MOLECULAR SURVEILLANCE OF *STAPHYLOCOCCUS AUREUS* ON FREQUENTLY TOUCHED SITES IN PUBLIC HOSPITALS IN KWAZULU-NATAL, SOUTH AFRICA

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This is a dissertation submitted to the School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, Westville, for the degree of Master of Medical Science in Medical Microbiology.

This is a dissertation by manuscript with an overall introduction and final summary.
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DECLARATION

I, Miss Siyethaba Mkhize, declare as follows:

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

2. That my contribution to the project was as follows:
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Signed:  Date: ____12/07/2021_____

Siyethaba Mkhize

Student number: 213509331

DEDICATION

This work is dedicated to my mother, Nomathamsanqa Virginia Cele, Ngiyabonga Mama wami. My father, Sibusiso Cele, Ndosi, Magaye.

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Any omissions and shortcomings that may be identified in this piece of work remains the sole responsibility of the researcher

S. Mkhize

Durban

2020

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LIST OF ACRONYMS AND ABBREVIATIONS

AMR	Antimicrobial-resistant
AST	Antimicrobial susceptibility testing
BRU	Biomedical Resource Unit
CA-MRSA	Community-acquired methicillin-resistant *S. aureus*
CHIPS	Chemotaxis inhibitory protein of *S. aureus*
CoNS	Coagulase-negative staphylococci
ClfA and ClfA	Fibrinogen- binding protein
CLSI	Clinical and Laboratory Standards Institute
CnA	Collagen-binding protein
DNA	Deoxyribonucleic acid
EPIC II	Extended prevalence of infection in intensive care
ERIC-PCR	Enterobacterial repetitive intergenic consensus PCR
EU	European Union
Eap	Extracellular adherence protein
FLIPr	Formyl peptide receptor-like 1 inhibitory protein
FnbpA and FnbpB	Fibronectin- binding protein
GLASS	Global antimicrobial resistance surveillance system
HA-MRSA	Hospital-acquired methicillin-resistant *S. aureus*
HAI	Hospital-acquired infection
HTSs	High touch surfaces
ICU	Intensive care unit
IDSA	Infectious Diseases Society of America

IgG	Immunoglobulin
IPC	Infection, prevention, and control
IV	Intravenous
KZN	KwaZulu-Natal
LA-MRSA	Livestock-acquired MRSA
MALDI-TOF MS	Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
MDR	Multi-drug resistant
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol salt agar
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NaCl	Sodium chloride
NHS	National Health Service
NRF	National Research Fund
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PPL	Priority pathogen list
PVL	Panton-Valentine leucocidin
REP-PCR	Repetitive extragenic palindromic PCR
S. aureus	*Staphylococcus aureus*
SAB	*S. aureus* bacteraemia
SaPIs	*S. aureus* pathogenicity islands

SCC	Staphylococcal chromosomal cassettes
SCIN	Staphylococcal complement inhibition
SCT	Slide coagulase test
SpA	Staphylococcal binding protein
Tn	Transposons
TCT	Tube coagulase test
TSST-1	Toxic shock syndrome toxin
UK	United Kingdom
USA	United States of America

ABSTRACT

There is an escalation in the prevalence of hospital-acquired infections (HAI) due to the transfer of pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), within the hospital environment. Pathogens contaminate inanimate objects and are transmitted in the hospital environment through direct hand contact, of which healthcare workers and patients act as vectors. This study aimed to conduct surveillance of methicillin-resistant *S. aureus* (MRSA) on frequently touched hospital environment sites of selected public hospitals from different healthcare levels in KwaZulu-Natal, South Africa. Eleven predetermined frequently touched sites in the general, paediatric and ICU wards were swabbed viz. occupied and unoccupied beds, ward telephones, drip stands, nurses' tables, door handles of laundry rooms, mops, sinks, ventilators, blood pressure machines and patient files. The swabs were plated on selective chromogenic media for *Staphylococcus aureus* (*S. aureus*) isolation and phenotypic identification. Total genomic DNA was extracted using the conventional boiling method. The presence of the *S. aureus* thermo-nuclease *nuc* gene was confirmed using the polymerase chain reaction (PCR). Antibiotic susceptibility tests were conducted by performing the Kirby-Bauer disk diffusion assays, according to CLSI guidelines, to determine the isolates' resistance profiles to nine antibiotics. The *mecA* gene, an MRSA indicator, and genes encoding resistance and virulence were identified through PCR. Genomic DNA was extracted using the Quick-DNA^{TM} Miniprep Plus kit, and ERIC-PCR was conducted to determine the clonality of the isolates. Pearson's correlation, Fisher's exact test and Chi-Square test were implemented using the SPSS software version 25 (IBM SPSS Statistics) for statistical analyses. The statistical significance determined by a probability value that was less than 0.05 ($p < 0.05$). An overall prevalence of 12.7 % (99/777) for *S. aureus* isolates was obtained. Of these, 89.9 % (89/99) were MRSA, and only 10.1 % (10/99) of the total collected isolates were identified as methicillin-susceptible *S. aureus* (MSSA). The sites with the highest prevalence were the occupied beds (16.2 % (16/99)), unoccupied beds (16.2 % (16/99)), patient files (14.1 % (14/99)), ward telephones (13.1 % (13/99)) and nurses' tables (14.1 % (14/99)). The sites with the lowest prevalence were the blood pressure machines (6.1 % (6/99)), drip stands (6.1 % (6/99)), ventilator (6.1 % (6/99)), door handle (4 % (4/99)), mop (3.0 % (3/99)) and sink (1.0 % (1/99)). The Pearson's Chi-square and Fischer's exact test indicated a significant relationship ($p < 0.05$) between the *mecA* gene and the collection site. A significant relationship ($p < 0.05$) was identified between the hospital and the *tetK*, *ermC*, *aac (6')-aph (2")* and *LukS/F-PV* genes. ERIC-PCR produced bands for 87.8 % (87/99) of the isolates; 12.1 % (12/99) were non-

typeable. Our findings have highlighted the *S. aureus* contamination on frequently touched hospital sites, virulence and resistance, and the clonal diversity of *S. aureus* isolates in the hospital environment of four KwaZulu Natal public hospitals in the eThekwini district. Our findings may be used as a baseline for future surveillance initiatives to improve hospital hygiene through IPC strategies centred around *S. aureus* in KwaZulu-Natal public hospitals

Keywords: hospital environment; IPC; contamination; *S. aureus*; MRSA; inanimate objects

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The hospital environment is often neglected as a contributing factor to hospital-acquired infections. However, the cleanliness of a hospital environment is a critical contributor to the proliferation of pathogenic organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), that cause hospital-acquired infections (Mitchell *et al.*, 2014). *Staphylococcus aureus* (*S. aureus*) was the most isolated bacteria in a study conducted by Ramsamy *et al.* (2018). During a five-year study (2011-2015), the mentioned research group evaluated the antibiotic resistance trends of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (ESKAPE pathogens) from public sector hospitals in KwaZulu-Natal, South Africa (Ramsamy *et al.*, 2018). *S. aureus* is frequently found colonizing the anterior nares of the nasal passages and skin of humans and animals (domestic animals, livestock and wild animals) (Khan *et al.*, 2015). The Infectious Diseases Society of America (IDSA) defined ESKAPE pathogens as being difficult to eradicate using standard antibiotics due to most being multi-drug resistant (MDR) (Ramsamy *et al.*, 2018). Vancomycin-resistant, intermediate and methicillin-resistant *S. aureus* is classified as a high priority under the International Priority Pathogen list (PPL) alongside *E. faecium* (Ramsamy *et al.*, 2018).

There is a scarcity of studies reporting on the rate of hospital-acquired infections (HAIs) in South Africa, mainly due to underdeveloped surveillance systems (Mahomed *et al.*, 2017). The initial reports on MRSA in South Africa were in the 1980s. Shittu and Lin (2006) reported that the prevalence of hospital-acquired MRSA in KwaZulu-Natal (KZN) was 26.9 % (61/227); however, there is still currently insufficient data on hospital-acquired MRSA (HA-MRSA) isolates from around KZN. In addition, the mechanisms of HA-MRSA emergence and distribution are not well understood. MRSA prevalence studies conducted in South Africa, KwaZulu-Natal, to be specific, have mainly focused on clinical isolates (Shittu and Lin, 2006; Amoako *et al.*, 2016) and livestock-associated *S. aureus* (Dweba & Zishiri, 2019). The sanitation of a hospital environment is one of the fundamental requirements to minimise transmission of hospital-acquired infections (HAIs) as hospital environments act as reservoifor

potential pathogens (Mitchell *et al.*, 2014). Direct contact is the primary mechanism for transmitting pathogenic organisms in the hospital setting (Squeri *et al.*, 2012). Therefore, a crucial practice of HAI management is proper disinfection of the hospital environment, hospital equipment, hospital visitors and healthcare workers aids in minimising the spread of HAI (Revelas, 2012).

1.2 Literature Review

This literature review provides knowledge relevant to the contamination of the hospital environment, hospital-acquired infections (HAIs), pathogenicity of *S. aureus* in humans, virulence, drug resistance and transmission of *S. aureus*. This review also contains knowledge of the South African public healthcare sector and how *S. aureus* prevalence may be a health risk.

1.3 Hospital environment

The environment used for hospital care consists of three features: the building or space used for healthcare; the equipment used; and the people (healthcare workers, patients and visitors) who use the space and equipment (Suleyman *et al.*, 2018). The patient environment is the proximity that surrounds an admitted patient and is utilized by both patients and healthcare workers (Suleyman *et al.*, 2018). High touch surfaces (HTS) are defined by Huslage *et al.* (2014) as the surfaces that a patient has direct contact with, such as the hospital bedrails, cart or trolley for medical supplies, the patient table, and the intravenous (IV) pump. Suleyman *et al.* (2018) further elaborated on this list to include the doorknobs, light switches, surfaces in patient toilets, and the edges of curtains that separate patients. Hospital environmental surfaces were identified as playing a crucial role in transmitting pathogenic bacteria that can cause HAIs (Otter *et al.*, 2013; Huslage *et al.*, 2014). The contamination of the hospital environment depends on the texture of the surfaces (rough, smooth, or porous) and whether they are wet or dry, new or old (Chaoui *et al.*, 2019). The transmissibility of pathogenic bacteria in hospitals is suspected to occur via healthcare workers interacting with a contaminated hospital environment and infected patients or carriers (Otter *et al.*, 2013).

Hospitals spend large amounts of resources to ensure that infections within hospitals are contained and prevented from occurring between patients, the environment and hospital healthcare workers (Price *et al.*, 2017). However, even though infection control and prevention (IPC) protocols exist in hospitals- the transmission of pathogens still occurs (Price *et al.*, 2017). Pathogens such as *S. aureus* have low infection dosages, and there is a high risk of *S. aureus* being present on HTSs as it is commonly found on the skin of infected patients and carriers (Dancer, 2008; Otter *et al.*, 2014). As a result, any pathogen on the surface of inanimate objects in the hospital environment is a transmission risk (Otter *et al.*, 2013). Transmission of pathogens in the hospital environment mainly occurs on HTSs such as patient files, hospital beds and rails, drip stands, and is passed around to different hospital sites where patients, healthcare workers and visitors interact. Thus, there is an increased risk of pathogens spreading from the hospital to the community (as illustrated in Figure 1.1). There is variability associated with the isolation of pathogenic bacteria on hospital surfaces. This is linked to several factors that include the ability to culture the organism, the extent of the shedding of the patients, the method that is used for sample collection, the effectiveness of the cleaning methods that are implemented and the presence of an outbreak during sample collection (Otter *et al.*, 2013).

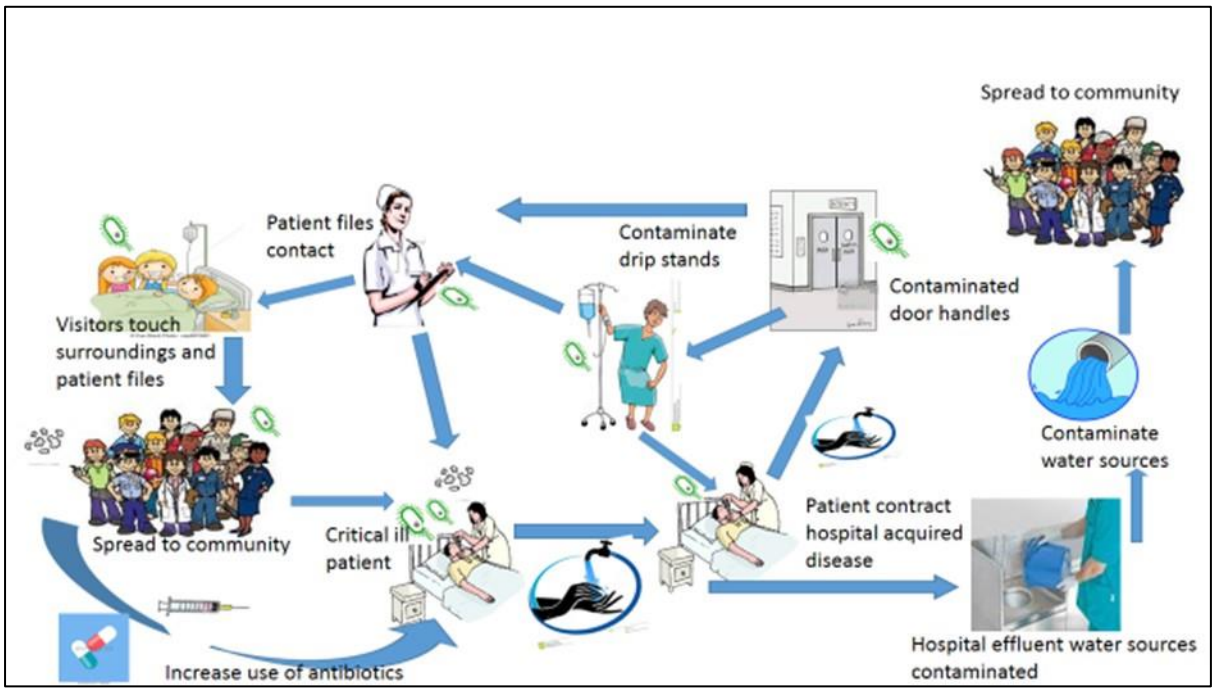

Figure 1.1: Illustration showing possible transmission routes of pathogenic organisms in the hospital (designed by Dr L.A Bester)

1.4 Hospital-acquired infections

Nosocomial infections/hospital-acquired infections occur in patients within 48 hours after hospital admission or during the period of in-patient care (Revelas, 2012). These infections are due to patient exposure to potentially pathogenic organisms such as bacteria, viruses, fungi, and parasites (McQuoid-Mason, 2012). Hospital-acquired infections are classified as endogenous or exogenous (Chaoui *et al*., 2019). Endogenous infections occur when the pathogens are present on the patient and are the source of the infection. Exogenous infections result from patient exposure to pathogens present in the hospital environment (surfaces, water and air), hospital staff, or other patients (Chaoui *et al*., 2019). The increase of hospital-acquired infections is a global threat for a variety of reasons. These include increased extended periods of hospitalization of patients, rising medical costs, admittance into the intensive care unit (ICU) and lengthy antimicrobial treatments (Babikerldris *et al*., 2017). Healthcare devices such as catheters and ventilators are most commonly associated with HAIs (Khan *et al*., 2017). HAIs are prone to both adult and paediatric wards (Revelas, 2012). Hospital populations at significant risk of exposure are patients in intensive care units (ICU), patients recovering from burns, patients undergoing organ transplants or surgery, and newborn babies (Khan *et al*., 2017). One in seven patients is at risk of acquiring at least one HAI in developed countries and a 10 % chance in developing countries (WHO, 2016). In the ICU, 51% of patients are often infected with a HAI (Khan *et al*., 2017). Children younger than one year have the highest rates of nosocomial infections (Revelas, 2012).

Bacteria cause 90 % of hospital-acquired infections (HAIs), while mycobacterial, protozoal or fungal micro-organisms are less commonly reported (Bereket *et al.*, 2012). The bacteria frequently associated with HAIs are *Staphylococcus aureus*, *Legionella, Streptococcus* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Bacillus cereus*, coagulase-negative *staphylococci*, and *enterococci*, (Khan *et al.*, 2015). The list also includes *Klebsiella pneumonia*, *Proteus mirabilis, Escherichia coli* and *Serratia marcescens*- classified under the Enterobacteriaceae family (Khan *et al.*, 2015). *Staphylococcus aureus* (*S. aureus*) is a significant pathogen that is responsible for HAIs (Khan *et al.*, 2015). Pathogenic bacteria are of global concern as they cause worldwide common hospital and community-acquired infections. Many bacterial strains have become multi-drug resistant, resulting in multi-drug resistant strains such as vancomycin-resistant *enterococci,* methicillin-resistant *S. aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (Khan *et al.*, 2015).

1.5 The presence of *S. aureus* in the hospital environment

S. aureus has been isolated in the hospital environment of both developed and developing countries globally; however, there is variation in the prevalence based on the geographic location. In Africa, there have been reports on the prevalence of *S. aureus* in the environmental sites of hospitals in Tunisia (12 %; 12/100) (Gharsa *et al*., 2016), Nigeria (50.8 %; 32/63) (Hammuel *et al*., 2014), and Ghana (39 %; 47/104) (Saba *et al*., 2017). Also, in Asia, reports represent Palestine (29.6 %; 58/196) (Adwan *et al*., 2015), Iran (7.4 %; 78/1051) (Ekrami, 2011), Nepal (20.9 %; 38/188) (Mukhiya *et al*., 2012), and Japan (27.9 %; 12/43) (Kurashige *et al*., 2016). *S. aureus* can survive on surfaces for as long as 12 months (Otter *et al*., 2014). A study conducted in a hospital in France by Pinon *et al*. (2013) noted *S. aureus* contamination of bed linen after washing of sheets and pillowcases (Pinon *et al*., 2013). In addition, *S. aureus* has seemed to persist on fabrics and materials such as cotton, cotton-terry, cotton-polyester blend, polyester, and polypropylene plastic for up to 90 days (Neely & Maley, 2000). *S. aureus* and MRSA have also been isolated from the hospital equipment (Mulvey *et al.*, 2011), hospital linen (Pinon *et al*., 2013), telephones (Nwankwo *et al*., 2014), and air (Creamer *et al*., 2014) in the hospital wards. A retrospective study conducted by Lei *et al*. (2020) which used data from various existing international studies on methicillin-resistant *S. aureus* in the ICU, showed that high-touch surfaces (HTSs) contributed significantly in transmitting MRSA in the hospital environment when proper cleaning practices were not implemented (Lei *et al*., 2020).

1.6 *Staphylococcus aureus'* role as a HAI

The European Centre for Disease Prevention and Control (European Centre for Disease Prevention and Control, 2013) reported that close to 3.2 million patients developed an HAI after receiving healthcare from European Union (EU) hospitals (Salge *et al.*, 2017). The report also stated that *Staphylococcus aureus* was the most prevalent Gram-positive organism, and 41 % of the isolates from patients showed antibiotic resistance, and methicillin-resistant *S. aureus* was the most pervasive (Salge *et al.*, 2017). In 2014, the World Health Organization (WHO) reported that the prevalence of MRSA infections in hospitals was at 20 %, leading to increased medical costs and high morbidity and mortality rates compared to patients affected by methicillin-susceptible *S. aureus* (MSSA). A study conducted by Klein *et al. (*2007) in the United States of America (USA) (1999-2005) to determine the yearly number of

hospitalizations and deaths due to *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA). They reported that the estimated number of *S. aureus*-related hospitalizations increased by 62 %, and the MRSA-related hospital admissions increased during this period. Thus, they concluded that *S. aureus* and MRSA had to be prioritized nationally for disease control. As a result, there has since been a notable decrease in MRSA infection rates in the USA, as Dantes *et al.* (2013) reported.

There is variation in the prevalence and molecular epidemiology of *S. aureus* from hospitals in South Africa. The variation is dependent on the provincial geography, population density and the state of the provincial healthcare system. A study conducted by Perovic *et al.* (2015) conducted in South Africa (2010-2012) assessed the *S. aureus* bacteraemia (SAB) trends and prevalence in patients. A total of 2709 clinical *S. aureus* isolates were obtained, 46.0 % (1231/2709) were methicillin resistant. The results indicated that the Gauteng province had the highest *S. aureus* isolates, 59.5 % (1612/2709), compared to the Free State, Western Cape, and KwaZulu-Natal provinces. South Africa has an underdeveloped antimicrobial surveillance system for pathogenic microbes. However, no institution or country can declare to have resolved this global challenge (Khan *et al.*, 2017).

Control against HAIs requires a multi-faceted approach as there is not one definite approach that can be applied. Consequently, effective infection, prevention and control (IPC) methods ensure that a clean hospital environment exists (Mitchell *et al.*, 2014). A surveillance study conducted by Adwan *et al.* (2015) assessed MRSA strains on the hospital environment and anterior nares of patients in two Northern Palestinian hospitals. The study further aimed to determine the link between hospital environmental contamination and hospital-acquired infections by investigating the clonal relatedness of the samples. This study highlighted a link between contaminated surfaces of the hospital environment and the transmission of multi-drug resistant (MDR) pathogens, thus an increased risk of hospital-acquired infections (Chaoui *et al.*, 2019; Otter *et al.*, 2014).

1.7 *S. aureus* genus characterization

The *Staphylococcus* genus has Gram-positive bacteria that are 0.5-1.5 µm in diameter. The genus is also characterized by DNA with a low G+C content (Malachowa & DeLeo, 2010). The *Staphylococcus* genus is further categorised into coagulase-positive and coagulase-negative staphylococci based on the presence or absence of the coagulase enzyme that causes blood clotting (Costa *et al.*, 2013). The coagulase-positive staphylococci group includes *Staphylococcus aureus*. The coagulase-negative staphylococci (CoNS) are present as commensal bacteria on the skin (Costa *et al.*, 2013). *S. aureus* is a non-spore-forming, immotile, facultative anaerobe that is catalase and coagulase-negative (Khan *et al.*, 2015). The *S. aureus* cell wall has one lipid membrane enclosed by an outer layer of peptidoglycan and lipoteichoic acid that is attached by diacylglycerol (Oliveira, 2018). The peptidoglycan makes up 50 % of the cell wall and is the main component. In addition, the cell wall also offers this bacterium protection against many toxic molecules (Peacock & Paterson, 2015; Oliveira, 2018). The name *aureus*, meaning "golden", refers to the colour formed by *S. aureus* colonies on solid rich media in the presence of carotenoids thus forming golden circular colonies (Costa *et al.*, 2013).

1.8 Phenotypic identification of *S. aureus* and MRSA

Developing countries mainly use phenotypic tests to detect and diagnose staphylococcal infections (Kateete *et al.*, 2010). Two methods of coagulase testing are primarily utilised; these are the slide coagulase (SCT) and tube coagulase (TCT) methods (Kateete *et al.*, 2010. Many diagnostic laboratories in developing countries use mannitol salt agar (MSA) or DNase tests as a presumptive test to detect *S. aureus* (Kateete *et al.*, 2010). MSA is a selective and differential media that grows specifically halophilic bacteria due to its high sodium chloride (NaCl) content (Shields & Tsang, 2016). It contains a sensitive dye and mannitol, which is a sugar alcohol. Bacteria that can ferment mannitol produce acidic waste products that alter the pH and cause the pH-sensitive dye to change to a bright yellow colour (Shields & Tsang, 2016). Halophilic bacteria that do not ferment mannitol show growth, but the medium remains pink. Even though coagulase tests help identify *S. aureus,* few studies have analysed their use in routine practice.

Organisms that possess biochemical characteristics that cannot be characterized into a known species or genus are problematic for diagnostic laboratories (Kateete *et al.*, 2010). The use of

chromogenic media has made phenotypic identification easier. Chromogenic media is also used in the phenotypic identification of *S. aureus* and MRSA. Chromogenic media contains synthetic chromogenic enzyme substrates that, in the presence of a particular target enzyme, are processed and produce bacterial colonies of a specific colour, resulting in phenotypic identification (Lee *et al.*, 2018). The Kirby-Bauer disk-diffusion method can be used to screen pure *S. aureus* cultures for methicillin resistance (Lee *et al.*, 2018). Oxacillin was previously used for MRSA detection; however, the Clinical Laboratory Standards Institute (CLSI) guidelines recommend using cefoxitin (M100-S27, 2017). Cefoxitin produces a more evident phenotypic result because it induces *mecA* and *mecC* better than oxacillin (Lee *et al.*, 2018). Other methods of *S. aureus* identification exist. These include matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) and VITEK 2 (bioMerieux, Durham, NC, USA) (Lee *et al.*, 2018).

1.9 *S. aureus* virulence factors

As a pathogen, *S. aureus* affects humans by causing skin and soft-tissue infections (Tong *et al.*, 2015). These infections cause septicemia and bacteremia when *S. aureus*, or the toxins it releases, enter the patient's bloodstream (Tong *et al.*, 2015). Naturally, a pathogen causes infection by evading the immune defences of the host organism (Oliveira, 2018). The virulence of *S. aureus* is defined by many virulence factors, and secreted toxins are significant (Otto, 2014). The *S. aureus* virulence factors and their functions are explained further in Table 1.1 that follows. Many toxins of *S. aureus* damage the hosts' cellular membranes, leading to cell death (Otto, 2014). This occurs by inhibiting the complement cascade or *S. aureus* recognition by the hosts' defences (Otto, 2014). *S. aureus* virulence factors are characterized into cell-surface factors and secreted proteins (Costa *et al.*, 2013). *S. aureus* has different cell surface factors that determine its virulence, namely: microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsular polysaccharides and carotenoid pigment (staphyloxanthin) (Costa *et al.*, 2013). Secreted factors are divided into three groups: exoenzymes (tissue degrading enzymes) such as proteases and lipases; cytotoxins (Panton-Valentine Leucocidin (PVL) and α, β, γ, δ–haemolysin), miscellaneous proteins and superantigens (Costa *et al.*, 2013).

Table 1.1: Indicating the functions of virulence factors that are involved in *S. aureus* pathogenesis (adapted from Costa *et al.* 2013)

Cell surface factors	**MSCRAMMS**	
	Name	**Function**
	Fibronectin- binding protein (*FnbpA* & *FnbpB*)	Attach to fibrinogen and fibronectin, and cause the inflammation and invasion of endothelial cells (Tong *et al.*, 2015)
	Fibrinogen- binding protein (*ClfA* & *ClfA*)	Binds to the extracellular matrix proteins and allow *S. aureus* to attach to host tissue (Lee *et al.*, 2018)
	Collagen-binding protein (*CnA*)	Facilitates *S. aureus* binding to collagen and cartilage in host tissue
	Staphylococcal binding protein (*SpA*)	Binds to the Fc region of Immunoglobulin (IgG), prevents opsonization, and causes phagocytosis inhibition (Lee *et al.*, 2018)
Secreted factors	**Superantigens**	
	Name	**Function**
	Staphylococcal enterotoxins (*sea*, *seb*, *sec*, *sed*, *see*, *seg* and *seq*)	Superantigens are immune-stimulatory proteins that cause food poisoning when *S. aureus* is ingested, causing host organisms to vomit and have diarrhoea (Grumann *et al.*, 2014).
	Toxic shock syndrome toxin (TSST-1)	
	Cytolytic toxins	
	α- haemolysin (coded by the *hla* gene)	These toxins target T-lymphocytes, white blood cells, and epidermal skin cells and make them vulnerable to other toxins (Oliveira, 2018).
	β- haemolysin	
	γ- haemolysin	
	Panton-Valentine Leukocidin (coded by the *LukS/F-PV*)	Involved in the breakdown of white blood cells (Costa *et al.*, 2013).
	Exoenzymes	
	Lipases	Involved in the inactivation of nucleic acids (Costa *et al.*, 2013)
	Nucleases	Cut nucleic acids (Costa *et al.*, 2013)
	Proteases:	Involved in neutrophil inactivation, thus disrupting host organism's immune defences (Costa *et al.*, 2013)
	Hyaluronidase	Involved in the breakdown of hyaluronic acid (Costa *et al.*, 2013)
	Miscellaneous proteins	
	Staphylococcal complement inhibition (SCIN)	Drastically hinder the innate and adaptive immune responses of host organisms by disrupting chemotaxis and neutrophil activity (Costa *et al.*, 2013)
	Extracellular fibrinogen binding protein	
	Chemotaxis inhibitory protein of *S. aureus* (CHIPS)	
	Formyl peptide receptor-like 1 inhibitory protein (FLIPr)	
	Extracellular adherence protein (Eap)	

1.10 Pathogenesis of *S. aureus*

Staphylococcus aureus is a versatile commensal bacteria that causes different diseases such as respiratory and skin infections in patients (Otto, 2014). Lung infections are predominantly of nosocomial origin, whereas skin infections are of a community-acquired nature (Otto, 2014). These infections in patients range from minor skin infections to life-threatening diseases. They include abscesses on soft tissue, toxic shock syndrome, pneumonia, septicaemia, skin infections, infective endocarditis, and pleuropulmonary infections. Soft tissue and skin infections result from intravascular devices such as intravenous (IV) drips and catheters (Liu, 2009; Tong *et al.*, 2015).

The pathogenesis of *S. aureus* infection usually begins when the bacteria attaches to the epithelial cells, mainly in the nasal cavities located on the mucous membranes (Lee *et al.*, 2018). The bacteria gains access into the host's body through open wounds and uses cell surface factors (MSCRAMMs) (as shown in Table 1.1 and Figure 1.2) to attach to proteins (e.g. collagen, fibronectin, and cytokeratin 10) in the extracellular matrix (Lee *et al.*, 2018). This occurs by *S. aureus* recognising the fibronectin-binding protein A (*FnPBA)*, a glycoprotein that causes attachment of cells in the extracellular matrix. Fibrinogen is a protein that produces fibrin which is found in blood plasma (Tong *et al.*, 2015). The neutrophil response is the primary defence mechanism to stop an *S. aureus* infection in humans (Tong *et al.*, 2015). Macrophages and neutrophils travel to the site of the infection (Tong *et al.*, 2015). *S. aureus* uses an elaborate tactic to evade the host's immune defences. The bacteria cell blocks the chemotaxis of white blood cells, evades detection by forming a biofilm or a polysaccharide capsule, and evading phagocytosis by producing an inhibitor (Tong *et al.*, 2015; Lee *et al.*, 2018). When exposure to the host's tissues has occurred, virulence genes are upregulated, thus infection occurs (Oliveira, 2018). The (*spA*) gene, which enables the cells to form a capsule with staphylococcal protein A, is also upregulated (Lee *et al.*, 2018). Staphylococcal protein A attaches to the Fc subgroup on IgG (immunoglobulin G), a protein active in humoral immunity, thus *S. aureus'* recognition for destruction via phagocytosis is prevented (Lee *et al.*, 2018).

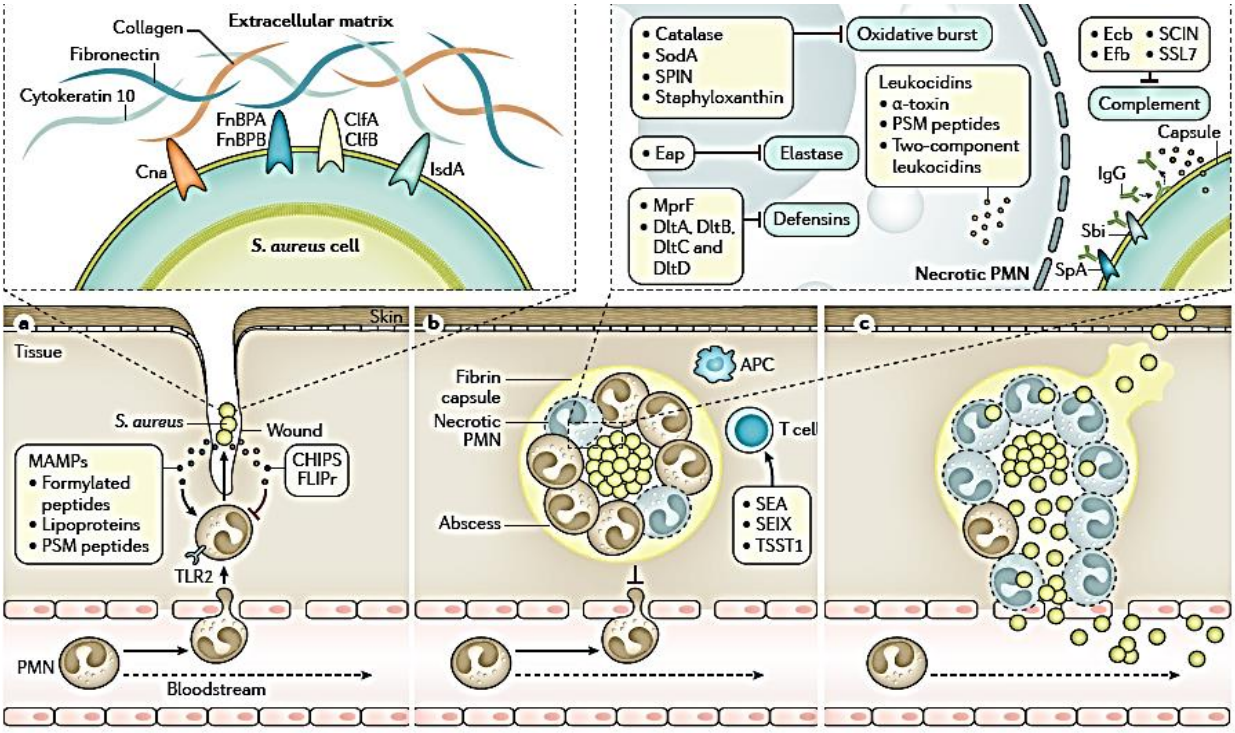

Figure 1.2: Illustrating the stages of infection and the virulence factors (cell surface and secreted factors) involved in *S. aureus* pathogenesis (Lee *et al.*, 2018).

1.11 Antibiotic resistance of *S. aureus*

The emergence of antibiotic-resistant phenotypes is linked to the clinical use of antimicrobial agents intended to kill bacteria, but the bacteria manifests resistance (Graffunder and Venezia, 2002). *S. aureus* antibiotic resistance occurs through a gene mutation or the bacteria acquiring a resistance gene from other bacteria via mobile genetic elements (MGEs). *S. aureus* resistance to penicillin and newer narrow-spectrum β- lactamase-resistant penicillin antibiotics such as methicillin and oxacillin was reported soon after the drugs were used as a form of treatment (Klein *et al.*, 2007). Penicillin was initially used as an antibiotic to kill *S. aureus*; however, *S. aureus* strains resistant to penicillin and methicillin were first reported in 1948 and 1961, respectively (Shittu and Lin, 2006).

S. aureus strains have become resistant to several antibiotic classes, which affects how this pathogenic bacterium is managed in hospitals, therefore making it the primary cause of infectious diseases in hospitals (Blair *et al.*, 2014). These antibiotic classes, to name a few, include β-lactams, fluoroquinolones, aminoglycosides, lipopeptides, lincosamides, streptogramins, ansamycins and macrolides (Blair *et al.*, 2014). The β-lactams are a class of

antibiotics that are categorised by the presence of a beta-lactam ring. They covalently attach to the *Ser* active site of penicillin-binding proteins, thus inhibiting peptidoglycan synthesis (Blair *et al.*, 2014). Carbapenems, cephalosporins, penicillins, monobactams and clavams are characterized as β-lactam sub-classes (Blair *et al.*, 2014). *S. aureus*' resistance to β-lactams is a result of the bacteria acquiring a plasmid that carries a gene that codes for the penicillin-binding protein 2a (PBP2a) enzyme. PBP2a can hydrolyse the β-lactam ring that causes antibiotic resistance to β-lactam antibiotics like penicillin and its derivatives (Otto, 2012). Penicillinase is the enzyme that cuts the β-lactam ring indicative of β-lactam antibiotics like penicillin and its derivative, thus causing resistance (Otto, 2012).

Fluoroquinolones are an antibiotic class that is involved in inhibiting DNA gyrase and topoisomerase IV in bacteria (Blair *et al.*, 2014). These synthetic compounds include ciprofloxacin, nalidixic, levofloxacin and moxifloxacin (Gold & Pillai, 2009). Aminoglycosides are antibiotics that inhibit protein synthesis. These antibiotics bind to the 30s ribosomal subunit, thus inhibiting translation (Blair *et al.*, 2014). This antibiotic class includes gentamicin, tobramycin, streptomycin, and kanamycin (Blair *et al.*, 2014). When the ribosomal subunit is modified, via methylation, the bacteria acquire aminoglycoside resistance (Blair *et al.*, 2014). The macrolides-lincosamide-streptogramin class of antibiotics that are clinically used to treat staphylococcal infections are macrolides (erythromycin, clarithromycin, and azithromycin), lincosamides (clindamycin), and quinupristin which is classified as a streptogramin B antibiotic (Gold & Pillai, 2009). This antibiotic is used together with dalfopristin, a streptogramin A antibiotic, in the antibiotic Synercid (Gold & Pillai, 2009).

Antibiotic resistance is regarded as a form of virulence. Virulent strains that acquire antibiotic resistance become life-threatening pathogens that are a considerable concern to modern medicine. After the first MRSA reported in 1961, it has become a global threat in hospitals and has made treatment using β-lactam class antibiotics futile (Diep and Otto, 2008). *S. aureus* strains origination from Africa are associated with resistance to tetracycline and co-trimoxazole. This is due to these drugs being frequently prescribed to patients (Schaumburg *et al.*, 2014). *S. aureus* resistance to vancomycin and teicoplanin has been reported in KwaZulu-Natal, South Africa (Shittu and Lin, 2006). Vancomycin, which is less efficient compared to β-lactams, has been used as an antibiotic to treat multi-drug resistant *S. aureus* (Otto, 2012). However, *S. aureus* species of high vancomycin resistance have been identified (Otto, 2012). A study conducted by Ramsamy *et al.* (2018) evaluated, over five years (2011-2015), the

antibiotic resistance trends of *Staphylococcus aureus* from public sector hospitals in KwaZulu-Natal, South Africa (Figure 1.3). The study reported a decrease in the *S. aureus* resistance to clindamycin, rifampicin, erythromycin, and ciprofloxacin when the study was conducted. In addition, the *S. aureus* isolates did not show vancomycin resistance (Ramsamy *et al.*, 2018). Although this study is clinical in nature, it provides information relevant to *S. aureus* isolates obtained from the healthcare sector and offers antibiotic trends of *S. aureus* isolates collected from KwaZulu-Natal hospitals.

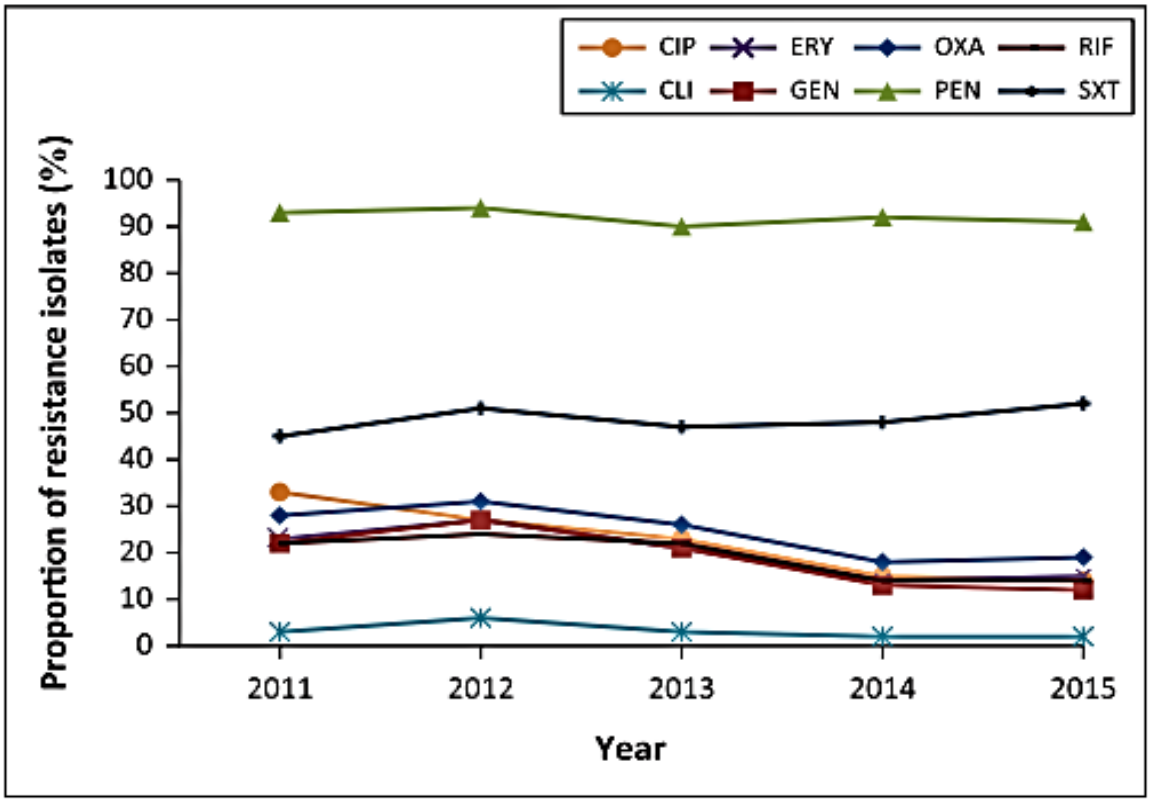

Figure 1.3: Illustrating the antibiotic resistance profiles of *S. aureus* (2011-2015) from public sector hospitals in KwaZulu-Natal, South Africa (sourced from Ramsamy *et al.* (2018)). Key: CIP, ciprofloxacin; ERY, erythromycin; OXA, oxacillin; RIF, rifampicin; CLI, clindamycin; GEN, gentamicin; PEN, penicillin; SXT, trimethoprim-sulfamethoxazole.

1.12 Mobile genetic elements of *S. aureus*

Mobile genetic elements (MGEs) are fragments of DNA that carry genes involved in antibiotic resistance and virulence (Malachowa & DeLeo, 2010). They are involved in transmitting genetic information between prokaryotes and eukaryotes (McCarthy & Lindsay, 2012). MGEs of *S. aureus* mainly encode resistance, virulence, and superantigen (Sag) genes (Grumann *et*

al., 2014). Genetic information is exchanged between multiple *Staphylococcus* strains through horizontal gene transfer (HGT), which plays a cardinal role in the evolution of this pathogen (see Figure 1.4) (Liu *et al.*, 2016). MGEs are also transmitted via vertical gene transfer; this is the transmission of genes from the parent cell to the progeny cell (Malachowa & DeLeo, 2010). HGT in *S. aureus* occurs via phage transduction, conjugation and, as reported, through direct uptake of "naked" DNA by genetic competence (Morikawa *et al.*, 2012). MGEs, which will be further explored in this review, include plasmids, bacteriophages, *S. aureus* pathogenicity islands (SaPIs), staphylococcal chromosomal cassettes (SCC) and transposons (Grumann *et al.*, 2014).

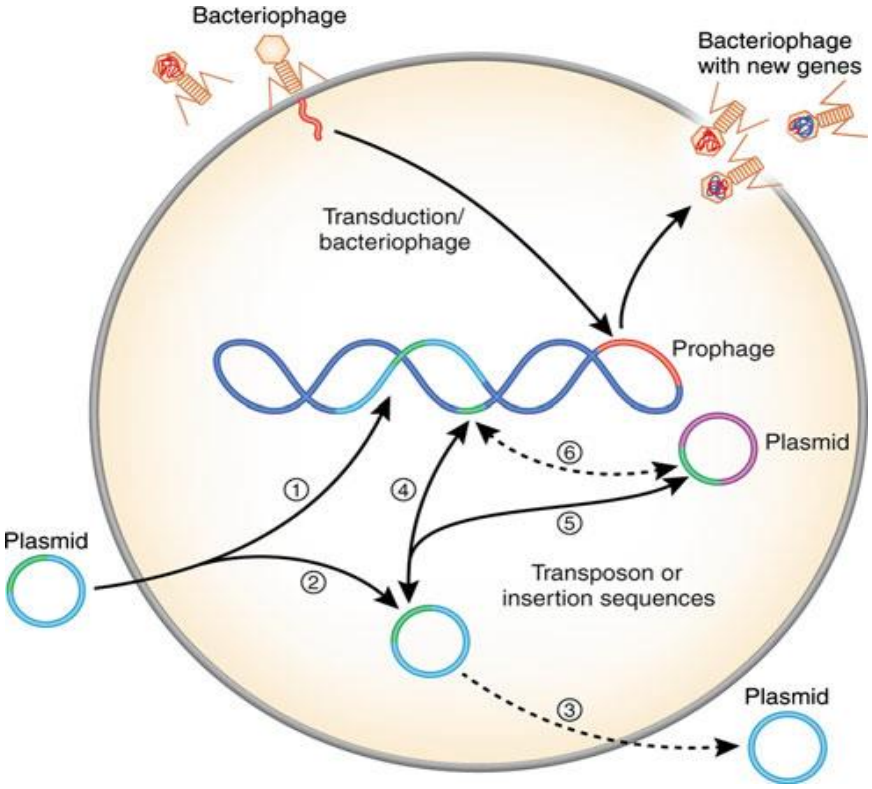

Figure 1.4: The illustration above (sourced from Malachowa & DeLeo (2010) shows how MGEs and plasmids are acquired by *S. aureus* through horizontal gene transfer. (1) Plasmids are linearly integrated into the hosts' genomic DNA, or (2) The plasmids may remain in the host as circular DNA. (3) the plasmid may be released from the host as a suicide plasmid. (4) Genetic information (DNA fragments or transposons) is exchanged between the plasmid and genomic DNA. (5) Genetic information (DNA fragments or transposons) is exchanged between the plasmid and another plasmid within the cell. (6) The exchange may also occur between the genomic DNA and another plasmid in the cell.

1.12.1 *S. aureus* plasmids

S. aureus plasmids carry a variety of antibiotics and virulence genes which can be transferred between *Staphylococcus* species through HGT (Blair *et al.*, 2014). *S. aureus* plasmids also carry genes involved in heavy metal and penicillin resistance (e.g. plasmid pI258) (Malachowa & DeLeo, 2010). In addition, these plasmids carry resistance to macrolides, aminoglycosides and β-lactams (McCarthy & Lindsay, 2012). Furthermore, *S. aureus* plasmids carry toxin genes such as exotoxin B (ETB), which causes skin blistering, and toxins *EntA*, *EntP*, *EntG* and *EntJ* (McCarthy & Lindsay, 2012). The plasmid *pT181* encodes genes (e.g. *tetk* and *tetM*) responsible for tetracycline resistance (Malachowa & DeLeo, 2010).

1.12.2 *S. aureus* transposons

Transposons (Tn) are small DNA fragments that are also carried on the *S. aureus* chromosome. They encode various antibiotic resistance genes for β-lactam antibiotics, gentamicin and vancomycin (Malachowa and DeLeo, 2010; McCarthy and Lindsay, 2012). The *Tn 554* transposon encodes MLSB (macrolides, erythromycin, lincosamides, clindamycin, streptogramin B) and streptomycin/spectinomycin (*aad9*) resistance genes (Malachowa & DeLeo, 2010). The *Tn552* transposon contains the *blaZ* penicillinase gene, and Tn4001 encodes the *aacA-aphD* gene (Malachowa & DeLeo, 2010).

1.12.3 Staphylococcal chromosomal cassettes

The staphylococcal cassette chromosomes *(*SCCs) are key mobile genetic elements essential for transmitting genes between staphylococcal species. They are large DNA fragments inserted into the orfX gene on the *S. aureus* chromosome and classified into two groups; staphylococcal cassette chromosome *mec* (SCC*mec*) and non-SCC*mec* (Liu *et al.*, 2016). SCC*mec* is located near the replication origin of the *Staphylococcus* chromosome (Malachowa & DeLeo, 2010) and is only present in methicillin-resistant *S.aureus* and absent in methicillin-susceptible *S.aureus* (Peacock & Paterson, 2015). The integration and excision of SCC*mec* by recombinases occurs at the *3'* end of *orfX* (Malachowa & DeLeo, 2010). The *mec* genes (*mecA, mecB* and *mecC*) for *methicillin* resistance are encoded on the SCCs. All MRSA strains have SCC*mec*, thus confer resistance to methicillin and *β-lactam* antibiotics (Blair *et al.*, 2014). The

mecC gene is commonly detected in livestock-acquired MRSA (LA-MRSA) (Blair *et al.*, 2014). A recent study conducted in KwaZulu-Natal, South Africa, by Dweba & Zishiri (2019), has reported the *mecC* gene in LA-MRSA. The detection of the *mecB* and *mecC* genes is still low in clinical isolates and has mainly been from patients that frequently interact with animals (Palavecino, 2014). SCC*mec* also encodes the repressor *mecI*, transmembrane *β-lactam* signal transducer *mecR1*, recombinases *CcrAB*, *CcrC* and joining regions J, which may encode additional antibiotic resistance (Malachowa & DeLeo, 2010). *MecI* and *MecR1* regulate the gene expression of the *mec* genes (Liu *et al.*, 2016). SCC*mec* elements are classified by the properties of the *mec* and *ccr* gene complexes and are grouped into different subtypes, types I to XI, based on their J region DNA segments (Liu *et al.*, 2016).

1.12.4 *S. aureus* pathogenicity islands (SaPIs)

Staphylococcal pathogenicity islands (SaPIs) are a group of MGEs that are 14-17 kb in size (Malachowa & DeLeo, 2010). SaPIs are highly diverse genetic elements located in one of six different chromosomal (8', 9', 18', 19', 44', and 49') (Sato'o *et al.*, 2012), and always have the same orientation (Malachowa & DeLeo, 2010). These genetic elements carry one or more super-antigen genes (*tst-1, seb, sec, selk, sel, and selq*) encoding for toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins and staphylococcal enterotoxin-like-toxin. SaPis are also involved in facilitating the evolution of the staphylococcal group (Sato'o *et al.*, 2012).

1.13 Mechanism of methicillin resistance in *S. aureus*

Treatment of infections caused by *S. aureus* has proven to be rather difficult due to the evolved resistance to antibiotic drugs (Blair *et al.*, 2014). The term MRSA is used for methicillin-resistant *S. aureus* strains. MRSA is identified by the presence of the *mecA* gene, which indicates the resistance to methicillin (Otto, 2012). Methicillin is a semisynthetic antibiotic that was developed to be used against penicillin-resistant *S. aureus* (Otto, 2012). Most literature indicates that methicillin resistance resulted within a year after Beecham clinically introduced methicillin in 1959 (Otto, 2012); however, there is now evidence that methicillin-resistant *S. aureus* emerged before clinical use (Harkins *et al.*, 2017). Harkins *et al.* (2017) have hypothesised that MRSA resulted from penicillin over-use instead of the clinical use of

methicillin. Whole-genome sequencing of 209 novel MRSA isolates from the UK suggested that the emergence of MRSA occurred in the mid-1940s (Harkins *et al.*, 2017).

The mechanism of resistance for methicillin-resistant strains differs from penicillin-resistant strains. The methicillin-resistant strains are protected from all β-lactams with the inclusion of penicillins, caphalosporins and carbapenems. The resistance to methicillin is due to the bacteria's ability to acquire the *mecA* gene that codes for a Penicillin-binding protein (PBP2a). This protein has a low affinity to β-lactams (Peacock & Paterson, 2015). The *mecA* gene is located on a mobile genetic element referred to as SCC*mec*. The expression of *mecA* occurs through a proteolytic signal transduction pathway that is carried out by a sensor protein (MecR1) and a repressor protein (Mec I) (Peacock & Paterson, 2015). Recent studies have reported the presence of a *mecA* variant called *mecC*. This variant was identified in livestock and human isolates from Ireland, Denmark and the United Kingdom (Blair *et al.*, 2014). This allele is located on a type XI SCC*mec* element and has a 70 % nucleotide similarity to *mecA* (Blair *et al.*, 2014). Standard PCR assays used for *mecA* identification do not readily identify the *mecC* gene. The antibiotic resistance of the two genes also differs- *mecC* MRSA isolates are more susceptible to oxacillin compared to *mecA* MRSA isolates, although still showing cefoxitin resistance (Blair *et al.*, 2014).

1.14 Community-acquired MRSA versus hospital-acquired MRSA

MRSA is divided into groups based on the source of origin, namely: Livestock-associated MRSA (LA-MRSA), Community-acquired MRSA (CA-MRSA), and Hospital-acquired MRSA (HA-MRSA). LA- MRSA is mainly found colonizing and infecting people that interact with livestock such as cattle, poultry, and bovine (Lee *et al.*, 2018). For the sake of this study, this section only compares community-acquired MRSA and hospital-acquired MRSA.

A comprehensive understanding of the gene content is necessary to understand better the genetic basis of HA-MRSA (Hospital Acquired-MRSA) strains and CA-MRSA (Community Acquired-MRSA) and the differences between CA-MRSA lineages that show specific epidemiological characteristics (Sader *et al.*, 2016). Five major ancestral hospital clones of MRSA have been reported in Africa (Breurec *et al.*, 2011). Thus, this suggests that the acquisition of the staphylococcal cassette chromosome (SCC*mec*), the genetic element that contains the genes that result in methicillin resistance, has occurred infrequently (Breurec *et*

al., 2010). Many of the HA-MRSA clones are found globally; however, their frequencies show variation between countries. Many global CA-MRSA infections are caused by strains that belong to only six CA-MRSA clones (Coombs *et al.*, 2004; Diep and Otto, 2008).

CA-MRSA differs from HA-MRSA lineages by its genotypic and phenotypic characteristics (Diep and Otto, 2008). There was a distinct difference between CA-MRSA and HA-MRSA due to their continental distribution; however, international travel routes have distorted this. A prime example would be the CA-MRSA clone USA300, initially identified in the United States of America (USA) but presently observed in many other countries (Breurec *et al.*, 2010). CA-MRSA can be treated with antibiotics as it has the staphylococcal cassette chromosome methicillin-resistant locus (SCC*mec*) type IV or V (Babikerldris *et al.*, 2017). Additionally, differences between CA-MRSA and HA-MRSA include epidemiology, microbiology, and the observed clinical manifestation (Babikerldris *et al.*, 2017). HA-MRSA has been problematic for in-patients since the 1960s, and CA-MRSA has been associated with healthy and young individuals (Albrich and Harbarth, 2008). CA-MRSA has spread from the communities where outbreaks have been observed to the hospital environment (Albrich and Harbarth, 2008). Initially, MRSA detection was infrequent in patients from the community (Graffunder and Venezia, 2002); however, reports of incidence have increased in the last few years, suggesting that the epidemiology has changed. Resistance to penicillin was first restricted to a small number of admitted patients; however, resistance increased as the use of penicillin became frequent in hospitals and the community (Klein *et al.*, 2007). Despite the general view that nosocomial pathogens are spreading from the hospital to the community, community-associated MRSA strains could be disseminating from the community environment into the hospital environment rather than the other way around (Klein *et al.*, 2007).

1.15 MRSA nosocomial outbreaks

The existence of a methicillin resistant *S. aureus* strain was first discovered at a hospital in the United Kingdom in 1961 (Sweswe *et al.*, 2015). A few years later, the first MRSA outbreak was reported at a Boston City hospital (USA) in 1968 (Bereket *et al*., 2012). In South Africa, the earliest report of an MRSA outbreak occurred in 1989 at a neonatal intensive care unit in KwaZulu-Natal (Sweswe *et al.*, 2015). MRSA outbreaks occur in approximately 40-60 % of *S. aureus* outbreaks (Alfatemi *et al.*, 2014). Recently, Pannewick *et al.* (2021) conducted a study by systematically analysing literature on international MRSA hospital outbreak reports.

The study included 104 outbreaks from 18 various countries. Most of these outbreaks were reported in eight countries (Figure 1.5). The US accounted for majority of reported outbreak cases (n = 21). The UK reported (n = 15), France (n = 14), Canada (n = 8), and Japan (n = 8) (Pannewick *et al.*, 2021). In addition, the most frequent MRSA outbreaks were the neonatal (n = 32), surgical ward (n = 27), burn units (n = 10), intermediate medicine (n = 10), and gynaecology (n = 5). The study further highlighted that 40 % of the reported outbreak cases had occurred in the ICU (Pannewick *et al.*, 2021). University hospitals reported the most hospital outbreaks (Pannewick *et al.*, 2021).

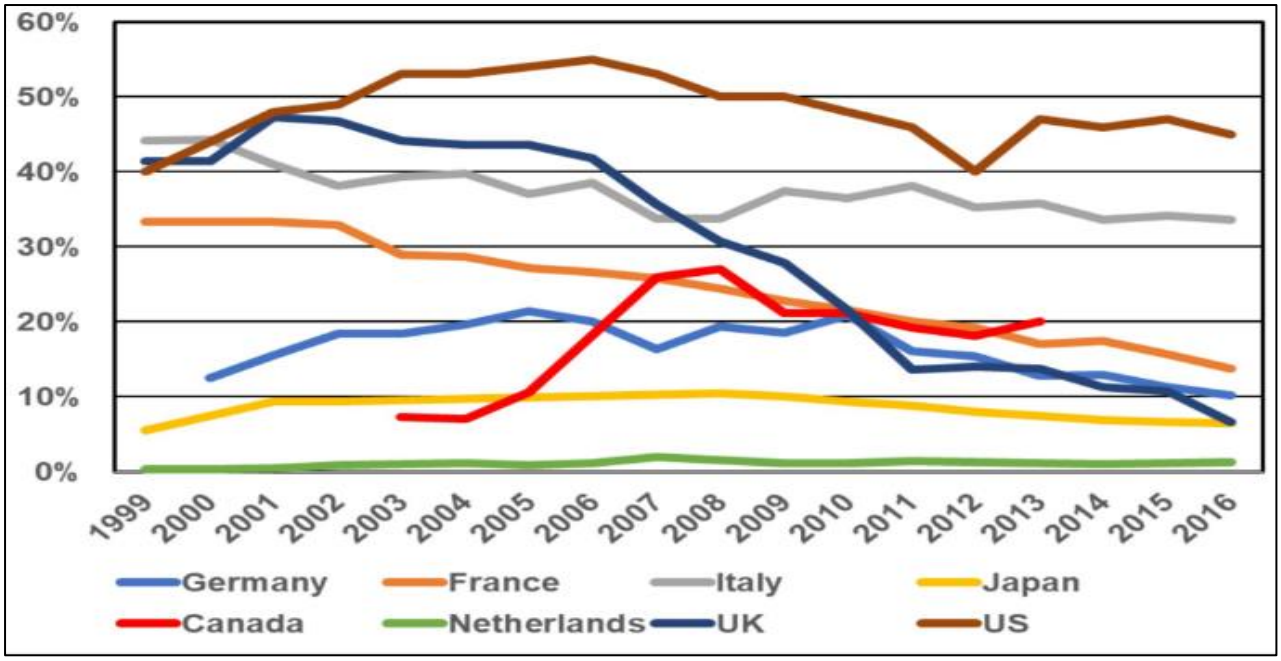

Figure 1.5: Indicating the reported MRSA outbreak cases from countries with the most prevalent outbreaks between 1999-2016 (sourced from Pannewick *et al.* (2021))

1.16 Molecular typing methods for *S. aureus*

Typing methods play an essential role in monitoring the epidemiology and spread of pathogens (Ciao, 2016). These typing methods include (to name a few): multilocus sequence typing (MLST), pulse-field gel electrophoresis (PFGE), *spa*-typing, enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP)-PCR, *SCC*mec-typing and Whole-genome sequencing (WGS). PFGE, ERIC-PCR, MLST, REP-PCR and *spa*-typing. Some of these are discussed further.

1.16.1 Pulse-field gel electrophoresis (PFGE)

PFGE is a whole-genome sequencing method used to analyse the genetic diversity of bacterial strains. It is referred to as the best method for molecular typing compared to other methods such as whole-genome sequencing (WGS), *spa*-typing, multilocus sequence typing (MLST), and SCC*mec* typing (Olive & Bean, 1999). An illustration of the steps involved in PFGE for *S. aureus* isolates (He *et al.*, 2014) is represented in (Figure 1.4). PFGE is conducted by combining bacterial cells with agarose gel. The mixture is poured into moulds to form agarose plugs (Melles *et al.*, 2007). The agarose plugs with embedded bacterial cells are treated with a restriction enzyme, *Smal* is used for *S. aureus* to digest the recognition sites of chromosomal DNA (Moodley *et al.*, 2010). The agarose plugs containing digested bacteria are placed into an agarose gel for electrophoresis. The electrophoresis is conducted in a device that manipulates the voltage/ electric field to separate the DNA fragments (Olive & Bean, 1999). A macro-restriction banding pattern is produced with DNA fragments from between 10kb-800kb in size (Olive & Bean, 1999). Moodley *et al.* (2010) have reported a study on *S. aureus* with DNA fragment sizes ranging from 50-1000kb. A fluorescent dye such as ethidium bromide is used to visualise the bands. The gels are viewed using computerised software such as the Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium) (Melles *et al.*, 2007) or GelCompar II (Applied Maths, Kortrijk, Belgium) (Moodley *et al.*, 2010) to produce a dendrogram.

Olive & Bean (1999) had reported that PFGE was the most efficient typing method for separating different MRSA strains. This is in contrast to a study conducted by Melles *et al.* (2007). They stated that PFGE was less reproducible, less efficient for epidemiological surveillance studies or evaluating the phylogenetic relatedness of *S. aureus* strains. PFGE has been used to analyse isolates from the hospital environment. Sweswe *et al.* (2015) conducted a surveillance study aimed to investigate an MRSA outbreak in a renal and dermatology ward at a central academic hospital in KwaZulu-Natal, South Africa. The PFGE results indicated that three isolates were identical (showed no band differences under PFGE conditions). The isolates were from an air sample, the curtain and the blood-pressure cuff from the dermatology ward (Sweswe *et al.*, 2015). Interestingly, four isolates from a nurse's hand and nose swab were identical (showed no band difference) to isolates from a blood-culture sample and nose swab of a patient (Sweswe *et al.*, 2015).

1.16.2 MLST

Multilocus sequence typing (MLST) is used to examine the epidemiology and genetic relatedness of various bacterial strains (Liu & Ji, 2020). This method uses housekeeping genes and an online database (http://www.mlst.net) to classify the genotypes of micro-organisms (Liu & Ji, 2020). The seven housekeeping genes analysed for *S. aureus*, and MRSA strains are *arcC*, *aroE*, *glp*, *gmk*, *pta*, *tpi*, *and yqiL*. These genes are 402 to 516 base pairs (bp) in length and code for carbamate kinase, shikimate dehydrogenase, glycerol kinase, guanylate kinase, phosphate acetyltransferase, triosephosphate isomerase, and acetyl coenzyme A acetyltransferase, correspondingly (Liu & Ji, 2020). However, MLST is very expensive to conduct and requires a PCR step, followed by the sequencing of amplicons (Palavecino, 2014). Therefore, it is not the best typing method to determine clinical strains' epidemiology and genetic relatedness but rather more suited for analysing the global spread of clones (Palavecino, 2014).

1.16.3 *Spa*-typing

The *spa*-typing method has been used to determine the phylogenetic and genotypic features of *S. aureus* strains from various sources (Wang, 2020). The staphylococcal protein A (spA) is made up of three components: The Fc-partial region, X-region, and the C terminal (Wang, 2020). *Spa*-typing depends on the presence of very polymorphic 24 bp repeated sequences on the X-region (Wang, 2020). Therefore, *Spa*-typing produces highly sensitive results similar to those produced by PFGE and is often used for isolates that show no bands (are non-typeable under PFGE conditions) (Wang, 2020). The steps used for spa-typing are the same as the steps for MLST. This entails a PCR step followed by sequencing the PCR amplicons using an automated sequencer (Palavecino, 2014). However, *spa*-typing only analyses one gene locus (*spa*) instead of seven loci as surveyed by MLST (Palavecino, 2014). The results are then analysed using software such as SpaServer (**http://www.spaServer.ridom.de/**) (Wang, 2020).

1.16.4 Enterobacterial repetitive intergenic consensus PCR (ERIC-PCR)

A study conducted by Versalovic *et al.* (1991) reported on using repetitive DNA sequences to determine the genetic relatedness of bacterial strains. The study presented two typing methods.

The first typing method analysed the repetitive extragenic palindromic (REP) sequences found in bacterial genomes (Olive & Bean, 1999). These sequences are 38bp in size and consist of six degenerate locations and a variable loop (Olive & Bean, 1999). The second typing method analysed enterobacterial repetitive intergenic consensus (ERIC) sequences (Olive & Bean, 1999). These sequences are found in the extragenic regions of bacterial genomes, are 126bp in size, and have a conserved central inverted repeat (Olive & Bean, 1999). ERIC-PCR and REP-PCR are easy to use and interpret, cost-efficient, produce results in a relatively short period, and have a high power of discrimination (Candan & Idil, 2013).

ERIC-PCR and REP-PCR have been effectively used to analyse the genetic relatedness of *S. aureus* strains. Candan & Idil (2013) compared the sensitivity and results produced by ERIC-PCR and REP-PCR. In addition, the study aimed to examine the epidemiology and genetic relatedness of *S. aureus* strains from hospital samples. The results indicated that ERIC-PCR and REP-PCR could be successfully used to examine the genetic relatedness of *S. aureus* strains. The study further reported that ERIC-PCR and REP-PCR could be used in the surveillance of *S. aureus* for infection, prevention and control (Candan & Idil, 2013). A similar study, using only ERIC-PCR, was done by Adwan *et al.* (2015). The results indicated that two isolates out of 265 collected swabs collected from the environmental sites of two hospitals in Iran were non-typeable under ERIC-PCR conditions. They further conducted *spa*-typing on the non-typeable isolates (Adwan *et al*., 2015).

1.17 The South African public healthcare sector

The South African hospital sector is divided into the private and public sectors. The private sector is usually well resourced and financed and caters for a minority of citizens, mainly the middle class and high net worth individuals (Ranchod *et al.*, 2017). The public healthcare sector caters to 84 % of the South African population even though it is faced with inadequate funding, old and rundown infrastructure/ buildings, a shortage of resources, is understaffed, and is often over-crowded (Ranchod *et al.*, 2017). These two sectors are divided by the socio-economic inequalities in South Africa post-apartheid (Ranchod *et al.*, 2017). Bhagwanjee & Scribante (2007) conducted a study to determine the bed ratio in ICU/ high care wards of South African public and private hospitals. The results indicated that the provinces with the highest ICU bed ratios were KwaZulu-Natal, Western Cape, and Gauteng. The ICU bed ratio was strikingly

lower (1.7 %) in public hospitals than private sector hospitals (8.9 %). A study conducted by Scribante & Bhagwanjee (2007) aimed to determine the number of nurses working in South African ICU wards and the nurses' ratio to hospital beds. The results indicated that there was one nurse for every bed (ratio 1:1); however, the study reported that nurses are overworked and work more than 40 hours per week. In addition, low work morale was reported amongst nurses in South African ICU wards (Scribante & Bhagwanjee, 2007).

South Africa has a total of 337 public hospitals. The public sector works on a referral system. In 2012, according to Section 35 combined with Section 90 of the National Health (Act No. 61 of 2003), the then Minister of Health amended specific regulations on the classification of South African public hospitals. South African public hospitals are grouped into five categories (tertiary, district, central, specialized and regional hospitals) based on the healthcare services they provide (Department of Health, 2017). A tertiary hospital has between 400 to 800 beds available. It provides intensive care services, which are supervised by a specialist or a specialist intensivist. It offers specialist level services such as those offered by a regional hospital and receives referrals from regional hospitals; however, referrals are not restricted to provincial boundaries (Department of Health, 2017). A tertiary hospital may also provide training for health care workers and service providers. A district hospital is characterized as small, medium or large based on the number of beds it has. A district hospital must typically have between 50 beds and 600 beds (Department of Health, 2017). District hospitals offer general surgery paediatric health services, internal medicine, obstetrics and gynaecology, and a family physician. General specialists that are based at regional hospitals offer support and outreach to District hospitals (Department of Health, 2017). The beds in a central hospital must be a maximum of 1200. A central hospital must provide tertiary services, central referral services, and national referral services. District hospitals also receive patients that are referred from different provinces. In addition, it must be attached to a medical school as a primary teaching platform. Central hospitals provide highly specialized care and expensive services (Department of Health, 2017). Regional hospitals have a bed capacity of 200-800 beds and offer at least one of these specialities: healthcare services: orthopaedic surgery, psychiatry, anaesthetics, and diagnostic radiology. These hospitals receive referrals from district hospitals and are assisted by tertiary hospitals (Department of Health, 2017). Specialized hospitals have 600 beds and provide specialised services such as psychiatric services, infectious diseases, and rehabilitation services (Department of Health, 2017).

1.18 IPC practices in the healthcare sector

The cleanliness or standards of hygiene in hospitals is one of the fundamental requirements to minimize transmission of HAIs as hospital environments act as a reservoir for potential pathogens (Mitchell *et al.*, 2014). Jenkins (2017) classified infection, prevention and control (IPC) as 'four Ps': pathogens, patients, practice, and place. The interaction of the 'four Ps' must be prioritized to manage and mitigate pathogens in the hospital setting (Jenkins, 2017). Direct contact is the primary mode for transmitting pathogenic organisms in the hospital setting (Squeri *et al.*, 2012). Squeri *et al.* (2012) reported a high prevalence of staphylococci present on the hands of medical and paramedical staff, which suggested an increased risk of transmission via direct contact. In the same study, the hospital environment also indicated a high prevalence which determined that IPC procedures were not properly adhered to; or were unsatisfactory. The disinfection of the hospital environment, hospital equipment, hospital visitors and healthcare workers also aids in alleviating the spread of HAIs (Revelas, 2012). Hospital environments being a possible source of methicillin-resistant *S. aureus* (MRSA) contamination and infection has brought forth many opposing views (Dancer *et al.*, 2014). One being that thorough cleaning of hospital environments minimizes the presence of the pathogenic bacteria. In contrast, some pathogens may persist and proliferate on surfaces even after cleaning has been implemented. A study conducted by Mulvey *et al.* (2011) highlights the effectiveness of three methods for monitoring the cleanliness of a hospital and reported that detergent-based cleaning only managed to reduce the levels of organic soil; however, it failed to eliminate staphylococci which survived the cleaning process. There is a gap in literature concerning the link between heavy-metal resistance and the use of detergent-based cleaners in the hospital environment and this should be addressed in future studies.

Hand hygiene is a cost-effective way to reduce pathogens in the hospital environment. The failure of compliance with IPC strategies dramatically affects the transmission of pathogens in hospitals (Khan *et al.*, 2017). Ensuring improved compliance to hand washing and hand hygiene post patient assessments and contact with a patient's surroundings would drastically decrease pathogens that are transmitted between patients by healthcare workers. (Otter *et al.*, 2013). The containment of patients' shedding of pathogens into the hospital environment could also minimise hospital transmission (Otter *et al.*, 2013). Incorporating chlorhexidine in patients' daily bathing routines has been reported to reduce the shedding of pathogens, such as MRSA and further reduce the transmission (Otter *et al.*, 2013). Hospital managers and IPC

officers could ensure that the methods used to clean the hospital environment meet regulated standards. Hydrogen peroxide vapour has been suggested to effectively sanitize wards that had previously occupied by patients carrying virulent and resistant organisms (Jenkins, 2017).

A longitudinal study was conducted by Salge *et al.* (2017) in England during April 2004-March 2009 to determine the outcome after the compulsory national MRSA surveillance initiative was implemented during October 2005. The study used various data from the English National Health Service (NHS) obtained from the England healthcare sector. The results indicated a decrease in MRSA infections due to frequent cleaning, IPC training and increased hand hygiene and surveillance. Durlach *et al.* (2012) defined surveillance as routine and continuous monitoring of the distribution and occurrence of an infection in a population and the factors which influence the threat associated with an infection. Surveillance includes data analysis and the spread of results to implement appropriate solutions (Durlach *et al.*, 2012). Asymptomatic carriers of pathogenic bacteria can be identified before patients are infected and show symptoms (Lee *et al.*, 2018). Active screening for MRSA of all patients has been suggested as a preventative solution; however, this method would not be cost-effective (Lee *et al.*, 2018).

1.19 Study Rationale

South African public hospitals experience a large burden of communicable diseases, particularly with KwaZulu-Natal being an epicentre for tuberculosis (TB) and human immune-deficiency virus (HIV) (Mahomed *et al.*, 2017). As a result, hospital-acquired infections are often neglected and not made a priority, the hospital environment contribution in infections is not well explored (Mahomed *et al.*, 2017) due to understaffing, lack of healthcare-worker training and insufficient funds to maintain IPC protocols. In addition, South African public hospitals also face the challenge of not having a standardized surveillance system for hospital-acquired infections (Steinhaus *et al.*, 2018). There is also a shortage of trained and dedicated healthcare workers that mainly focus on IPC strategies and protocols, and many South African public hospitals lack antibiotic stewardship programs (Steinhaus *et al.*, 2018). Most South African surveillance studies in the healthcare sector, private and public hospitals, focus on clinical isolates. These studies are often not focused on the hospital environment (Lowman,

2016). Studies on *S. aureus* that have previously been conducted in KwaZulu-Natal hospitals have mainly focused on samples collected from patient specimens such as blood, pus, and mucus (Shittu and Lin, 2006; Amoako *et al.*, 2016). A few studies have selectively focused on the hospital environment in the transmission of *S. aureus*. The main outcome of the study is to provide data on *S. aureus* that can be used as a baseline for future surveillance studies.

1.20 Aim and Objectives

Aim of the study

To conduct molecular surveillance of methicillin-resistant *S. aureus* on frequently touched hospital environment sites of four public hospitals of different healthcare levels in the eThekwini district, KwaZulu-Natal, South Africa.

Objectives

- To obtain samples from eleven pre-determined environment sites in the hospital environment viz. ward telephone, nurse's table, drip stands, bp machine, sink, patient file, occupied and unoccupied bed, ventilator, mop and the laundry room door handle from the ICU, general ward, and paediatric wards at four public hospitals of different levels of healthcare in the eThekwini district, KwaZulu-Natal, South Africa.
- To isolate and phenotypically screen for *S. aureus* isolates using chromogenic media and PCR detection of the *nuc* gene
- To ascertain the antibiotic resistance of *S. aureus* to the following antibiotics: erythromycin, cefoxitin, penicillin, tetracycline, linezolid. clindamycin, quinupristin-dalfopristin, ciprofloxacin and rifampicin using the Kirby-Bauer disk diffusion method according to CLSI guidelines
- To identify the presence of *mecA* gene for methicillin-resistant isolates using PCR
- To confirm the presence of antimicrobial resistance (*aac (6')–aph* (2''), *blaZ, tetK, tetM* and *ermC)* and virulence (*LukS/F-PV, hla* and *hld)* genes using PCR
- To analyse the genotypic diversity of *S. aureus* isolates using the enterobacterial repetitive intergenic consensus sequences polymerase chain reaction (ERIC-PCR)

1.21 Study Outline

This study has been presented in three chapters and is in the manuscript format according to the College of Health Sciences requirements at the University of KwaZulu-Natal. Chapter one contains a review of existing literature relating to the study- presented as the introduction and literature review. The aims and objectives, study rationale and a brief description of the study methodology are also included in this chapter. The second chapter is titled "Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) on frequently touched sites in selected public hospitals in Kwazulu-Natal, South Africa". It outlines the prevalence, antibiotic susceptibility testing, analysis of *S. aureus* resistance and virulence genes, statistical analysis, and analysis of the genetic diversity of *S. aureus* isolates on the hospital environment of four public hospitals in KwaZulu-Natal, South Africa. Chapter three presents the conclusions, limitations, and recommendations arising from the study.

1.22 The general methodology

This study defines the molecular surveillance of *S. aureus* on frequently touched hospital environment sites, of four public hospitals, of different levels of healthcare in KwaZulu-Natal, South Africa. Eleven frequently touched sites (ward telephone, nurse's table, drip stands, sink, blood pressure machine, patient file, occupied and unoccupied bed, ventilator, mop, and the laundry room door handle) in the hospital environment were swabbed for the presence of *S. aureus* isolates. Ethical approval was obtained from the Biomedical Research and Ethics Committee (BREC) of the University of KwaZulu-Natal (reference number: **BE606/16)**. Chromogenic media was used for the phenotypic identification of *S. aureus*. Molecular confirmation of the species-specific *nuc* gene was conducted using PCR. The presence of the methicillin resistance gene (*mecA*) was evaluated using PCR to determine methicillin-resistant *S. aureus* (MRSA) isolates. Antibiotic susceptibility testing was conducted using the Kirby-Bauer disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) M100-S27 guidelines. Virulence and antibiotic resistance genes were detected using PCR. Statistical analysis was performed with the Fisher's exact and Pearson Chi-Square tests to determine the significance of the relationship between the virulence genes detected and the site from which the samples originated. The relationships between the virulence genes and the

hospitals as well as virulence genes and wards were determined. The enterobacterial repetitive intergenic consensus (ERIC-PCR) was used to determine the genetic diversity of *S. aureus* isolates collected.

1.23 References

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CHAPTER 2

PREVALENCE OF *METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS* (MRSA) ON FREQUENTLY TOUCHED SITES IN SELECTED PUBLIC HOSPITALS IN KWAZULU-NATAL, SOUTH AFRICA

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Running title: Characterisation of MRSA on hospital surfaces in South Africa

ABSTRACT

Introduction: The hospital environment acts as a reservoir in the transmission of pathogens, such as MRSA, that may cause hospital-acquired infections. The study aimed to evaluate the prevalence of MRSA on some frequently touched hospital sites.

Methods: A total of 777 swabs were randomly collected from frequently touched sites (viz. ward telephone, nurses' tables, drip stands, sinks, bp machines, patient files, occupied and unoccupied beds, ventilators, mops and the laundry room door handles) in the hospital environment of three wards; intensive care unit, paediatrics and general, of four public hospitals in the KwaZulu-Natal province of South Africa. Isolation of *S. aureus* and confirmation was done using chromogenic selective media and PCR amplification of the thermo-nuclease (*nuc*) gene. MRSA isolates were determined by the presence of the *mecA* gene. Virulence and resistance genes (*hla*, *hld*, *luks/F-PV*, *ermC*, *tetk*, *tetM*, *aac (6')-aph (2")*, and *blaZ*) were detected using standard monoplex PCR, antibiotic susceptibility testing was performed with nine selected antibiotics using the Kirby-Bauer disk-diffusion method, The SPSS software version 25 (IBM SPSS Statistics) was used for statistical analyses. Fisher's exact- and Pearson Chi-Square tests were used to determine the significance of the relationship between the virulence genes detected and the site from which the samples originated. ERIC-PCR was conducted to evaluate the genetic relatedness of the isolates.

Results: An overall prevalence of 12.7% (99/777) for *S. aureus* isolates was obtained. Out of these, 89.9% (89/99) were confirmed to be MRSA using PCR. Only 10.1% (10/99) isolates were identified as methicillin-susceptible *S. aureus* (MSSA). The sites with the highest prevalence were the occupied bed 16.2 % (16/99), unoccupied bed 16.2% (16/99), patient file 14.1 % (14/99), phone 13.1 % (13/99) and nurses table 14.1 % (14/99). The sites with the lowest prevalence were the blood pressure machines 6.1 % (6/99), drip stand 6.1 % (6/99), ventilator 6.1 % (6/99), door handle 4 % (4/99), mop 3.0 % (3/99) and sink 1.0 % (1/99). The Pearson's Chi-Square and Fischer's exact test indicated that there was a statistically significant relationship ($p<0.05$) between the *mecA* gene, and the hospital environment sites sampled. A statistically significant relationship ($p<0.05$) was identified between the hospital and the *tetK*, *ermC*, *aac (6')-aph (2")* and *LukS/F-PV* genes. ERIC-PCR produced bands for 87 of the isolates; 12 were non-typeable.

Conclusion: Our findings have led to the conclusion that frequently touched sites in the hospital environment act as reservoirs in the transmission of MRSA in KwaZulu-Natal public hospitals.

Keywords: Methicillin-resistant *Staphylococcus aureus*, hospital-acquired infection, hospital-environmental contamination

2.1 Introduction

A hospital-acquired infection (HAI) or nosocomial infection develops during hospitalisation or within 48 hours after the patient has been discharged. In most cases, this is not the initial cause of hospital admission (Revelas, 2012). *Staphylococcus aureus* is considered one of the most important pathogens responsible for hospital-acquired infections (HAIs) (Liu *et al.*, 2016). HAIs are a financial burden in developed and developing countries by causing significant strain on the economy due to the high cost of treatments, increased mortality and morbidity rates that are associated with these types of infections (De Angelis *et al.*, 2010; Rosenthal *et al.*, 2011, Rahmqvist *et al.*, 2016). In addition, *S. aureus* is one of eight significant pathogens listed by the Global Antimicrobial Resistance Surveillance System (GLASS) alongside *Shigella* spp., *Salmonella* spp., *Streptococcus pneumoniae, Klebsiella pneumoniae*, *Neisseria gonorrhoeae, Acinetobacter* spp., and *Escherichia coli* (WHO, 2017-2018).

S. aureus is a versatile pathogen that causes a variety of diseases, from respiratory to skin infections (Otto, 2014), as it colonises the skin and mucosal membranes of animals and humans (Malachowa & DeLeo, 2010). Lung infections are predominantly of nosocomial origin. In contrast, skin infections are primarily of a community-acquired nature (Otto, 2014). The severity of the disease ranges from minor skin infections to life-threatening conditions (soft tissue abscesses, toxic shock syndrome, pneumonia, septicaemia, bacteraemia and endocarditis) (Tong *et al.*, 2015). Skin infections generally originate from intravascular devices such as catheters and intravenous (IV) drips (Tong *et al.*, 2015). *S. aureus* strains have become resistant to a diverse range of antibiotics resulting in multi-drug resistant (MDR) strains. There is an increasing shortage of antibiotics that can be used to treat these infections (Blair *et al.*, 2014). Methicillin is one of the antibiotics used to treat *S. aureus* infections, the term MRSA

is used for methicillin-resistant *S. aureus* strains. MRSA is a global multi-drug resistant human pathogen present in hospital and community environments (Milheiriço *et al.*, 2017).

Environmental sampling of the hospital surfaces indicated that ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) pathogens are shed into the hospital environment by patients and survive on surfaces for an extended period as they are difficult to destroy through cleaning and disinfection (Otter *et al.*, 2013). Within the hospital, MRSA has been isolated from hospital equipment (Mulvey *et al.*, 2011), hospital linen (Pinon *et al.*, 2013), telephones (Nwankwo *et al.*, 2014), and air (Creamer *et al.*, 2014). There is a dearth of published scientific information on the prevalence of MRSA within the hospital environment in South Africa. Therefore, there is a need to determine if current infection, prevention and control (IPC) strategies are adequate to minimise MRSA in the hospital environment of public hospitals. The study aimed to determine the prevalence of *S. aureus* on frequently touched sites in three hospital wards, the ratio of methicillin-resistant isolates, the presence of resistance and virulence genes, antibiotic susceptibility testing and clonality of *S. aureus* isolates from the hospital environment of four public hospitals classified as central, tertiary, regional and district hospital respectively, in the eThekwini district, KwaZulu-Natal province, of South Africa.

2.2 Methods and Materials

2.2.1 Ethical considerations

The study was approved by the Biomedical Research and Ethics Committee (BREC) of the University of KwaZulu-Natal (reference number: **BE606/16)** and listed on the Health Research and Knowledge Management Database (HRKM 098/17) of the Department of Health (reference no. **KZ 2017RP24 630**). In addition, the district health department and hospital management provided gatekeepers approval.

2.2.2 Sample site

Swab samples were collected over three months (September to November 2017) from four provincial public hospitals classified under the South African National Health Act of 2003 (Department of Health, 2017) according to their different levels of healthcare viz. Central

(hospital A), Tertiary (hospital B), Regional (hospital C) and District (hospital D). Central/Specialized hospitals, with bed sizes between 800-1200, accept referrals from both district and regional hospitals. Tertiary hospitals have 400 to 800 beds available and provide specialised services such as those offered by a regional hospital. They also receive referrals from regional hospitals; however, referrals are not limited to provincial boundaries. Regional hospitals, have between 200 and 800 beds, receive referrals from several district hospitals and provide services to a specific regional population. District hospitals have between 50- 600 beds: depending on their classification of either being small, medium or large. These hospitals receive outreach and support from specialists based at regional hospitals. Samples were collected from three wards viz. intensive care unit (ICU), paediatrics ward and general male ward of the studied hospitals. The Infection, prevention and control (IPC) staff stated that the male general ward had the highest occupation of patients, therefore was chosen instead of the female ward.

2.2.3 Sample Collection

A total of 777 swabs were collected from eleven predetermined and frequently touched surfaces in the hospital environment viz. ward telephone, nurses' tables, drip stands, sinks, blood pressure (bp) machines, patient files, the linen of occupied and unoccupied beds, ventilators, mops, and the laundry room door handles. The stratified random sampling method was used. Samples were collected by gently swabbing approximately a 5 cm circumference of the surfaces using capped Amies agar swabs in translucent transport media (Thermo Fisher Scientific, Waltham, MA USA). The swabs were then placed in a cooler box with ice for transportation to the laboratory and processed within four hours upon arrival.

2.2.4 Phenotypic identification and isolation of *Staphylococcus aureus*

For enrichment purposes, swabs were broken off into labelled 50 mL blue-capped centrifuge tubes containing 20 mL of tryptic soy broth (TSB) (Sigma-Aldrich, Germany) and incubated at 37 °C for ± 24 hrs while stirred (Steyn Scientific, USA). After incubation, a loopful of broth was aseptically inoculated and streaked onto plates containing chromogenic agar, HiChrome ™ *Aureus* Agar Base (HIMEDIA, India), and supplemented with 2 % egg-yolk tellurite

emulsion (HIMEDIA, India). The plates were incubated (Shel Lab, Sheldon Manufacturing Inc., USA) at 37 °C for ± 24 hrs. Single black colonies with clear zones were presumed to be *S. aureus* and stored in cryovials containing TSB supplemented with 10 % glycerol (VWR international life sciences, Amresco, Parkway) until further analyses.

2.2.5 Deoxyribonucleic acid (DNA) extraction

Total genomic DNA was extracted using the conventional boiling method (Millar *et al.*, 2000). Colonies from pure *S. aureus* cultures were suspended in 300 µL of TE (Tris-EDTA) (10mM Tris-HCl pH 8.0 with 1mM EDTA) buffer and vortexed to homogenise the cells. The suspension was boiled in a heating block at 100 °C for 10 minutes with immediate cooling on ice for 5 minutes. The tubes were centrifuged (Beckman Coulter Microfuge 16, United States) at 14000 × *g* for 5 minutes. The supernatant was transferred into a new sterile tube and stored at -20 °C for PCR use. Nanodrop readings were taken to measure the concentration and purity of the isolated DNA using the Thermo Scientific Nanodrop 2000, UV-Vis spectrophotometer (Wilmington, Delaware, USA). Readings within 1.8-1.9 at the ratio of 260/280 were regarded as pure DNA. Nuclease-free water (Thermo Fisher Scientific, Waltham, MA USA) was used to adjust the DNA concentration accordingly for PCR reactions.

2.2.6 Molecular confirmation of *S. aureus*

Primers for the polymerase chain reaction (PCR) were synthesised at Inqaba Biotechnologies (South Africa). The primer sequences, annealing temperatures, and product sizes are indicated in Table 2.2.8. The species-specific thermo-nuclease *nuc* gene (Brakstad *et al.*, 1992) was used for the positive identification of *S. aureus.* The positive control *S. aureus* ATCC 25913 and negative control (nuclease-free water) were used for all reactions. The Bio-RAD, T100^{TM} Thermal Cycler (Singapore), was used to carry out the PCR assays. A 15 µL reaction contained 7 µL of DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA USA), 0.5 µL of both the reverse and forward primers of a 20 µM primer concentration, 3 µL of template DNA and 4 µL of nuclease-free water making a total reaction volume of 15 µL. Gel electrophoresis was carried out by loading the PCR products into a 1.5 % (w/vol) agarose gel with 5 µL of 10 mg/ml ethidium bromide (Thermo Fisher Scientific, Waltham, MA USA) and run at 100 volts for 30 minutes in a tank containing Tris-borate-EDTA (pH 8.3, 1X). A

100bp DNA ladder (New England Biolabs, Ipswich, USA) was used as the molecular weight marker. Gels were viewed on the ChemiDoc™ Imaging System (Bio-Rad Laboratories, Inc., USA).

2.2.7 Antimicrobial susceptibility testing of isolated bacteria.

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method, according to the Clinical and Laboratory Standards Institute M100-S27 guidelines (M100-S27, 2017), using Mueller-Hinton agar (Oxoid, England). An inoculum was suspended in sterile water and developed using the 0.5 McFarland standard (Den-1B McFarland, Biosan, Latvia). All antibiotics were sourced from (Oxoid, England). The following antibiotics were used: erythromycin (macrolides) (15 µg), cefoxitin (penicillins) (30 µg), penicillin (penicillins) (10units), tetracycline (tetracyclines) (30 µg), linezolid (lipopeptides) (30 ug), clindamycin (lincosamides) (2 µg), quinupristin-dalfopristin (streptogramins) (15 µg), ciprofloxacin (fluoroquinolones) (5 µg) and rifampicin (ansamycins) (5 µg). These antibiotics were selected based on a review conducted by Gold and Pillai (2009) for their clinical use in human therapy. *S. aureus* ATCC 25923 was used as the control strain. Methicillin resistance of *S. aureus* isolates were identified using cefoxitin testing following CLSI guidelines (M100-S27, 2017).

2.2.8 Genotypic identification of resistance and virulence genes

DNA extraction and volumes used to conduct singleplex reactions were as described previously. The PCR primers and conditions followed were indicated in Table 2.2.8 The resistance genes: *aac (6')–aph* (2"), *blaZ*, *tetK*, *tetM* and *ermC* that confer resistance to aminoglycoside, macrolide-lincosamide-streptogramins B [MLS_B], tetracycline and erythromycin, respectively, were identified. Virulence genes for Panton-Valentine leucocidin (*LukS/F-PV*), alpha and delta haemolysin (*hla* and *hld)* were also identified. The positive control *S. aureus* ATCC 25913 and negative control (nuclease-free water) were used for all the reactions conducted. Analysis of PCR conditions were conducted as previously stated.

2.2.9 Analysis of *S. aureus* genetic diversity using enterobacterial repetitive intergenic consensus (ERIC) PCR

Genomic DNA was extracted using the Quick-DNA^{TM} Miniprep Plus kit (ZymoResearch, USA) according to the manufacturer's instructions. The purity and concentration were measured using the Thermo Scientific Nanodrop 2000, UV-Vis spectrophotometer (Wilmington, Delaware, USA). A reaction volume of 25 µL was set up; 12.5 µL of DreamTaq Green PCR Master mix 2X (Thermo Fisher Scientific, Waltham, MA USA), 9.3 µL of nuclease-free water, 3 µL of template DNA and 0.1µL of 100mM ERIC 1 (5'-CACTTAGGGGTCCTCGAATGTA-3') and ERIC 2 (5'-AAGTAAGTGATGGGGTGAGCG-3') primers (Inqaba Biotechnologies, South Africa). The PCR conditions were as follows: 95 °C for 2 minutes, 35 cycles of 30 s of denaturation at 90 °C, 1 min. of annealing at 52 °C, 8 min. of extension at 65 °C and final elongation at 65 °C for 16 min. Gel electrophoresis was performed at 70V for 75 min. The agarose gel was prepared as previously mentioned. The ERIC-PCR gels were captured (ChemiDoc^{TM} Imaging System (Bio-Rad Laboratories, Inc., USA) and analysed using BioNumerics software version 6.6 Applied Maths NV (BioMérieux, Sint-Martens-Latem, Belgium). A dendrogram was produced using an unweighted pair group with arithmetic mean (UPGMA) method and dice coefficient parameters of 1% tolerance and 0.5% optimisation.

2.2.10 Statistical Analysis

The SPSS software version 25 (IBM SPSS Statistics) was employed for statistical analyses using a probability value of less than 0.05 ($p<0.05$) for significance. Fisher's exact and Pearson Chi-Square tests were used to determine the significance of the relationship between the virulence genes detected and the site from which the samples originated. In addition, the relationships between the virulence genes and the hospitals, as well as virulence genes and wards, were determined. For each statistical model, the dependent variable was the virulence gene presence (0 = absent; 1 = present).

Table 2.2.8: Cycling conditions and primer sequences for genes presented in this study

	Description	Gene	Primer Sequence	PCR conditions	Bp size	Reference
Species-specific gene	Thermonuclease	*Nuc*	**F**- GCGATTGATGGTGATACGGTT **R**- AGCCAAGCCTTGACGAACTAAAGC	30 s 95 °C, 30 s 55 °C, 1 min 72 °C	270	(Brakstad *et al.*, 1992)
Virulence genes	Haemolysin	*Hla*	**F**- CTGATTACTATCCAAGAAATTCGATTG **R**- CTTTCCAGCCTACTTTTTTATCAGT	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	209	(Hoseini-Alfatemi *et al.*, 2014)
		Hld	**F**-AAGAATTTTTATCTTAATTAAGGAAGGAGTG **R**- TTAGTGAATTTGTTCACTGTGTCGA	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	111	(Hoseini-Alfatemi *et al.*, 2014)
	Panton-Valentine leukocidin	*lukS/F-PV*	**F**- ATCATTAGGTAAAATGTCTGGACATGATCCA **R**- GCATCAAGTGTATTGGATAGCAAAAGC	30 s 95 °C, 45 s 60 °C, 1 min 72 °C	443	(Hoseini-Alfatemi *et al.*, 2014)
Resistance genes	Erythromycin	*ermC*	**F**- CTTGTTGATCACGATAATTTCC **R**- ATCTTTTAGCAAACCCGTATTC	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	190	(Kuntová *et al.*, 2012)
	Tetracycline	*tetK*	**F**- TCGATAGGAACAGCAGTA **R**- CAGCAGATCCTACTCCTT	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	169	(Kuntová *et al.*, 2012
		tetM	**F**- GTGGACAAAGGTACAACGAG **R**- CGGTAAAGTTCGTCACACAC	50s 95 °C, 1 min 55°C, 1 min 72°C	657	(Thong *et al.*, 2012)
	Aminoglycosides	*aac (6')-aph (2'')*	**F**- TAATCCAAGAGCAATAAGGGC **R**- GCCACACTATCATAACCACTA	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	227	(Kuntová *et al.*, 2012
	Methicillin	*mecA*	**F**- AACAGGTGAATTATTAGCACTTGTAAG **R**- ATTGCTGTTAATATTTTTTGAGTTGAA	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	174	(Kuntová *et al.*, 2012)
	β-lactamase	*blaZ*	**F**- ACTTCAACACCTGCTGCTTT **R**- TGACCACTTTTATCAGCAAC	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	173	(Kuntová *et al.*, 2012)

2.3 Results

2.3.1 Prevalence of *Staphylococcus aureus*

The *S. aureus* species-specific *nuc* gene was identified in 12.7 % (99/777) isolates. The prevalence of *S. aureus* isolates per hospital was as follows: the central hospital (hospital A) had a majority of 23.2% (23/99), the tertiary hospital (hospital B) had a prevalence of 23.2% (23/99), the regional hospital (hospital C) had a prevalence of 33.3 % (33/99), and lastly, the district hospital (hospital D) had a prevalence of 20.2 % (20/99) (Figure 2.3.1). The general ward revealed the highest prevalence with 41.4 % (41/99) isolates, followed by the paediatric ward 34.3 % (34/99) and ICU 24.2 % (24/99), no statistical significance was observed ($p \geq 0.05$). Isolates were recovered from all the eleven sites sampled. The sites with the highest prevalence (indicated in Figure 2.3.2) were the occupied beds 16.2% (16/99), unoccupied beds 16.2 % (16/99), patient files 14.1% (14/99), telephones 13.1% (13/99) and nurses' tables 14.1 % (14/99). The sites with the lowest prevalence were the blood pressure machines 6.1 % (6/99), drip stands 6.1 % (6/99), ventilators 6.1 % (6/99), door handles 4 % (4/99), mops 3.0 % (3/99) and sinks 1.0 % (1/99). The *mecA* gene was identified in 89.9% (89/99) of the *S. aureus* isolates. Only ten isolates were identified as methicillin-susceptible *S. aureus* (MSSA) (Figure 2.3.2). There was no statistical significance ($p > 0.05$) observed between the presence of the *mecA* gene and the hospital levels (supplementary Table 1).

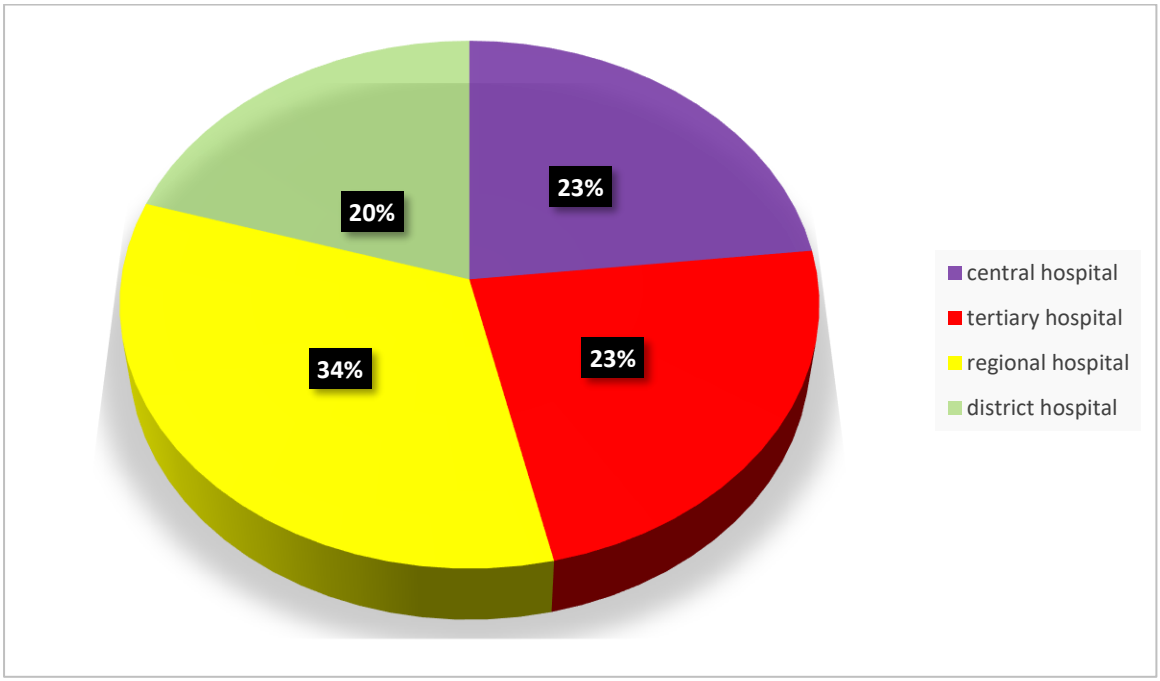

Figure 2.3.1: The *S. aureus* isolate prevalence obtained from four public hospitals in the eThekwini district, KwaZulu-Natal, South Africa

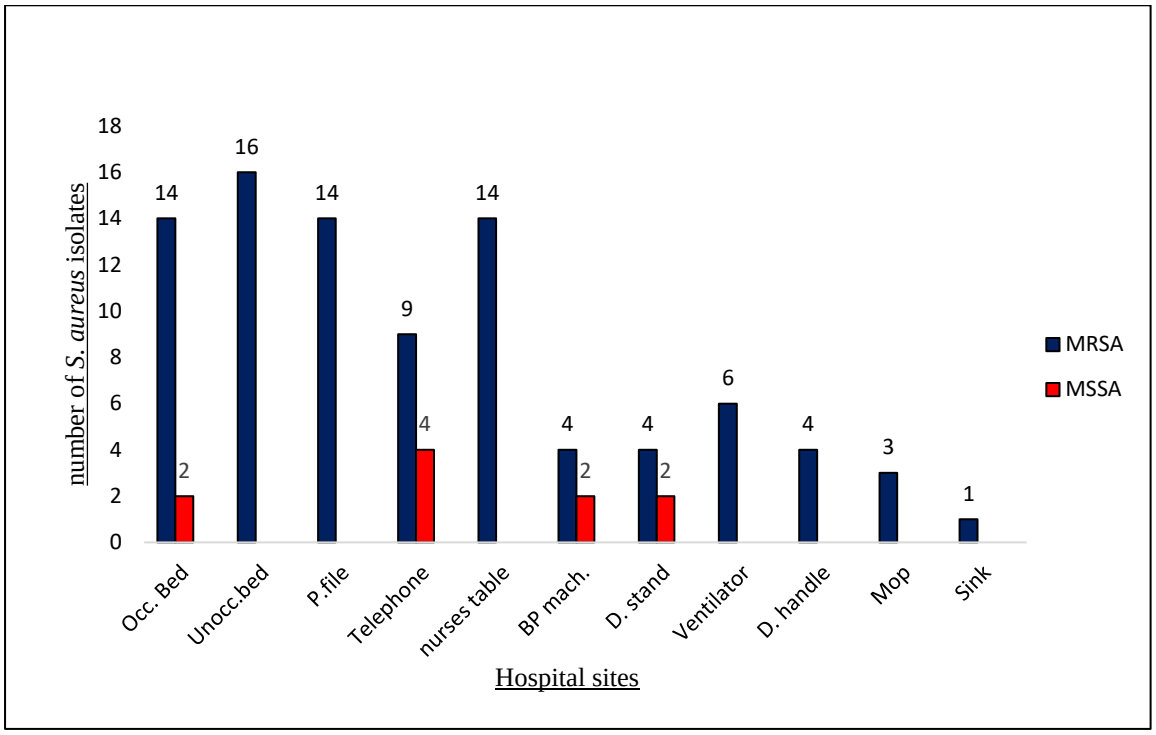

Figure 2.3.2: Prevalence of *S. aureus* (MRSA and MSSA) isolates sampled on eleven hospital sites within the wards and public hospitals investigated.

2.3.2 Antibiotic susceptibility tests

The Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S27, 2017) were used to interpret the zone outcomes of the antibiotic susceptibility tests. Antimicrobial susceptibility testing was performed on all the 99 isolates collected. The isolates showed the highest resistance to penicillin (60; 60%), followed by cefoxitin (46; 46%) and erythromycin (42; 42 %). Less resistance was observed for quinupristin-dalfopristin (29; 29%), clindamycin (27; 27 %), ciprofloxacin (18; 18%), tetracycline (10; 10%), rifampicin (13; 13%) and linezolid (3; 3 %). A total of 24/99 (24%) of the isolates showed multi-drug resistance, resistance to three or more antibiotic classes. Three isolates were resistant to six of the antibiotic classes incorporated in this study. Two isolates were from the ward telephones in the ICU and paediatric wards of the regional hospital (C) and central hospital (A). One isolate was from the ventilator in the ICU of the district hospital (A).

2.3.3 Virulence and resistance genes

The gene with the highest frequency observed was the haemolysin gene *hld* 87 (87.9%). The *tetM* and *tetK* genes encoding tetracycline resistance were detected in 60 (60.6%) and 57 (57.6%) of the isolates, respectively. The *blaZ*, *ermC* and *LukS/F-PV* genes were present is 53 (53.5%) of the isolates. The genes with the lowest frequencies were the *aac (6')-aph (2")* and *hla* with a frequency of 29 (29.3%) and 45 (45.5%), respectively. The Pearson's correlation analyses (Supplementary Table 2) demonstrated that the *mecA* gene had a significant ($p < 0.05$) positive correlation with the *tetK* (25.5%), *ermC* (36.0%), *blaZ* (22.5%), *aac (6')-aph (2")* (21.6%), *tetM* (41.6%) and *LukS/F-PV* (22.5%) genes. The study also identified that the *aac (6')-aph (2")* gene had a positive correlation ($p < 0.05$) to *tetM* (24.6%), *tetK* (28.3%), *ermC* (33.3%), *blaZ* (37.7%) and *LukS/F-PV* (19.9%) genes. The *blaZ* gene had a significant positive correlation ($p < 0.05$) with *ermC* (47.2%) and *tetM* (20.2%). The *tetK* gene had a strong and positive correlation ($p < 0.05$) with *ermC* (30.7%), *hla* (-24.3%), *tetM* (35.4%) and *LukS/F-PV* (34.8%). *ErmC* had a strong and positive correlation ($p < 0.05$) with *tetM* (36.8%) and *LukS/F-PV* (39.1%). The relationships between the genes and site, genes and ward as well as the genes and hospital were examined. The Pearson's Chi-Square and Fischer's exact test indicated that there was a significant relationship ($p<0.05$) between the *mecA* gene and the site. A significant relationship ($p<0.05$) was identified between the hospital and the *tetK*, *ermC*, *aac (6')-aph (2")* and *LukS/F-PV* genes.

2.3.4 ERIC-PCR

The enterobacterial repetitive intergenic consensus (ERIC-PCR) was used to determine the genetic diversity of *S. aureus* isolates collected from frequently touched surfaces in the hospital environment of four public hospitals in KZN, South Africa. Bands were produced for 87 of the isolates recovered; 12 of the isolates were non-typeable (these isolates showed no bands under ERIC-PCR conditions). The BioNumerics software (BioMérieux, Sint-Martens-Latem, Belgium) assigned 87 isolates into 54 different ERIC-types, namely A-BC, based on a similarity index, indicated by the solid red line, of ≥ 60% (Figure 2.3.4). The results obtained indicated a high level of genetic diversity. The software grouped (18/87; 21%) of the *S. aureus* isolates into six major ERIC-types: F (n = 3), G (n = 3), P (n = 3), W n = 3), AC (n = 3), AG (n = 3) (Figure 2.3.4). It was observed that out of the six major ERIC-types, three major ERIC-

types (P, W and AC) were shared between two hospitals, viz. district hospital (D) and regional hospital (C). The AG (n = 3) cluster indicated that isolates from the ward telephone (n=2) and unoccupied bed (n = 1) within the paediatrics ward of the tertiary hospital (B) belonged to the same ERIC-type. A 100 % similarity and the same antibiogram was observed for the isolates belonging to the BA_1 (n=2) ERIC-type. These isolates were from the blood pressure machines in the general ward of the regional hospital (C) and the door handle in the paediatrics ward from the tertiary hospital (B).

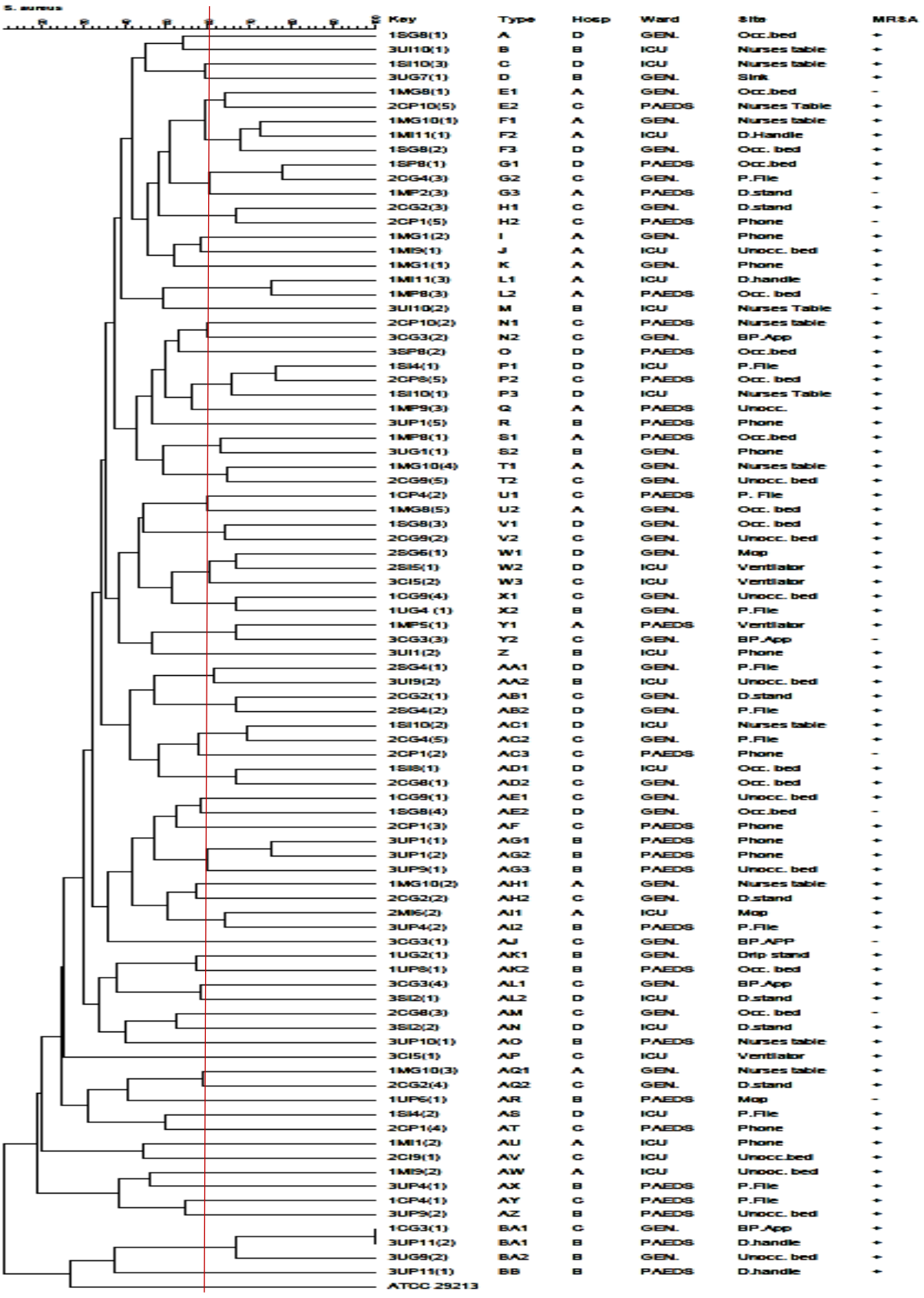

Figure 2.3.4: ERIC-PCR fingerprinting of *Staphylococcus aureus* ERIC-type groups of isolates recovered from four public hospitals in KZN, South Africa. An index of 60%, indicated

by the solid red line, was used respectively to determine similarity and clustering. *S. aureus* ATCC 29213 was used as the quality control strain.

2.4 Discussion

The sanitation of a hospital environment plays a crucial role in limiting the spread of pathogenic organisms such as MRSA (Mitchell *et al.*, 2014). MRSA can be transferred from person to person or from person to frequently touched objects in the hospital environment and vice versa (Nwankwo *et al.*, 2014). The overall prevalence of *S. aureus* obtained was 12.7 % (99/777) which comprised of 89 isolates of MRSA (methicillin-resistant *S. aureus*) and ten isolates of MSSA (methicillin-susceptible *S. aureus*). The result obtained indicated a low prevalence of MSSA. Although the reported MSSA prevalence was low, MRSA and MSSA do not differ in the diseases they cause (Otto, 2012). Due to a shortage in published studies of a similar nature in South Africa, the MRSA prevalence obtained in this study was compared to internationally reported prevalence rates. In Africa, a lower prevalence rate of 2.7 % (1/37) was reported by Adekunle *et al.* (2019), who had studied environmental isolates collected from a general hospital in Nigeria. In addition, a prevalence of 17% (8/47), mainly from door handles, was reported in a study conducted in three government hospitals in Ghana (Saba *et al.*, 2017).

The prevalence rate obtained in this study was higher than a survey by Mukhiya *et al.* (2012), who obtained 40.7 % (11/27) from environmental isolates collected from hospitals in Nepal; a developing country. The level of healthcare provided by these hospitals had not been specified. The prevalence in our study was also higher than that of Ekrami *et al.* (2011), who obtained an MRSA prevalence of 60.0 % from hospital environmental isolates collected from hospitals in Iran which is also a developing country. South African public hospitals lack funding, a shortage of resources, are understaffed and often overcrowded (Ranchod *et al.*, 2017). These factors greatly affect IPC implementation and may contribute to a lack in hospital-hygiene management. The regional hospital (hospital C) had the highest number of isolates compared to the other three hospitals. However, patients may act as vectors that translocate hospital-acquired pathogens between hospitals (Donker *et al.*, 2012). This was evident in a study by Donker *et al.* (2012), who reported a positive correlation (33.0 %) between patient referrals and the incidence of hospital-acquired pathogens such as MRSA in hospitals in England and the Netherlands.

One of the transmission routes of MRSA is through direct skin contact and shedding of epidermal skin cells (Stiefel *et al.*, 2011). Infected patients or patients that are carriers of MRSA may shed their skin onto the hospital beds. The results also revealed that there was a significant relationship ($p<0.05$) between the *mecA* gene and the site (shown in Table 2). These results are similar to Adwan *et al.* (2015), who also reported a significant relationship. It is interesting to note that the highest prevalence of MRSA was obtained from the unoccupied beds compared to other sites in the study (see Figure 2.3.2). These results are an indication that IPC protocols pertaining to the laundry or hospital bed disinfection were unsatisfactory as the presence of MRSA may have emanated from a previously admitted patient. The results showed that the occupied and unoccupied beds accounted for 32.4 % of the total isolates. These results indicate substandard cleaning agents or improper execution of IPC protocols. Contamination of unoccupied beds may occur before the patient has had direct contact with the site. These results are consistent with the results obtained by Pinon *et al.* (2013), who conducted a study in a hospital in France as *S. aureus* has been reported to contaminate bed linen even though the sheets and pillowcases had been washed (Pinon *et al.*, 2013). A possible reason may be attributed to the survival abilities of *S. aureus* for one to 90 days or more on fabrics and materials such as cotton, cotton terry, cotton-polyester blend, polyester, and polypropylene plastic (Neely & Maley, 2000).

S. aureus has been associated with a low infectious dosage indicating that *S. aureus* is highly contagious even in small amounts, only 15 cells of *S. aureus* introduced into experimental lesions were enough to result in infection (Otter *et al.*, 2014). Therefore, patients through direct contact with the surface may be exposed to the pathogen through open wounds or post-surgical procedures. Thus, suggesting that contaminated surfaces may be an essential and underappreciated source of MRSA transmission (Stiefel *et al.*, 2011). Transmission of pathogens is dependent on a range of factors which are but not limited to: the viability of the pathogen on that environmental site; relative humidity; the frequency of contact between patients; healthcare workers, and contaminated surfaces; ambient temperature; and the dose of the transmitted pathogen (Adwan *et al.*, 2015).

The antibiotic susceptibility testing results showed that only 46% of the isolates were resistant to cefoxitin, the antibiotic used as an indication for methicillin resistance of *S. aureus* (MRSA) isolates, according to CLSI recommendations (M100-S27, 2017). The results obtained were

contrary when compared to the high *mecA* gene presence of 89 % indicated by the PCR results. *S. aureus* strains that are methicillin-resistant carry either the *mecA*, *mecB* or *mecC* genes. These genes are acquired genetic determinants that encode for PBP2a or PBP2a' - low-affinity penicillin-binding proteins (Otto, 2012). PBP2a has a very low affinity for most β-lactam antibiotics (Otto, 2012).

The level of *mecA* transcription or presence in isolates does not predict the level of phenotypic methicillin resistance. A possible explanation is found in the work of Lee *et al.* (2018), who identified three attributing factors. The first factor is stringent stress response (the bacteria's reaction to different stress conditions, such as amino acid, fatty acid, iron limitation, and heat shock) (Lee *et al.*, 2018). The work of Boyle-vavra *et al.* (2006) presented the second factor. The inactivation of *VraS* was shown to have induced the transcription of *mecA* but did not increase the level of PBP2a activity (Boyle-vavra *et al.*, 2006). *Vras* is a part of the regulatory system made up of the sensor protein *VraS* and response regulator protein *VraR*. These proteins are involved in controlling the cell wall peptidoglycan biosynthesis (Lee *et al.*, 2018). The third factor is the chaperone foldase protein, *PrsA*, which changes the amount of correctly folded PBP2a that is found in the membrane. As a result, this would intern affect the methicillin resistance without hindering the transcription of the *mecA* gene. Thus, these factors are an indication that the gene presence does not determine antibiotic resistance (Lee *et al.*, 2018)

The presence of the *LukS/F-PV* gene is commonly associated with strains of community-acquired MRSA; however, this varies based on geographic locations (Bhatta *et al.*, 2016). The virulence gene for Panton-Valentine leucocidin, *LukS/F-PV*, was detected in 53,5 % of the isolates. This percentage was higher than previously reported by Adwan *et al.* (2015), who obtained an incidence of 14.3 % in a similar study. The presence of this gene indicates the possible production of a toxin, which induces the formation of virulence pores in leukocytes (Adwan *et al.*, 2015). If patients are exposed to these strains, this may result in severe chronic skin infections or necrotising pneumonia with an extremely high mortality rate even in young and healthy patients. The presence of the *LukS/F-PV* gene obtained in this study is contrary to the results of Bhatta *et al.* (2016), who reported that there was no presence of this gene among hospital environment isolates collected in Nepal; which may have been an indication of the gene not being associated with isolates from the hospital environment.

There are limited studies that have implemented ERIC-PCR to evaluate the clonality of *S. aureus* isolates from the hospital environment. The ERIC-PCR results obtained in this study showed a high diversity between the *S. aureus* isolates collected. The results showed that 12 of the isolates were non-typeable under ERIC-PCR conditions. These results were compared to the study conducted by Adwan *et al.* (2015). Their results indicated that two isolates out of 265 swabs collected from two hospitals in Iran were non-typeable under ERIC-PCR conditions. They further conducted *spa*-typing on the non-typeable isolates. Due to study budget constraints, *spa*-typing could not be performed on the 12 isolates which did not produce bands. Other studies have also reported the occurrence of non-typeable isolates under ERIC-PCR. The ERIC-PCR results indicated three of the six major ERIC-types were shared between district hospital (D) and regional hospital (C). District hospitals fall under level 1 of the referral system of South African hospitals (KZN Department of Health, 2014). Patients are referred from the district hospitals to the local, regional hospital whenever the correct health service cannot be offered. These results further highlight the transmission risk of pathogens between hospitals due to referrals. The spread of *S. aureus* was observed between two sites of the same hospital ward (Figure 2.3.4).

2.5 Conclusion

In summary, our findings have concluded that hospital environments act as reservoirs in the transmission of MRSA in KwaZulu-Natal public hospitals. This paper has investigated the prevalence, antibiotic resistance, characterized resistance and virulence genes, and the *S. aureus* isolates' genetic relatedness of the hospital environment isolates. We have confirmed the presence of the *LukS/F-PV* gene in the hospital environment associated with *S. aureus*. More emphasis on IPC protocols needs to be placed on the laundry procedures carried out for bedding and laundry materials. Our study further highlights the spread of *S. aureus* within the same hospital ward. Limitations exist in our work. Despite this, we conceive that our work can be used as a framework for future surveillance initiatives to improve hospital hygiene through IPC strategies centred around *S. aureus* to minimize the presence of *S. aureus* and other pathogenic micro-organisms that are present in the hospital environment. This would minimise hospital-acquired infections, due to contaminated sites, and provide a safe environment for patients and healthcare workers.

2.6 Limitations

The hospital staff and management had to be informed before arriving at the hospital and sampling conducted. In addition, it is suspected that the hospital cleaning staff cleaned the sites in some instances as they feared that they were under evaluation. This may have influenced the findings of this study.

2.7 Conflict of Interest

None

2.8 Funding

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2.10 Author contribution

Co-conceptualized the study: LB, CS, OZ. Sample collection: SM. Performed the laboratory work: SM. Analysed the data: SM, LB, DA. Vetting of the results: SM, LB. Wrote the paper: SM. Undertook critical revision of the manuscript: SM, DA, LB, OZ.

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Supplementary Tables

Table 1: Fischer's exact test and Pearson's Chi-Square test for virulence and resistance genes from eleven frequently touched sites, wards and hospitals

		Asymptotic significance (2-sided)								
	Statistical Tests	**Resistance Genes**						**Virulence Genes**		
		mecA	*tetM*	*tetK*	*ermC*	*aac (6')-aph (2'')*	*blaZ*	*LukS/F-PV*	*hla*	*hld*
Site	Pearson's Chi-Square Tests	**0.031***	0.831	0.06	0.255	0.887	0.590	0.577	0.185	0.361
	Fisher's exact test	**0.02***	0.825	**0.05***	0.215	0.916	0.639	0.646	0.170	0.424
Ward	Pearson's Chi-Square Tests	0.158	0.831	0.062	0.255	0.887	0.590	0.577	0.185	0.361
	Fisher's exact test	0.125	0.054	0.163	0.761	0.375	0.067	0.107	0.314	0.362
Hospital	Pearson's Chi-Square Tests	0.124	0.057	**0.000***	**0.010***	**0.003***	0.111	**0.000***	0.191	0.061
	Fisher's exact test	0.104	0.051	**0.000***	**0.008***	**0.003***	0.116	**0.000***	0.196	0.072

The statistical significance of the relationship between the virulence genes detected and the site, ward and hospital were examined. The highlighted values with an asterisk (*) indicated a probability value of less than 0.05 *($p < 0.05$).*

Table 2: Comparison of Pearson's correlation for virulence and resistance genes detected in *S. aureus* environmental isolates from four public sector hospitals

		mecA	tetK	ermC	blaZ	aac (6') aph (2")	hla	hld	tetM	lukS/F-PV
mecA	Pearson Correlation	1	.255*	.360**	.225*	.216*	-.098	-.013	.416**	.225*
	Sig. (2-tailed)	-	.011	.000	.025	.032	.335	.898	.000	.025
tetK	Pearson Correlation	.255*	1	.307**	.102	.283**	-.243*	.084	.354**	.348**
	Sig. (2-tailed)	.011	-	.002	.316	.005	.016	.411	.000	.000
ermC	Pearson Correlation	.360**	.307**	1	.472**	.333**	-.126	.054	.368**	.391**
	Sig. (2-tailed)	.000	.002	-	.000	.001	.215	.596	.000	.000
blaZ	Pearson Correlation	.225*	.102	.472**	1	.377**	-.085	.054	.202*	.188
	Sig. (2-tailed)	.025	.316	.000	-	.000	.403	.596	.045	.063
aac (6') aph (2")	Pearson Correlation	.216*	.283**	.333**	.377**	1	-.186	-.194	.246*	.199*
	Sig. (2-tailed)	.032	.005	.001	.000	-	.065	.055	.014	.048
hla	Pearson Correlation	-.098	-.243*	-.126	-.085	-.186	1	.068	-.219*	-.166
	Sig. (2-tailed)	.335	.016	.215	.403	.065	-	.505	.029	.100
hld	Pearson Correlation	-.013	.084	.054	.054	-.194	.068	1	.041	.184
	Sig. (2-tailed)	.898	.411	.596	.596	.055	.505	-	.688	.070
tetM	Pearson Correlation	.416**	.354**	.368**	.202*	.246*	-.219*	.041	1	.285**
	Sig. (2-tailed)	.000	.000	.000	.045	.014	.029	.688	-	.004
lukS/F-PV	Pearson Correlation	.225*	.348**	.391**	.188	.199*	-.166	.184	.285**	1
	Sig. (2-tailed)	.025	.000	.000	.063	.048	.100	.070	.004	-

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

CHAPTER 3

OVERVIEW OF SIGNIFICANT FINDINGS AND IMPLICATIONS OF THE STUDY, LIMITATIONS AND FUTURE RECOMMENDATIONS

The study describes the prevalence, antibiotic resistance profiles, virulence genes, and the genetic relatedness of *S. aureus*, isolates collected from frequently touched environmental sites in four public hospitals in the KwaZulu-Natal district, South Africa. The most striking results, the study limitations and recommendations are presented in this chapter.

3.1 Significant Findings and Implications

A series of analyses were performed in this project to isolate and detect methicillin-resistant *S. aureus* isolates from frequently touched sites in the hospital environment of four public hospitals. Samples were successfully collected from eleven hospital environment sites viz. ward telephone, nurse's table, drip stands, sink, bp machine, patient file, occupied and unoccupied bed, ventilator, mop, and the laundry room door handle. *S. aureus* isolates were isolated using chromogenic media and PCR amplification of the *nuc* gene. The overall prevalence of 12.7 % (99/777) of *S. aureus* recovered from 777 swabs was relatively low. This could be due to *S. aureus* being commonly found in the anterior nares and the skin of carriers and infected patients (Tong *et al.*, 2015). The presence of the *mecA* gene for methicillin-resistant *S. aureus*, was confirmed in 89/99 of the *S. aureus* isolates using PCR. Ten isolates lacked the presence of the *mecA* gene and were identified as methicillin-susceptible *S. aureus*. The infections caused by MRSA and MSSA do not differ; however, MRSA infections are associated with increased costs due to extended hospitalization of patients and higher mortality (Otto, 2012). The evidence of this study suggests that environmental sites of hospitals in a KwaZulu-Natal district are a reservoir for MRSA.

The regional hospital (hospital C) was observed to have the highest prevalence of *S. aureus* isolates compared to the district, central and tertiary hospitals. KwaZulu-Natal regional hospitals offer district hospitals relief by accepting referred patients when these hospitals cannot provide assistance (KZN Department of Health, 2014). Referrals increase the movement and number of patients in hospitals. The movement of patients creates a transmission risk of MRSA between public hospitals and, as a result, may increase the prevalence, as reported by (Donker *et al.*, 2012). These results highlight that hospitals should not be viewed in isolation

but as part of a system in creating IPC strategies. The male general ward obtained the highest number of isolates, 41.4 % (41). These results were an indication that the male general ward is an increased risk for *S. aureus* contamination. In addition, the results interestingly identified that the hospital beds accounted for 32.4 % (32) of the isolates collected. The relationship between the presence of the *mecA* gene and the site was found to be significant ($p < 0.05$). These results highlight that more emphasis needs to be placed on IPC strategies for occupied and unoccupied beds. The antibiotic susceptibility testing (AST) results revealed that 24 % (24/99) of the isolates were multi-drug resistant (MDR). The isolates were mainly resistant to the penicillins, lincosamides, fluoroquinolones, macrolides, tetracyclines, streptogramins and ansamycins antibiotic classes. β-lactams, fluoroquinolones, macrolides and lincosamides are commonly prescribed for *S. aureus* treatment (Moodley *et al.*, 2010). Multi-drug resistant organisms lead to increased treatment costs, and there is a shortage of antibiotics to treat these organisms (Blair *et al.*, 2014).

Monoplex PCR was used to detect the presence of five antimicrobial resistance genes (*aac (6')–aph* (2"), *blaZ, tetK, tetM* and *ermC*) and three virulence genes (*LukS/F-PV, hla* and *hld).* The Pearson's correlation results demonstrated that the presence of the *mecA* gene had a strong correlation ($p< 0.05$) with the presence of four genes: *tetK, blaZ, aac (6')-aph (2")* and *LukS/F-PV* genes. The *blaZ* gene confers resistance to ampicillin and penicillin, the *aac (6')-aph (2")* gene confers resistance to aminoglycosides. The study also identified that the *aac (6')-aph (2")* gene had a positive correlation ($p< 0.05$) to *tetM* and *LukS/F-PV* genes. The coexistence of genes present in MRSA isolates has previously been reported in a study by (Hoseini-Alfatemi *et al.*, 2014). The results indicated that the presence of the *LukS/F-PV* was high (53.5 %). Bhatta *et al.* (2016) reported that the Panton-Valentine Lukocidin gene, *LukS/F-PV,* is associated with community-acquired MRSA strains. There was no presence of the gene in isolates collected from the hospital environment. This was contrary to our findings as the gene was present in this study. The enterobacterial repetitive intergenic consensus (ERIC) PCR results identified that the isolates collected were genetically diverse. Six major ERIC types were observed (Figure 2.3.4). Interestingly, three major ERIC types were shared between the district hospital (D) and the regional hospital (C). The ERIC-PCR results also grouped major ERIC-type AG, three isolates that belonged to different sites of the same hospital ward. Two isolates, the BA_1 (n=2) ERIC-type, showed 100 % similarity but belonged to different hospitals viz. regional hospital (C) and tertiary hospital (B).

In summary, this study has highlighted the methicillin-resistant *S. aureus* contamination on frequently touched hospital sites, virulence, and resistance; and the clonal diversity of *S. aureus* isolates in the hospital environment of four KwaZulu Natal public hospitals. Our findings may be used as a baseline for future surveillance initiatives, with focus on unoccupied beds, to improve hospital hygiene through IPC strategies centred around *S. aureus* in KwaZulu-Natal public hospitals.

3.2 Limitations of the study

The hospital staff and management had to be informed prior to arrival at the hospital and sampling being conducted. It is suspected that the hospital cleaning staff cleaned the sites in some instances as they feared they were under IPC evaluation. As a result, this would have affected the prevalence of *S. aureus* isolates. In addition, the hospitals included in this study were only from the eThekwini district in KZN. Therefore, the results obtained in this study are not a representation of all public hospitals in KwaZulu-Natal or South Africa. Another limitation is the number of hospital wards and the number of hospital sites that were collected. Thus, this study also does not represent all the wards present at each hospital. Lastly, due to cost constraints, sequencing and *spa*-typing of isolates that did not produce bands under ERIC-PCR condition could not be conducted.

3.3 Recommendations

- Future studies should analyse the role seasonality may play in the prevalence of MRSA in the hospital environment. The time of year and season that samples are collected may affect the results obtained.
- The number of hospitals and wards studied should be increased in future studies. This would increase the sample size.
- The samples should be taken unexpectedly without the hospital cleaning staff being made aware prior to sampling. Sampling should be conducted in different hospitals across the province and country; therefore, the results can serve as a national surveillance system.
- Based on the study findings, IPC protocols should include laundry surveillance on unoccupied beds prior to patient admission

- Further studies need to be conducted to determine the relationship between *mecA* gene presence and cefoxitin resistance in MRSA isolates. The percentage of cefoxitin resistant isolates obtained contrasted with the presence of the *mecA* gene in our study was unexpected.
- Further studies can be conducted to analyse heavy-metal resistance in hospital environmental *S. aureus* isolates due to detergents used. There is a scarcity of studies that have evaluated the presence of heavy-metal resistance genes from isolates in the hospital environment.
- Other molecular techniques for typing such as pulse-field gel electrophoresis (PFGE), repetitive intergenic consensus (REP) PCR and multilocus sequence typing (MLST) can be used apart from ERIC-PCR to evaluate the genetic relatedness of *S. aureus* in the hospital environment.
- Future studies should collect samples simultaneously from the hands of healthcare workers and the hospital environment. This would expand the study further to evaluate the link between the hands of healthcare workers and the hospital environment in KwaZulu-Natl public hospitals

3.4 REFERENCES

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APPENDIX

Appendix A: Ethical clearance for this study obtained from the Biomedical Research and Ethics Committee (BREC) of the University of KwaZulu-Natal **(reference number: BE606/16).**

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
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Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

16 March 2018

Dr LA Bester
Biomedical Resource Unit
School of Laboratory Medicine and Medical Sciences
besterl@ukzn.ac.za

Dear Dr Bester

Protocol: To ascertain the nature and extent of infection, prevention and control (IPC) programs at different levels of care in eThekwini district, KwaZulu-Natal. **Degree:** Non-degree
BREC reference number: BE606/16

We wish to advise you that your application for Amendments received on 01 February 2018 for the above study has been **noted and provisionally approved** by a sub-committee of the Biomedical Research Ethics Committee subject to a response to the following:

1. Site permissions that the amendment is acceptable.
2. An info sheet and consent form for the questionnaire component.
3. Postgraduate approval for new students that are going to be enrolled in the study.

The PI should consider making this a BCA (BREC Class Approval) if the study will be ongoing and if more students are going to be added into the study.

Yours sincerely

[redacted]

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

Appendix B: Research ethics certificates for the completion of the TRREE course addressing introduction to research ethics and evaluation, good clinical practice, HIV vaccine trials, informed consent, and the involvement of adolescents in HIV prevention trials.


TRREE
Zertifikat
Certificat
Certificado
Certificate
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Promoting the highest ethical standards in the protection of biomedical research participants
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The University of Hong Kong
Certificat de formation - Training Certificate
Ce document atteste que - this document certifies that
siyethaba mkhize
a complété avec succès - has successfully completed
Research Ethics Evaluation
du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation
February 17, 2016
Professeur Dominique Sprumont
Coordinateur TRREE Coordinator
FMH
FPH
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TRREE

Zertifikat
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Certificado
Certificate

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

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

siyethaba mkhize

a complété avec succès - has successfully completed

Informed Consent

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

[redacted]

February 17, 2016
CID : g0HXFUBaMZ

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de formation continue (5 Crédits)

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Continuing Education Programme
Programme de formation continue

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TRREE

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Certificat de formation - Training Certificate

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Good Clinical Practice (GCP)

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

[redacted]

February 17th, 2016
CID : Hh2m540.r6T

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation Continue (5 Crédits)

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[REV : 20140328]

Appendix C: Raw data indicating the site, ward and hospital, and the virulence and resistance genes for the samples collected and included in this study

Sample no.	Sample name	site	Ward	Hospital	nuc	mecA	tetM	tetK	ermC	aac(6') aph(2")	blaZ	lukS/F-PV	hla	hld
1	1SI4(1)	Patient file	ICU	District	+	+	+	+	+	+	+	+	+	+
2	2CP10(2)	nurses' table	PAEDS	Regional	+	+	+	+	+	-	+	-	-	+
3	2CP10(1)	nurses' table	PAEDS	Regional	+	+	+	+	-	-	-	-	-	+
4	1MG3(1)	Bp machine	GENERAL	Central	+	+	+	-	+	+	+	-	-	-
5	2SG3(2)	Bp machine	GENERAL	District	+	+	+	-	+	+	+	-	-	-
6	2CG2(1)	Drip stand	GENERAL	Regional	+	+	+	-	-	-	-	-	-	+
7	2CG2(4)	Drip stand	GENERAL	Regional	+	-	-	-	-	-	-	-	-	+
8	2CP8(5)	Occ. Bed	PAEDS	Regional	+	+	-	-	-	-	-	+	+	+
9	1MI9(4)	unocc. Bed	ICU	Central	+	+	-	-	-	-	-	-	-	-
10	2CG4(3)	Patient file	GENERAL	Regional	+	+	-	-	-	-	-	-	+	+
11	2SG4(2)	Patient file	GENERAL	District	+	+	+	-	-	-	-	-	-	+
12	2SI5(1)	ventilator	ICU	District	+	+	+	+	-	+	-	-	+	+
13	1MG10(1)	nurses' table	GENERAL	Central	+	+	-	-	-	-	+	-	+	+
14	1MI11(3)	door handle	ICU	Central	+	+	-	+	-	-	+	+	+	+
15	3SI2(1)	Drip stand	ICU	District	+	+	+	-	-	+	-	+	-	+
16	2SG5(2)	ventilator	GENERAL	District	+	+	-	-	-	-	+	-	+	+
17	2CG9(5)	unocc. Bed	GENERAL	Regional	+	+	+	+	-	-	-	-	+	+
18	2CG8(1)	Occ. Bed	GENERAL	Regional	+	+	+	-	+	-	-	-	+	+
19	3CI5(1)	ventilator	ICU	Regional	+	+	-	+	+	-	+	-	+	+
20	3SP8(2)	Occ. Bed	PAEDS	District	+	+	-	+	-	-	-	-	+	-
21	1MP9(3)	unocc. Bed	PAEDS	Central	+	+	-	-	+	-	+	+	+	+
22	1MI1(2)	Phone	ICU	Central	+	+	+	-	+	+	+	+	+	-
23	1MP8(3)	Occ. Bed	PAEDS	Central	+	-	-	-	-	-	-	-	+	-
24	2SG4(1)	Patient file	GENERAL	District	+	+	-	-	+	-	+	+	-	+
25	1MI9(*)	unocc. Bed	ICU	Central	+	+	+	+	-	-	+	+	+	+

26	1MI11(1)	door handle	ICU	Central	+	+	-	-	-	-	+	-	-	+
27	2CG2(2)	Drip stand	GENERAL	Regional	+	+	-	-	-	-	-	-	+	+
28	3CG3(1)	Bp machine	GENERAL	Regional	+	-	-	+	-	-	-	+	-	+
29	2CP10(5)	nurses' table	PAEDS	Regional	+	+	-	-	-	-	-	-	+	-
30	1MG10(3)	nurses' table	GENERAL	Central	+	+	-	+	+	-	-	-	+	+
31	1MG1(*)	Phone	GENERAL	Central	+	+	-	-	-	+	+	-	-	+
32	1SG8(1)	Occ. Bed	GENERAL	District	+	+	-	-	+	-	-	-	+	+
33	3SP8(*)	Occ. Bed	PAEDS	District	+	+	+	+	-	-	+	-	-	+
34	2CG9(2)	unocc. Bed	GENERAL	Regional	+	+	-	+	-	+	-	-	+	+
35	2CG10(5)	nurses' table	GENERAL	Regional	+	+	-	+	+	-	+	+	+	+
36	1SG8(2)	Occ. Bed	GENERAL	District	+	+	+	+	+	-	-	+	-	+
37	1MP5(1)	ventilator	PAEDS	Central	+	+	+	+	-	-	-	+	+	+
38	1SI10(2)	nurses' table	ICU	District	+	+	+	-	+	+	+	+	+	+
39	1MG8(1)	Occ. Bed	GENERAL	Central	+	-	-	-	-	-	-	-	+	+
40	1SG1(1)	Phone	GENERAL	District	+	-	-	-	-	-	-	+	-	+
41	3CG3(2)	Bp machine	GENERAL	Regional	+	+	-	-	-	-	-	+	+	+
42	2CG4(2)	Patient file	GENERAL	Regional	+	+	+	+	+	+	+	+	-	+
43	2CP1(4)	Phone	PAEDS	Regional	+	-	-	+	-	-	-	-	+	+
44	2MI6(2)	Mop	ICU	Central	+	+	+	+	+	+	+	+	-	+
45	1SI4(2)	Patient file	ICU	District	+	+	+	+	+	-	+	+	-	+
46	1SI10(1)	nurses' table	ICU	District	+	+	-	+	+	+	+	-	+	+
47	1MG10(2)	nurses' table	GENERAL	Central	+	+	-	-	+	-	+	+	-	+
48	3SI2(1)	Drip stand	ICU	District	+	+	+	-	-	-	-	+	+	+
49	3CG3(3)	Bp machine	GENERAL	Regional	+	-	-	-	-	-	-	-	+	+
50	1MG8(5)	Occ. Bed	GENERAL	Central	+	+	+	+	+	+	+	-	-	-
51	1MG1(2)	Phone	GENERAL	Central	+	+	+	-	+	-	+	-	+	+
52	1SI4(2)	Patient file	ICU	District	+	+	+	+	-	-	+	-	+	+
53	1SI8(1)	Occ. Bed	ICU	District	+	+	+	-	+	-	+	+	+	+
54	2CI9(1)	unocc. Bed	ICU	Regional	+	+	+	-	+	-	+	+	+	+
55	2CG9(1)	unocc. Bed	GENERAL	Regional	+	+	+	+	-	-	-	+	-	+
56	3CI5(2)	ventilator	ICU	Regional	+	+	+	-	-	-	+	+	+	+

57	1SP8(1)	Occ. Bed	PAEDS	District	+	+	+	-	+	-	+	-	+	+
58	2CP1(3)	Phone	PAEDS	Regional	+	+	+	-	+	-	-	-	+	+
59	2CP1(5)	Phone	PAEDS	Regional	+	-	-	-	-	-	-	-	+	+
60	2CP1(2)	Phone	PAEDS	Regional	+	-	-	-	-	-	+	-	+	+
61	1MG9(2)	unocc. Bed	GENERAL	Central	+	+	+	-	-	-	+	-	-	+
62	3CG4(1)	Patient file	GENERAL	Regional	+	+	-	-	-	-	-	-	-	+
63	1MP5(4)	ventilator	PAEDS	Central	+	+	+	-	+	-	+	-	+	+
64	2CG4(5)	Patient file	GENERAL	Regional	+	+	-	-	-	-	-	-	-	+
65	1MP8(1)	Occ. Bed	PAEDS	Central	+	+	+	+	-	-	-	-	-	-
66	1MP8(5)	Occ. Bed	PAEDS	Central	+	+	-	-	-	-	-	+	-	+
67	1MP2(3)	Drip stand	PAEDS	Central	+	-	-	-	-	-	+	-	-	+
68	1MI9	unocc. Bed	ICU	Central	+	+	+	+	-	-	-	-	-	+
69	2CG8(3)	Occ. Bed	GENERAL	Regional	+	+	+	+	+	+	+	+	-	+
70	3SP8(3)	Occ. Bed	PAEDS	District	+	+	-	+	+	+	+	-	-	+
71	3UI9(2)	unocc. Bed	ICU	Tertiary	+	+	+	+	+	-	+	+	-	+
72	1UP6(1)	Mop	PAEDS	Tertiary	+	+	+	+	+	-	-	+	-	+
73	3UP9(2)	unocc. Bed	PAEDS	Tertiary	+	+	+	+	+	+	+	+	-	+
74	1CP4(1)	Patient file	PAEDS	Regional	+	+	+	+	+	+	+	+	-	+
75	1CG9(1)	unocc. Bed	GENERAL	Regional	+	+	+	+	+	-	+	+	-	+
76	1CP4(2)	Patient file	PAEDS	Regional	+	+	+	+	+	-	+	+	-	+
77	3UG1(1)	Phone	GENERAL	Tertiary	+	+	+	+	+	-	+	+	-	+
78	3UP1 (1)	Phone	PAEDS	Tertiary	+	+	-	+	+	+	+	+	-	-
79	3UP4(2)	Patient file	PAEDS	Tertiary	+	+	+	+	+	+	+	+	+	+
80	3UP1(2)	Phone	PAEDS	Tertiary	+	+	+	+	+	+	+	+	+	+
81	3UP4(1)	Patient file	PAEDS	Tertiary	+	+	-	+	+	-	-	+	+	+
82	1CI10(2)	nurses' table	ICU	Regional	+	+	+	+	+	-	-	+	-	+
83	1UG6(1)	Mop	GENERAL	Tertiary	+	+	-	+	-	-	-	+	-	+
84	3UI10(1)	nurses' table	ICU	Tertiary	+	+	+	+	+	+	+	+	-	+
85	3UP9(3)	unocc. Bed	PAEDS	Tertiary	+	+	+	+	+	+	+	+	-	+
86	3UG9(2)	unocc. Bed	GENERAL	Tertiary	+	+	+	+	-	-	-	+	+	+
87	3UP10(1)	nurses' table	PAEDS	Tertiary	+	+	+	+	-	+	-	+	-	-

88	3UP11(2)	door handle	PAEDS	Tertiary	+	+	+	+	+	+	+	+	+	+
89	1UG11(1)	door handle	GENERAL	Tertiary	+	+	+	+	+	+	-	+	-	+
90	1UG4(1)	Patient file	GENERAL	Tertiary	+	+	+	+	+	-	-	-	-	+
91	3UP9(1)	unocc. Bed	PAEDS	Tertiary	+	+	+	+	-	+	+	-	-	+
92	3UG7(1)	Sink	GENERAL	Tertiary	+	+	-	+	+	+	+	+	-	+
93	1CG3(1)	Bp machine	GENERAL	Regional	+	+	+	+	+	-	-	+	-	+
94	3UI10(2)	nurses' table	ICU	Tertiary	+	+	+	+	+	-	-	+	-	+
95	1CG9(4)	unocc. Bed	GENERAL	Regional	+	+	+	+	+	-	+	+	-	+
96	3UI1(2)	Phone	ICU	Tertiary	+	+	+	+	+	+	+	+	-	+
97	1UP8(1)	Occ. Bed	PAEDS	Tertiary	+	+	+	+	+	+	+	+	-	+
98	3UP11(1)	nurses' table	PAEDS	Tertiary	+	+	+	+	+	-	+	+	-	+
99	3UP1(5)	Phone	PAEDS	Tertiary	+	+	+	+	+	-	-	+	+	+