligibility criteria, Participants, Test/s, Reference standard, Assessment of risk of bias, Methods of data synthesis; Results; Discussion.

Systematic Reviews are limited to 3500 words.

Narrative Review

We are happy to publish reviews that are helpful to our readers on relevant topics not recently reviewed. These should be short (2500 words or fewer and maximum 60 references), evidence-based, their search strategy explicit, and the implications of their results for patient management, health policy and future research thoughtfully discussed. We ask for a structured abstract with the following sections: Background; Objectives; Sources; Content; Implications (maximum 300 words in length).

CMI publishes "How to" reviews with an in-depth explanation on how to do things that are of interest to our readers. These topics of interest might be new laboratory techniques (in the clinical laboratory or in the research one); programs (e.g. how to establish an antibiotic stewardship effort in a hospital in which there is none); or analysis (e.g. how to use interrupted time-series analysis) (please see http://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(17)30290-2/fulltext). The structure of the "How to" review is similar to the Narrative Review, including the Abstract.

Theme Issues

Theme Issues consist of 3-5 Narrative Reviews addressing the different aspects of one topic. To serve as a Guest Editor of a theme issue please contact the CMI Editor-in-Chief, Leonard Leibovic: leibovic@post.tau.ac.il with an outline of the topic and of each of the reviews, including names of proposed authors for the reviews. The format of the reviews is that of Narrative Reviews (please see above). The reviews are accompanied by an Editorial written by the editor of the Theme Issue. For the Editorial's format, please see the instructions for Commentary.

Commentary

We are looking for personal viewpoints; opinions; novel or exciting ideas, criticism (or praise) on topics relevant to our readers. A Commentary should be a short contribution: 1400 words or less, maximum 10 references.

Commentaries are published without an Abstract. However, for the purpose of informing our peer-reviewers please upload 4–5 sentences describing the content of your Commentary in the box dedicated to Abstracts. These lines will not be published.

Guidelines

We publish the guidelines of the European Society of Clinical Microbiology and Infection (ESCMID). These guidelines are peer reviewed. In special cases we will consider the publication of other guidelines and position papers, if they fit the purpose of the journal and the aims of ESCMID. Our guide for reporting guidelines and position papers can be found here: https://www.elsevier.com/__data/promis_misc/CMI_Template reporting guidelines consensus position papers.docx. Please also see our Editorial on conflict of interest in guidelines: http://www.clinicalmicrobiologyandinfection.com/article/S1198-743X(15)00864-2/fulltext.

In addition to GRADE we require a separate risk of bias appraisal of the individual studies and explanation of the GRADE classifications based on the GRADE items.

Please also see the ESCMID EOPs for guidelines:

https://www.escmid.org/membership_organization/about_escmid/operating_procedures/eops_for_medical_guidelines/

Guidelines will be appraised using the AGREE-II tool.

Before submitting non-ESCMID guidelines, and preferably before writing them, please consult the Editor in charge of guidelines at the CMI, Professor Mical Paul: paulm@technion.ac.il.

Picture of a Microorganism

High-quality images of pathogens should be of special interest, have an informative title and be accompanied by no more than 250 words and 1 reference.

Letters

Letters will be considered for publication if they contain constructive criticism on articles published within the previous 3 months, the authors of which will be given the right of reply. Letters must specify the title and authors of the article they are writing about.

Items of topical interest, including case reports presenting a significant advance in therapy or highlighting substantial scientific advances in understanding the mechanism(s) of the disease process, will also be considered under this heading. Letters should not exceed 800 words; have no more than 5 references and only 1 figure or table.

Letters should begin 'To the Editor'. Letters are published without an Abstract. However, for the purpose of informing our peer-reviewers please upload 4–5 sentences describing the content of your Letter in the box dedicated to Abstracts. These lines will not be published. Letters should have no more than 6 authors.

BEFORE YOU BEGIN

Ethics in publishing
Please see our information on Ethics in publishing.

Human and animal rights
If the work involves the use of human participants, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans, https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-invol Uniform Requirements for manuscripts submitted to Biomedical journals, http://www.icmje.org/recommendations/browse/roles-and-responsibilities/protection-of-research-participant Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human participants. The privacy rights of human participants must always be observed.

In some countries observational studies do not require patients? consent; and in some, research done in anonymized databases does not require the approval of an ethics committee. If this is the case with your research, please say so in the Methods section. Please see specific points on ethical considerations in research published in the CMI: http://dx.doi.org/10.1016/j.cmi.2016.08.006.

Experimental studies in animals
CMI has formulated its policy and the type of studies that are within its scope: see here.

The EU Directive 2010/63/EU should be complied with. CMI encourages authors to submit their preclinical experimental animal work applying the standards on reporting, and to upload a completed ARRIVE checklist with their manuscript. CMI invites authors to submit with the checklist a 3R supplementary document, justifying the use of animals for their study and how the 3Rs were addressed.

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Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify compliance, your article may be checked by Crossref Similarity Check and other originality or duplicate checking software.

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Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of ethnicity, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

Reporting sex- and gender-based analyses
Reporting guidance

For research involving or pertaining to humans, animals or eukaryotic cells, investigators should integrate sex and gender-based analyses (SGBA) into their research design according to funder/sponsor requirements and best practices within a field. Authors should address the sex and/or gender dimensions of their research in their article. In cases where they cannot, they should discuss this as a limitation to their research's generalizability. Importantly, authors should explicitly state what definitions of sex and/or gender they are applying to enhance the precision, rigor and reproducibility of their research and to avoid ambiguity or conflation of terms and the constructs to which they refer (see Definitions section below). Authors can refer to the Sex and Gender Equity in Research (SAGER) guidelines and the SAGER guidelines checklist. These offer systematic approaches to the use and editorial review of sex and gender information in study design, data analysis, outcome reporting and research interpretation - however, please note there is no single, universally agreed-upon set of guidelines for defining sex and gender.

Definitions
Sex generally refers to a set of biological attributes that are associated with physical and physiological features (e.g., chromosomal genotype, hormonal levels, internal and external anatomy). A binary sex categorization (male/female) is usually designated at birth ("sex assigned at birth"), most often based solely on the visible external anatomy of a newborn. Gender generally refers to socially constructed roles, behaviors, and identities of women, men and gender-diverse people that occur in a historical and cultural context and may vary across societies and over time. Gender influences how people view themselves and each other, how they behave and interact and how power is distributed in society. Sex and gender are often incorrectly portrayed as binary (female/male or woman/man) and unchanging whereas these constructs actually exist along a spectrum and include additional sex categorizations and gender identities such as people who are intersex/have differences of sex development (DSD) or identify as non-binary. Moreover, the terms "sex" and "gender" can be ambiguous—thus it is important for authors to define the manner in which they are used. In addition to this definition guidance and the SAGER guidelines, the resources on this page offer further insight around sex and gender in research studies.

Authorship
All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

At the end of the text before the references please include a paragraph detailing the authors' contributions. Please use the CRediT taxonomy (Brand A, Allen L, Altman M, Hlava M, Scott J. Beyond authorship: Attribution, contribution, collaboration, and credit. Learn Publ. 205;28:151–5). Terms can be added according to context.

Changes to authorship
Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.
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Questions concerning the editorial process or an editorial decision should be addressed to Ms. Julia Friedman, manager of CMI Editorial Office (jlubom2@gmail.comleibovic).

Appeals

As can be expected we are not exempt from mistakes. You can address appeals on editorial decisions to the Editor-in Chief, Leonard Leibovici: leibovic@post.tau.ac.il. Good grounds for appeal are gross misunderstandings of methods or analysis by the Editor or peer-reviewers. If your manuscript was rejected because we could not assign it a high enough priority for publication, the chances of a successful appeal are low.

PREPARATION

Manuscript requirements

Text
Manuscripts must be submitted in English, using British spelling. Authors unsure of English usage should consult someone proficient in the English language.

Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's Webshop or visit our customer support site for more information.

Nonconformity to accepted standards of English usage or journal style may delay both the review and the editorial process. Brevity is an advantage as well as a requirement (see length restrictions under Manuscript Categories). In particular, text should not be repeated in more than one section; text and tables/figures should not be redundant. Consistency is important; contributions from different authors and text from different sources must be rewritten to create a coherent and homogenous text.

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All manuscripts must be double-spaced, with wide margins, and should have continuous line numbers throughout.

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All submissions in all manuscript categories must include a title page indicating the intended category, the title, the full names of the authors, the full names and institutional affiliations of each author, and a complete postal address, email address, and international telephone and fax numbers for the single Corresponding Author (telephone and fax numbers will not be published).

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Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site.

Illustrations and figures

All colour illustrations and figures are published online, free of charge to the author.

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General points
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• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.

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• Ensure that color images are accessible to all, including those with impaired color vision.

A detailed guide on electronic artwork is available.
You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
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Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
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Please do not:
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Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

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Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

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Example:
Liu J, Chen Y, Li R, Wu Z, Xu Q, Li Z, et al. Intravenous immunoglobulin treatment for patients with severe COVID-19: a retrospective multi-center study. Clin Microbiol Infect. 2021; 27:1488–93. DOI:https://doi.org/10.1016/j.cmi.2021.07.012

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List: Number the references in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:
[1] Gagyor I, Hummers E, Schmiemann G, Friede T, Pfieffer S, Afshar K, et al. Herbal treatment with Uva Ursi extract versus fosfomycin in women with uncomplicated urinary tract infection in primary care: a randomized controlled trial. Clin Microbiol Infect. 2021;27:1441–7. DOI:https://doi.org/10.1016/j.cmi.2021.05.032

Reference to a book:
[2] Ewert EW, Mitten DS, Overholt JR. Natural environments and human health. Wallingford, UK: CAB International; 2014. DOI:https://doi.org/10.1079/9781845939199.0000

Reference to a chapter in an edited book:
[3] Valls-Ferrer M, Mora JC. L2 fluency development in formal instruction and study abroad: The role of initial fluency level and language contact. In Perez-Vidal C., editor. Language acquisition in study abroad and formal instructional contexts. Amsterdam: John Benjamins; 2014, p. 111–36. DOI:https://doi.org/10.1075/aals.13.07ch5

Data reference:
[4] Oguro M, Imahiro S, Saito S, Nakashizuka T. Mortality data for Japanese oak wilt disease and surrounding forest compositions. Mendeley Data, v1; 2015. DOI:http://dx.doi.org/10.17632/xwj98nb39r.1

Note shortened form for last page number, e.g. 51–9, and that for more than 6 authors the first 6 should be listed followed by 'et al.'

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Please submit the material together with the article and supply a concise and descriptive caption for each file. If you wish to make any changes to supplementary data during any stage of the process, then please make sure to provide an updated file, and do not annotate any corrections on a previous version. Please also make sure to switch off the 'Track Changes' option in any Microsoft Office files as these will appear in the published supplementary file(s). For more detailed instructions please visit our artwork instruction pages at https://www.elsevier.com/artworkinstructions.

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If you wish to include a list of collaborators with your paper, you will need to provide a full list showing all of the detail that you would like to appear in your paper. We will also need to know if you wish the collaborators to be tagged for PubMed purposes or not.

If they do need to be tagged for PubMed then online they will appear as a secondary list within the main author list at the end, which can be expanded or hidden to just show the name of the collaborative group. In print the name of the collaborative group will appear at the end of the main author list with an asterisk that links to an appendix at the end of your paper or it can link to a footnote on the first page, which lists all of the names, if you would prefer.

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AFTER ACCEPTANCE

Availability of accepted article

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ANNEXURE F

Turnitin Report

BJ Mynhardt -Dissertation

ORIGINALITY REPORT

5%	5%	3%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	repository.up.ac.za Internet Source	2%
2	doctiktak.com Internet Source	1%
3	"Posters", Clinical Microbiology and Infection, 2009 Publication	1%
4	idoc.pub Internet Source	1%
5	Leta Guta, Seid Mohammed, Malaku Sombo. "Identification and Molecular Characterization of Antibiotics Resistance Staphylococcus species from of the Nasal Cavity of Goats in Adama, Ethiopia", Research Square Platform LLC, 2022 Publication	1%
6	upetd.up.ac.za Internet Source	1%

ANNEXURE G

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Faculty of Health Sciences **Research Ethics Committee**

13 April 2023

Approval Certificate
Annual Renewal

Dear Mr BJ Mynhardt,

Ethics Reference No.: 115/2019 – Line 5
Title: GENOTYPIC PROFILES OF EMERGING MULTIDRUG RESISTANT STAPHYLOCOCCUS CAPITIS ISOLATES FROM AN ONGOING OUTBREAK IN CRITICALLY ILL PATIENTS.

The **Annual Renewal** as supported by documents received between 2023-03-28 and 2023-04-12 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-04-12 as resolved by its quorate meeting.

Please note the following about your ethics approval:
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2024-04-13.
- Please remember to use your protocol number (115/2019) 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

_____
On behalf of the FHS REC, 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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