

ANTIBIOTIC RESISTANCE PROFILES OF *STAPHYLOCOCCUS* SPECIES ISOLATED FROM HUMAN CLINICAL SAMPLES IN MAFIKENG, NORTH WEST PROVINCE, SOUTH AFRICA

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Dissertation submitted in fulfilment of the requirements for the degree
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DECLARATION

I, Ashton Hetsa Bakwena, declare that the dissertation entitled “**Antibiotic resistance profiles of *Staphylococcus* species isolated from human clinical samples in Mafikeng, North West Province, South Africa**”, hereby submitted for the degree of Master of Science in Biology, has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

Signed at on this..... day of 2015

Ashton Bakwena Hetsa (Student)

Signed at on this..... day of 2015

Professor CN Ateba (Supervisor)

DEDICATION

I dedicate this study to my baby boy, Hetsa Aphiwe Neoentle Gilbert Hetsa, my late mother, 'Mmabakoena Agnes Hetsa, my father, Mr Gilbert Hetsa as well as my siblings for their endless love, support and encouragement.

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DEFINITION OF CONCEPTS

Bacteremia	: The presence of bacterial microorganisms in blood.
Colonisation	: The presence of a potential pathogen that can be cultured without causing symptoms or disease.
Group B <i>Streptococcus</i>	: Streptococci that cause complete haemolysis.
Infection	: The presence of clinical symptoms or illness due to a microorganism.
Meningitis	: Inflammation of the meninges, sometimes caused by <i>Neisseria meningitidis</i> .
Neonatal early-onset	: An infection that occurs within the first 7 days of life or after birth.
Pathogenicity	: The ability of a microbe to cause disease.
.	
Polymerase chain reaction	: A molecular method used to amplify specific regions of DNA many times over using primers.
Prophylaxis	: The prevention of the occurrence of a disease
Sepsis	: A syndrome characterised by signs of infection and accompanied by septicaemia.
<i>Staphylococcus</i>	: A genus of spherical gram positive bacteria, belonging to the phylum Firmicutes and the lactic acid bacteria group whose cells occur in chains or pairs.
Tandem Repeats Analysis	: An objective typing method amenable to automation used to type other bacterial pathogens.
Transmission	: The transmission of a potential pathogenic organism from one individual to another.
Typing	: A phenotypic and/or genetic analysis of bacterial isolates below the species level employed to generate strain specific fingerprints that

could be used to investigate cross contaminations, transmission patterns, sources of infections in humans and/or consumers of a particular food product.

Vertical transmission

: The transmission of an organism from mother to foetus.

LIST OF ABBREVIATIONS AND ACRONYMS

The following abbreviations have been used throughout this study and follow the style recommended by the American Society for Microbiology for Journals.

ATCC	: American Type Culture Collection
BA	: Blood agar
Bp	: Base-pairs
BH	: Brain heart infusion agar
CA-MRSA	: Community-associated methicillin-resistant <i>Staphylococcus aureus</i>
CA-MSSA	: Community-associated methicillin-sensitive <i>Staphylococcus aureus</i>
CDC	: Centre for Disease Control and Prevention
CLI	: Clindamycin
DNA	: Deoxyribonucleic Acid
ERM	: Erythromycin Ribosomal Methylase genes
ERY	: Erythromycin
ERIC-PCR	: Enterobacterial Repetitive Intergenic Consensus
HA-MRSA	: Hospital-associated methicillin-resistant <i>Staphylococcus aure</i>
<i>Mec</i> Genes	: Macrolide efflux genes

MLS _B	: Macrolide, Lincosamide and Streptogramin B component
MLVA	: Multi-locus Variable Number Tandem Repeats Analysis
MRSA	: Methicillin-resistant <i>Staphylococcus aureus</i>
MSCRAMM	: Microbial surface component recognising adhesive matrix molecules
MSSA	: Methicillin-sensitive <i>Staphylococcus aureus</i>
IAP	: Intrapartum antibiotics prophylaxis
TAE	: Tris, acetic acid and EDTA
GYN	: Gynaecology
GBS	: Group B <i>Streptococcus</i>
NCCLS	: National Committee for Clinical Laboratory Standards
NICU	: Neonatal intensive care unit
OB	: Obstetric
PBP2a	: Alternative penicillin-binding protein 2
PCR	: Polymerase chain reaction
PFGE	: Pulse Field gel electrophoresis
PVL	: Panton Valentine Leukocidin
RNA	: Ribonucleic Acid

RAPD	: Random Amplified Polymorphic DNA
REA	: Restriction Endonuclease Analysis
SCC <i>mec</i>	: Staphylococcal Chromosome Cassette methicillin resistance gene
SE	: Staphylococcal Enterotoxin
SEL	: Staphylococcal Enterotoxin–like
TSS	: Toxic Shock Syndrome
TSST	: Toxic Shock Syndrome Toxin

ABSTRACT

Vaginal colonisation with, coagulase-negative Staphylococci (CNS) and coagulase-positive Staphylococci (CPS) among pregnant women has resulted in greater usage of antibiotics and consequently an increase in the occurrence of multiple antibiotic resistance strains among the genus *Staphylococcus*. This has, however, resulted in serious limitations in the treatment of staphylococcal infections due to high levels of resistance to a range of antibiotics. The aim of the study was to isolate, identify and characterise antibiotic resistant Staphylococci strains from pregnant women in the Mafikeng area, North-West Province, South Africa using phenotypic and genotypic assays. Vaginal samples were collected from women who were in their 35 to 37th week of pregnancy and reported for routine pre-natal check-ups at two local clinics in the Mafikeng area. The samples were placed in transport medium, labelled properly and transported on ice to the laboratory for analysis. Selective isolation of staphylococci was performed using Mannitol Salt Agar and potential colonies subjected to both preliminary (Gram staining, catalase, haemolysis, mannitol fermentation and coagulase tests) and confirmatory (Latex agglutination test, and MALDI-TOF-MS and *nuc* PCR analysis) identification tests. The percentage of antibiotic resistant profiles and multiple antibiotic resistant phenotypes of the isolates were determined using (which method). The presence of the Penicillin Binding Protein 2a (PBP2a) was determined using a serological assay while the virulence potential of isolates was determined using *sea-to-see* genes specific multiplex PCR analysis. The genetic similarities of isolates was also determined using ERIC and RAPD PCR analysis.

All the sixty-eight 68 (100%) Gram positive cocci were catalase positive; a small proportion 32 (47%) produced the coagulase enzyme and 33 (49%) were positively identified based on the presence of the clumping factor. Of the 68 isolates, 36 (53%) were non-hemolytic, while 32 (47%) were beta-hemolytic. MALDI-TOF MS analysis revealed that only 33 (49%) were positively identified to the species level as belonging to staphylococci. Among these isolates, *S. aureus* was most frequently isolated 14 (43%)

than *S. vitulinus* 7 (21%), *S. epidermidis* 4 (12%), *S. cohnii* 3 (9%), *S. equorum*, 3 (9%) and *S. succinus* 2 (6%). Out of these, 25 (76%) isolates were positive for the *nuc* gene.

Antibiotic resistance data revealed that large proportions (76% - 100%) of isolates were resistant to the antibiotics tested. All the isolates were most often, resistant to penicillin and, ampicillin (100%), and variously resistant to oxacillin (97%), oxytetracycline (97%), vancomycin (97%), rifampin (85%), erythromycin (82%) and streptomycin (76%). In this study, a large proportion of these isolates were resistant to three (3) or more antibiotics and therefore, were termed multiple antibiotic resistant (MAR) strains. Despite this, isolates from different sample sites portrayed slight variations in antibiotic resistance patterns. The MARPs PG-AMP-OX-VA-GM-VA-ERY-RIP-OT, PG-AMP-OX-K-VA-ERY-RIP-OT and PG-AMP-OX-S-VA-ERY-RIP-OT were predominant among *S. aureus* while PG-AMP-OX-GM-VA-ERY-RIP-OT, PG-AMP-OX-VA-ERY-RIP-OT were common among *S. vitulinus*. In addition, PG-AP-OX-K-VA-OT and PG-AMP-OX-K-VA-ERY-RIP-OT were predominant among *S. cohnii* and *S. epidermidis* respectively. Despite the fact that only one *S. succinus* isolate possessed the PG-AP-OX-S-K-GM-VA-ERY-RIP-OT, a cause for concern is the fact that this isolate was resistant to all the ten antimicrobial agents tested. A large proportion 25 (76%) of isolates possessed the PBP2a. These isolates are of clinical importance since they are able to destroy β -lactam antibiotics.

Cluster analysis based on antibiotic inhibition zone diameter data revealed two major clusters (I–II) and when the clusters were further analysed for patterns of associations of the different species present in a given cluster, 21 of the these isolates were present in cluster II which was the larger cluster while a small proportion 12 (36 %) was present in cluster I. Cluster I was divided into three sub-clusters designated A-C and sub-cluster IA contained 2 (16.6%) *S. cohnii* and only 1(8.3%) *S. aureus*. On the contrary, cluster IB contained only *S. aureus* isolates whereas cluster IC was represented by 2 (17%) of *S. aureus* and *S. vitulinus* respectively, including (8.3%) *S. equorum* and *S. epidermidis* respectively.

Cluster II was sub-divided into 5 clusters designated A to E. Sub-clusters (cluster IIC and IIE), respectively contained the largest number of isolates. Sub-cluster IIC contained only the *S. aureus*, while sub-cluster IIE contained 2 (9.5%) isolates each of *S. aureus* and *S. vitulinus*. In addition, 1 isolate each of *S. epidermidis* and *S. cohnii* were present in this sub-cluster. In sub-cluster IIA, 2 *S. succinus* isolates clustered with 1 each of *S. aureus* and *S. vitulinus*. With the exception of sub-clusters IB and IIC that contained only *S. aureus* isolates, all the other sub-clusters mixed.

A total of 4 (12%) which were positive for the *seb* gene and all these were *S. aureus*. All 33 *Staphylococcus* isolates were subjected to non-specific PCR analysis using the primers S, AP7 and ERIC2 to determine their genetic relatedness. The band patterns of the isolates ranged from 0 to 11 per isolate. A total of 31 isolates that produced reliable band patterns were subjected to cluster analysis in order to determine their genetic relatedness. The clustering patterns generated with the ERIC-PCR protocol when the primers were combined revealed six main clusters (I-VI). Generally, isolates belonging to different species and isolated from different locations clustered together and were considered mixed clusters. The largest cluster was cluster one (I) with seven (23%) out of 31 *Staphylococcus* isolates and *S. aureus* was the predominant species (43%) within this cluster. In addition, a large proportion of these isolates were obtained from samples collected in Stad Clinic. Clusters III and VI respectively contained 6 isolates each.

Despite the fact that *S. aureus* was also the predominant species in cluster III, an interesting observation is that all these isolates were obtained from Montshioa clinic. In addition, one isolate each of *S. cohnii*, *S. equorum* and *S. vitulinus* were present in this cluster. The species composition of isolates in cluster VI was similar to that of cluster III since *S. aureus* was predominant. However, in this cluster, *S. cohnii*, *S. epidermidis* and *S. vitulinus* that were resistant to multiple antibiotics were also present. Overall, the remarkable similarities (65 to 95%) between the ERIC profiles of some isolates

from humans in the same clinic that also belonged to the same or different species indicated that the ERIC-PCR protocol used in this study could be very effective in differentiating between isolates.

CHAPTER ONE
INTRODUCTION AND PROBLEM STATEMENT

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION AND PROBLEM STATEMENT

Staphylococci are Gram-positive, catalase-positive, non-motile bacteria that are ubiquitous and very often exist as commensals on the skin and mucous membranes of warm-blooded animals and humans (Lowy, 1998; Von Eiff *et al.*, 2001; Ishii *et al.*, 2006; Irlinger, 2008; Piette and Verschaeegen, 2009). The widespread distribution and persistence of staphylococci in the environment is attributed to their cellular structure, which provides the cells with the ability to tolerate extreme dry conditions thereby, preventing dehydration (Kuroda *et al.*, 2001). In addition, staphylococci can also survive in dry conditions due to the fact that they have a low water activity. These non-motile cocci are also able to grow in aerobic and anaerobic conditions (Kuroda *et al.*, 2001). The cells occur singly, in pairs or as irregular grapelike clusters under the microscope which potentially serve as a valid preliminary identification criterion (Kuroda *et al.*, 2001).

The genus *Staphylococcus* contains a large number of species and sub-species divided into two main groups based on their ability to coagulate rabbit plasma (Lowy, 1998). These include the coagulase-negative staphylococci (CNS) and coagulase-positive staphylococci (CPS). Although these organisms form part of the normal flora of animals and are generally considered to be of low virulence, they have been implicated in the etiology of a variety of human and animal infections worldwide, and have recently established themselves as important pathogens showing increasing trends towards antibiotic resistance in the last decade (Von Eiff *et al.*, 2001).

Among the genus staphylococci, *S. aureus* has been frequently identified as the major species capable of causing severe human infections and a host of common and emerging syndromes. It is therefore frequently associated with serious community-acquired and nosocomial infections worldwide (Steinberg *et al.*, 1996; Lowy, 1998; Jahoda *et al.*, 2007). Nevertheless, other coagulase-negative staphylococci such as *Staphylococcus epidermidis*, *S. equorum*, *S. succinus*, *S. haemolyticus*, *S. warneri*, *S. hominis*, *S. vitulinus* and *S. saprophyticus* which were formally considered to be less virulent are now recognised as etiologic agents of a wide variety of human infections (York *et al.*, 1996; John *et al.*, 2007; Irlinger, 2008; Piette and Verscraegen, 2009). In addition, these strains have been identified to possess high levels of resistance against a variety of clinically important antimicrobial agents (York *et al.*, 1996; John *et al.*, 2007; Irlinger, 2008; Piette and Verscraegen, 2009). They are currently among the most widespread pathogenic and opportunistic organisms worldwide (Arslan *et al.*, 2012) and this therefore necessitates the need to evaluate the antibiotic resistance profiles.

Despite the ability of staphylococci to establish commensal relationships with humans and the potential to colonise other parts of the body, they also occur most often as opportunistic pathogens in immuno-compromised individuals where they are associated with life-threatening infections (Stanley *et al.*, 2013). However, risk factors that predispose humans to coagulase-negative staphylococci include the usage of a medical device or surgical implants (Becker *et al.*, 2014). Coagulase-negative staphylococci cause mostly nosocomial infections, such as catheter-related bloodstream infections, prosthetic valve endocarditis, central nervous system shunt infections and prosthetic joint infections (Von Eiff *et al.*, 2002; Andrews *et al.*, 2008; Deurenberg and Stobberingh, 2008). Not all staphylococci are clinically important even though

strains of *S. aureus*, *S. epidermidis*, *S. cohnii*, *S. haemolyticus*, *S. saprophyticus* and *S. hominis* are known to cause complications in humans (Piette and Verscraegen, 2009).

In females, colonisation of the vagina with staphylococci, especially strains that are resistant to multiple antibiotics may serve as a reservoir for the transmission of infections to the infant during child birth (Andrews *et al.*, 2008; Creech *et al.*, 2010). Moreover, obstetric and gynecological surgeries such as vaginal hysterectomy, sexual intercourse, or vertical transmission from mother to infant during birth may also facilitate the transfer of these organisms to healthy individuals (Laibl *et al.*, 2005; Chen *et al.*, 2006; Andrews *et al.*, 2008; Pinter *et al.*, 2009; Creech *et al.*, 2010).

Despite the fact that a number of studies have been conducted in other parts of world, alleged routes of transmission of the organism to neonate include contaminated hands and clothing of health care workers, parents and family members, medical equipment, breast milk and vertical transmission during birth (Gastelum *et al.*, 2005; McDonald *et al.*, 2007; Lazenby *et al.*, 2012). Staphylococci have been isolated from the vaginal tract and intra-amniotic fluid in humans (Sperling *et al.*, 1988; Andrews *et al.*, 2008; Creech *et al.*, 2010). Although there is very limited data on the occurrence of staphylococci and associated multiple antibiotic resistant strains among pregnant women, the risk of transmission and the potential to cause opportunistic infections, especially to infants demands for such investigation (Andrews *et al.*, 2009).

The treatment of infections caused by *Staphylococcus* species have been achieved through the administration of antimicrobial agents (WHO, 2001). However, it has resulted in the

development of antibiotic resistant strains (Lowy, 2003). It has been shown that community-associated MRSA strains are generally less resistant to multiple antibiotics compared to health care-associated strains (Naimi *et al.*, 2003; Vandenesch *et al.*, 2003). However, resistance to multiple antibiotics is known to pose serious challenges to both health care professionals and patients by limiting treatment options. Moreover, community-associated MRSA strains generally have chromosomal DNA restriction patterns that are different from health care-associated MRSA strains (Creech *et al.*, 2010) and this may enhance differentiation and source tracking. In the North West Province of South Africa, the source of multiple antibiotic resistant staphylococci, including strains resistant to Methicillin in humans and subsequent risk of infections among neonates in the neonatal intensive care unit (NICU) has not been investigated. The aim of the current study is therefore to isolate and identify staphylococci from human clinical specimens. A further objective is to characterise isolates using their antibiotic resistance profiles, virulence gene profiles and genotypic fingerprinting profiles.

1.2. AIM AND OBJECTIVES OF THE STUDY

1.2.1 Aim of the study

The aim of this study was to isolate, identify and characterise antibiotic resistant staphylococci from vaginal samples using phenotypic and genotypic assays.

1.2.2 Objectives of the study

The specific objectives of the study were to:

- isolate and identify staphylococci from vaginal swabs of pregnant women using preliminary tests.

- determine the haemolytic patterns of isolates using 5% (w/v) ox-blood agar);
- confirm the identities of isolates using *nuc* specific PCR assay;
- determine the proportion of isolates resistant ;
- determine the presence of Oxacillin resistant isolates using a phenotypic serological assay;
- screen isolates for the presence of staphylococcal virulence genes by multiplex PCR analysis; and
- determine polymorphic patterns of isolates using ERIC-RAPD PCR analysis.

CHAPTER TWO
LITERATURE REVIEW

CHAPTER TWO

LITERATURE REVIEW

2.1 MORPHOLOGY AND BIOCHEMICAL CHARACTERISTICS OF STAPHYLOCOCCI

Staphylococcus species are Gram positive, non-motile, non-spore-forming cocci of varying sizes that occur singly, in pairs or in irregular clusters under the microscope. They grow rapidly in both aerobic and anaerobic conditions on Mannitol Salt Agar, blood agar and Brain-Heart Infusion medium (Mauriello *et al.*, 2004). During growth, they produce opaque colonies and may be white or cream and occasionally yellow or orange depending on the biochemical profile of isolates. Optimum growth is usually achieved between 30°C and 37°C and these organisms have a fermentative metabolism. With the exception of *S. sciuri*, *Staphylococcus* species are usually catalase positive and oxidase negative. Molecular typing methods such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *SCCmec* typing and *spa* typing are commonly used for the differentiation and characterisation of isolates even though bacterial whole-genome sequencing has been found to be more discriminatory (Köser *et al.*, 2012).

2.2 COAGULASE POSITIVE STAPHYLOCOCCI (CoPS)

CoPS represent a group of major pathogenic *Staphylococcus* species, and *S. aureus* is the most common pathogenic species among them (Foster, 1996; Loeffler *et al.*, 2005). The pathogenicity of members belonging to this group of staphylococci is highly associated with the ability to produce a wide variety of virulence factors such as slime and haemolysins, leukocidins, exfoliative toxins and antibiotic resistance determinants that enables cells to colonise different

host tissues and escape phagocytosis (Lowy, 1998; Irlinger, 2008; Thammavongsa *et al.*, 2009; Berends *et al.*, 2010). Based on the aforementioned attributes, CoPS remain an important group of nosocomial pathogens known to cause a wide variety of infections in humans (Jahoda *et al.*, 2007). Furthermore, the potential of these isolates to serve as reservoirs for the transmission of antibiotic resistance genes and the current increasing resistance patterns to multiple antibiotics may lead to the emergence of highly virulent CoPS strains (Jahoda *et al.*, 2007; Park *et al.*, 2008). In addition, the emergence of resistant CoPS is most often associated with the excessive of antibiotics in both veterinary and human medicine (Vandenesch *et al.*, 2003).

2.3 COAGULASE-NEGATIVE STAPHYLOCOCCI (CoNS)

CoNS are ubiquitous and usually exist as commensals on the skin and mucous membranes of hosts but are not harmful (Otto, 2013). There are different *Staphylococcus* species and these organisms have historically been considered to have relatively low virulence potentials. However, considering the health complications of CoNS strains on both humans and animals (Štěpán *et al.*, 2004; Bayram and Balci, 2006). These organisms have gained increasing attention in recent years. In addition, CoNS represent a collection of opportunistic microorganisms frequently associated with infections in immuno-compromised patients. They are the main cause of nosocomial bloodstream infections, bacteremia, central nervous system shunt infections, endocarditis, urinary tract infections, surgical site infections and endophthalmitis (Piette and Verschraegen, 2009). They also account for high morbidity and mortality due to their ability to develop resistance against most of the commonly used antimicrobial agents (Marshall *et al.*, 1998; Davies and Davies, 2010). Multiple antibiotic resistance in CoNS occur during different processes such as mutation and DNA transfer (Szewczyk and Rozalska 1999; Stanley *et al.*,

2013). CoNS have therefore been found to be resistant to several classes of antibiotics such as Methicillin, Macrolides, Tetracycline, Aminoglycosides and Penicillins (Marshall *et al.*, 1998; Livermore, 2000; Irlinger, 2008; Rogers *et al.*, 2009). Against this backdrop, glycopeptide antibiotics were considered the drug of choice in the treatment of CoNS infections and may account for the recent increase in glycopeptide resistance among these strains (Tacconelli *et al.*, 2001; Srinivasan *et al.*, 2002).

2.4 HOST RANGE OF STAPHYLOCOCCI

Staphylococcus species are widely spread in nature, they inhabit various parts of the skin and mucous membranes of warm-blooded animals and humans (Irlinger, 2008). They have also been isolated from a wide range of foodstuffs and water samples (Longauerova, 2006). The distribution pattern of these organisms in their hosts and the environment greatly facilitates their transmission among different animal species and humans (Devriese *et al.*, 1985; Enright, 2003). Despite the fact that most staphylococci species have been isolated from milk obtained from cattle and goats (Kinge *et al.*, 2010), a number of studies have detected virulent strains in pregnant women, infants, immune-compromised individuals, hospital environments, hospital staff and hospitalised patients (Szewczyk and Rozalska, 1999). Based on preceding data, the host range of staphylococci may vary within different locations and/or species within the same region (Kinge *et al.*, 2010).

2.5 EPIDEMIOLOGY OF CoNS AND CoPS

Staphylococci associated with human infections are known to colonise various skin and mucosal surfaces of hosts (Piette and Verschraegen, 2009). Several CoNS species are currently

considered as emerging pathogens, especially in hospitalised patients who have undergone surgical procedures such as medical implants (Von Eiff *et al.*, 2002). The epidemiology of most CoNS and CoPS associated with clinical disease in humans is currently not well documented because of their ubiquitous nature. However, *S. epidermidis*, *S. haemolyticus* and *S. hominis* are the species that are most frequently encountered in clinical samples (Piette and Verschraegen, 2009). Despite the fact that *S. epidermidis* is the most prevalent CoNS species that accounts for approximately 60-70% of all infections caused by these strains (Favre *et al.*, 2005). In the North West Province of South Africa, CoPS *S. aureus* has been isolated from milk obtained from dairy cattle (Ateba *et al.*, 2010).

2.5.1 Transmission

Humans and animals are the natural hosts of *Staphylococcus* (Lowy, 1998; Von Eiff *et al.*, 2001; Berends *et al.*, 2010). Despite the fact that these species occur as normal flora in their hosts, a number of strains have been associated with diseases in some subjects (Jahoda *et al.*, 2007). . Individuals who have a history of staphylococci, especially MRSA carriage or infections and those who might have frequently visited hospitals or have been admitted to a hospital are at risk of cross contamination. Within hospitals and clinics, it is known that the predominant mode of transmission of staphylococci is through contact with contaminated hands of staff members (Mortimer *et al.*, 1962).

Infants may often acquire antibiotic resistant staphylococci strains and community-associated methicillin-resistant *S. aureus* (CA-MRSA) from colonised parents (Behari *et al.*, 2004; Lindberg *et al.*, 2004; Huijsdens *et al.*, 2006; Al-Tawfiq, 2006). Several studies in hospitals have

revealed that colonised and infected health care workers usually serve as potential sources of MRSA strains transmitted to infants (Matussek *et al.*, 2007). In addition, nosocomial transmission of *Staphylococcus* species among hospitalised infants may result from inadequate infection control practices within the facility or the use of inadequately sterilised equipment (Nguyen *et al.*, 2007). In South Africa, pregnant women who visit health clinics for antenatal purposes are screened by nurses to determine the expected time of delivery. During this process, the transmission of some nosocomial organisms such as staphylococci and streptococci into the birth canal is possible. The presence of these organisms in the birth canal of pregnant women may facilitate transfer to the infant during birth. The impact of these nosocomial pathogens in the infant may even be more severe if they harbour both antibiotic and virulence gene determinants. It is therefore suggested that appropriate control measures and good hygiene practices be implemented during these routine screening processes.

2.6 ANTIBIOTIC RESISTANCE AND SELECTION PRESSURE

An increase in bacterial resistance to antimicrobial agents has been observed and it is an increasing global health challenge (Dessen *et al.*, 2001; Vandenesch *et al.*, 2003; Hancock, 2005; Morris *et al.*, 2006; Zhou and Wang, 2013). The development of bacterial resistance to antibiotics is caused by rapid evolution of the bacterial genome and by antibiotic selective pressure of the environment. Therefore, the presence of antimicrobial residues in the environment may correlate with the antibiotic resistance pattern observed for a given microbial species and in a specific location (Kümmerer, 2004). In addition, the uncontrolled usage of clinically important antibiotics may also account for the increase in multiple antibiotic resistant strains (Dessen *et al.*, 2001; Morris *et al.*, 2006). Resistant mutants usually survive in an

environment in which several antimicrobials are present and may serve as a potential source for antimicrobial resistance determinants (Andersson, 2003; Chopra *et al.*, 2003).

2.6.1 Antimicrobial resistance of staphylococci

Resistance of staphylococcal strains to antibiotics may be attributed to overuse and indiscriminate use of antibiotics (Dessen *et al.*, 2001; Enright, 2003). Bacteria can develop resistance to antimicrobials in different ways (Hegreness *et al.*, 2008; Dwyer *et al.*, 2009). These include acquisition of resistance genes from other bacteria, through horizontal gene transfer, mutation due to low level of antibiotic treatment and increased mutagenesis in some bacterial strains (Andersson, 2003; Chopra *et al.*, 2003; Livermore, 2003).

Resistance to methicillin by strains of *S. aureus* developed rapidly after the introduction of the drug and these *Staphylococcus* strains have become increasingly resistant to a number of antimicrobial agents that belong to different classes and with varying modes of action (Casey *et al.*, 2007). Methicillin-resistant *S. aureus* (MRSA) have been isolated in both health care settings and in the community which accounts for frequent detection of methicillin resistance in patients in intensive care units (Weber, 2005). In addition, *Staphylococcus* species have been resistant to erythromycin, oxytetracycline, rifampin, oxacillin, ampicillin, penicillin, gentamycin, kanamycin, streptomycin, and vancomycin (Irlinger, 2008; Tavares *et al.*, 2010; Ali *et al.*, 2014; Guney *et al.*, 2014).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is defined as the presence of a large, heterogeneous genetic element known as “staphylococcal cassette chromosome *mec*” (*SCCmec*),

which harbours the gene *mecA* coding for methicillin resistance as well as its regulators *mecI* and *mecRI*, is currently the most common resistance profile in *Staphylococcus aureus* (Ito *et al.*, 1999; Katayama *et al.*, 2003). This resistance determinant is also found in other staphylococcal species, and it is thought that methicillin-resistant coagulase-negative staphylococci (MRCoNS) constitute a reservoir for *SCCmec* acquisition by *S. aureus* (Katayama *et al.*, 2003).

The *SCCmec* elements are usually characterised by the presence of terminal inverted and direct repeats, site-specific cassette chromosome recombinases (*ccr*), a *mecA* gene complex (*mecA*) and a specific chromosomal insertion site within a conserved open reading frame (*orfX*) whose function is not known (Oliveira *et al.*, 2006; Berglund *et al.*, 2008). The *ccr* gene complex encodes recombinases that are responsible for the mobility of the *SCCmec* resistance determinants to other related *Staphylococcus* species (Oliveira *et al.*, 2006; Berglund *et al.*, 2008). The *SCCmec* resistance determinants also contain the J regions that comprise J1, J2 and J3 sub-regions which introduce nonessential components of the cassette, although in some cases, they may carry additional antibiotic resistance determinants and variations in the J regions within the same *Mec-ccr* combination are usually utilised in the characterisation of *SCCmec* subtypes in staphylococci (Oliveira *et al.*, 2006; Berglund *et al.*, 2008)

Several *SCCmec* subtypes and variants have been defined based on the combination of the *Mec* and *ccr* gene complexes. Seven main designated types (I to VII) are currently recognised (Berglund *et al.*, 2008). The seven *SCCmec* types range in size from 20.9 to 66.9 Kb (Lee, 2010). Despite the fact that *SCCmec* types I, IV, V, VI and VII have been shown to carry only

the *mecA* gene and therefore are responsible for only β -Lactam antibiotic resistance profiles in staphylococci, *SCCmec* types II and III are known to induce resistance to several classes of antimicrobial agents resulting from the integration of additional drug resistance mobile genetic elements such as plasmids and transposons into *SCCmec* component (Ito *et al.*, 1999; Katayama *et al.*, 2003).

2.7 MECHANISM OF ACTION OF ANTIBIOTICS

2.7.1 Tetracyclines

Tetracyclines are broad-spectrum antimicrobial agents that exhibit activity against a wide range of gram-positive and gram-negative bacteria (Ateba *et al.*, 2010; Kinge *et al.*, 2010; Phokela *et al.*, 2011; Ali *et al.*, 2014). They are a family of antibiotics that reversibly inhibit protein synthesis (Chopra and Roberts., 2001). They achieve this by entering the bacterial cell, binding to the 30S ribosomal subunit and stopping the process of protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Brodersen *et al.*, 2000; Martineau *et al.*, 2000; Connell *et al.*, 2003). As a result, they are able to inhibit the elongation phase of protein synthesis in cells (Martineau *et al.*, 2000). The implication is that it will lead to the destruction of tetracycline susceptible cells in the environment where the drug is applied (Chopra and Brenna., 1998; Chopra and Roberts, 2001). The favourable antimicrobial properties of tetracyclines and the limited side-effects associated with these agents have led to their extensive use in therapy among humans and animals (Chopra and Roberts, 2001). This may therefore account for the increasing levels of resistance to tetracycline observed in many studies worldwide (Trzcinski *et al.*, 2000; Fluit *et al.*, 2005).

2.7.2 Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics, which increase the error rate of the ribosome in protein synthesis, causing misreading and hindering of the translocation step (Mccallum *et al.*, 2010). Although all aminoglycosides bind to the 30S ribosomal subunit, interactions for the different classes of aminoglycosides with their targets in bacteria cells vary (Wright, 2003). The uptake of aminoglycosides into bacterial cells are known to occur in different steps as follows: an initial energy-independent binding of a positively charged aminoglycoside to phospholipids; and teichoic acids in Gram-positive organisms followed by an energy-dependent transport across the cytoplasmic membrane (Taber *et al.*, 1987; Karasawa *et al.*, 2008). These antimicrobial agents cause membrane damage through the incorporation of defective proteins, and trigger a second energy-dependent phase resulting in increased aminoglycoside uptake (Karasawa *et al.*, 2008). An increase in the uptake of these antimicrobial agents and subsequent binding of the drug to the ribosomal RNA induce mis-translation and inhibition of protein synthesis resulting in the death of bacteria (Karasawa and Steyger, 2011).

2.7.3 Macrolides

Macrolides are antimicrobials agents whose structure is composed of a 14, 15 or 16-membered lactone ring substituted with several neutral or amino sugars (Tenson *et al.*, 2003). Erythromycin, a 14-membered ring is possibly the best known and commonly utilised macrolide (Alvarez-Elcoro *et al.*, 1999). Macrolides are clinically useful antibiotics which bind to the large (50S) ribosomal subunit situated close to the peptidyl transferase centre and composed entirely of RNA (Nissen *et al.*, 2000; Hansen *et al.*, 2002). This subunit catalyzes the formation of

peptide bonds during protein elongation and therefore, facilitates activity of these drugs against bacterial cells.

2.7.4 Rifamycins

Rifamycins belong to the family of Ansamycin and are made up of an aromatic moiety bridged at nonadjacent positions by an aliphatic chain (Rinehart *et al.*, 1977). Rifampin is a rifamycin antibiotic known or suspected to inhibit the action of bacterial DNA-dependent RNA polymerases. It interferes with the initiation phase of RNA synthesis leading to the death of bacterial cells. Bacterial DNA- dependent-RNA polymerase is an attractive drug target since the RNA chain elongation process is essential for bacterial growth (Calvori *et al.*, 1965; Jin *et al.*, 1988). Unfortunately, a number of pathogenic organisms are able to rapidly develop resistance mechanisms and determinants against rifampicins and this explains why the drug is always used in combination with other antibiotics (Gillespie, 2002). However, in some countries, including the United States of America, it is recommended that these drugs be used only in clinical emergencies (Gillespie, 2002; Aguado *et al.*, 2009).

2.7.5 Beta-Lactams

Beta-lactam antibiotics are a broad class of antibiotics consisting of components that contain β -lactam ring in their structure. They are generally bactericidal and kill the cell by an indirect consequence of the inhibition of bacterial cell wall synthesis (Wilke *et al.*, 2005). Beta-lactam antibiotics inhibit the growth of sensitive bacteria by inactivating enzymes located in the bacterial cell membrane involved in the third stage of cell wall synthesis. In the presence of these drugs, linear strands of the peptidoglycan layer that cross-link into a fishnet-like polymer,

surrounds the bacterial cell and confers osmotic stability in hypertonic environments not formed (Lambert, 2002). Enzymes known as penicillin binding proteins (PBPs) can be detected based on their ability to bind covalently to radioactively-labeled Penicillin or other Beta-lactams. Bacterial cells that produce PBPs are able to bind to penicillin and are therefore, destroyed in the presence of the drug and vice versa (Lambert, 2002; Wilke *et al.*, 2005).

2.7.6 Glycopeptides

Glycopeptides, such as vancomycin, bind to the terminal D-Ala-D-Ala dipeptide of bacterial peptidoglycan in the cell wall of Gram-positive bacteria thereby forming the glycopeptide-peptidoglycan complex. The formation of this complex bacteria inhibits the cell wall of transpeptidase maintains the integrity of the cell wall and halts the formation of a cell wall (Wright, 2003). In addition, glycopeptide antibiotics can also inhibit the growth of bacterial cells by directly blocking enzymes involved in the ultimate steps of peptidoglycan biosynthesis (Leimkuhler *et al.*, 2005).

2.8 MECHANISM OF RESISTANCE

Mechanisms of resistance in bacterial cells may occur through three varying resistance mechanisms categorised into different groups as follows: Firstly, the antibiotic may be rendered inactive mostly by enzymatic bacterial modification of the active chemical ingredient of an antimicrobial; secondly, the specific modification of the antimicrobial target in a cell by mutation; and lastly, by active antibiotic efflux which prevents the antibiotics from reaching the target site, or through decreased uptake (Walsh, 2000).

2.8.1 Beta-Lactam antibiotics

Staphylococci resistance to penicillin occurs due to the presence and expression of *blaz* gene that encodes β -lactamase (Rice, 2006; Cha *et al.*, 2007). Beta-lactamases are a group of enzymes that inactivate β -lactam antibiotics by covalently binding the carbonyl moiety of the β -lactam ring and hydrolysing its amide bond (Lowy, 2003; Cha *et al.*, 2007). This occurs when staphylococci are exposed to β -lactam antibiotics, and are responsible for the principal mechanisms in which bacterial strains develop resistance to β -lactam antibiotics (Rice, 2006). In addition, resistance due to *mecA* gene that encodes the altered penicillin-binding protein (PBP2a) is 78-kDa in size and confers resistance to other β -Lactam antibiotics including methicillin (Song *et al.*, 1987). In the presence of the altered PBP2a, the binding of β -Lactam antibiotics is impaired resulting in reduced affinity for β -Lactams (Mccallum *et al.*, 2010). The peptidoglycan is not disrupted and cells continue to thrive in the presence of the antibiotics (Mccallum *et al.*, 2010). The expression of the *mecA* gene is regulated by the repressor *MecI* and the transmembrane β -lactam-sensing signal-transducer *MecR1* are both transcribed in different directions (Cha *et al.*, 2007). In addition, the transcription of the *mecA* gene is influenced by the presence or absence of β -Lactam antibiotics and vice versa (Mccallum *et al.*, 2010).

2.8.2 Macrolide Antibiotics

Macrolides are antibiotics that have a biochemical alteration of the 50S subunit of the bacterial ribosome, namely N⁶-dimethylation of a single adenine residue at position 2058 in 23S rRNA (Leclercq and Courvalin., 1991; Leclercq, 2002). An alteration of the bacterial ribosome results in a reduction of the affinity of the ribosome for the antibiotic and the inability of the drug to inhibit synthesis of proteins in resistant microorganisms (Leclercq, 2002).

Staphylococci resistance to macrolides may occur due to the presence of macrolide ribosomal methylases involved in the methylation of macrolide-binding sites on the 23S rRNA through action of methylase enzymes encoded by *erm* genes *ermA*, *ermB* and *ermC* (Roberts *et al.*, 1999; Mingeot-Leclercq *et al.*, 1999; Lüthje and Schwarz, 2007). Methylation of the ribosome results in modification of the drug-binding site on the bacterial ribosome. This is mediated by ribosomal methylases which leads to reduced binding of macrolides and other structurally related antibiotics (Roberts *et al.*, 1999; Leclercq, 2002; Lüthje and Schwarz, 2007).

The common efflux system that plays an important role in erythromycin resistant staphylococci is mediated by macrolide and streptogramin B resistance protein A *MsrA*, belonging to the ABC transporter family. This protein expels 14-membered macrolides and streptogramin B antibiotics from bacterial cells (Roberts *et al.*, 1999). The active efflux of antimicrobial agents from the bacterial cell, due to energy-dependent pump usually occurs through the macrolide efflux pump that is mediated by a membrane bound protein encoded by a *mef* gene (Luna *et al.*, 1999). The *mef* pump usually confers a lower level of resistance only to the 14 and 15-membered lactone ring macrolides (Chancey *et al.*, 2011).

2.8.3 Tetracyclines

Tetracyclines are a group of broad-spectrum antibiotics generally considered to prevent bacterial growth by inhibiting protein synthesis. This results from binding of antibiotic to a single site in the 30S ribosomal subunit which prevents attachment of aminoacyl-tRNA to the ribosomal acceptor site (Chopra *et al.*, 1992; Chopra and Roberts, 2001). Movement of the antibiotic to

successfully reach the ribosome is achieved when these antibiotics pass through the hydrophobic lipid bilayer of the bacterial cytoplasmic membrane (Chopra and Roberts, 2001).

The principal resistance mechanisms against tetracycline in *Staphylococcus* species are ribosomal protection by elongation factor-like proteins *tetM* or *tetO* (Connell *et al.*, 2003), and active efflux by *tetK* or *tetL*, encoded by mobile *tet* genes (Yee, 2011). At sub-inhibitory concentrations of Tetracycline, both efflux and ribosomal protection in staphylococci are inducible (Trzcinski *et al.*, 2000). Induction of *tet K* and *tet L* by tetracycline occurs through translational attenuation and these gene determinants are constitutively expressed (Roberts, 1996). In addition, tetracycline resistance in staphylococci is also mediated by a newly identified efflux pump, that is, a chromosomally encoded transporter known as *tet38* (Truong-Bolduc *et al.*, 2005; Poole, 2007). *MgrA* is a transcriptional regulator and directly regulates activities of *tet 38*. An increase in *tet 38* transcription also results in a 32 fold increase in minimum inhibitory concentration (MIC) of Tetracycline (Truong-Bolduc *et al.*, 2005; Poole, 2007).

2.8.4 Aminoglycosides

The most common resistance mechanism against aminoglycosides is the enzymatic modification of the drug either by acetylation, phosphorylation or adenylation. These processes are catalysed by phosphotransferases (APH), acetyl-transferases (AAC) and nucleotidyl-transferases (ANT) respectively and interfere with binding of the antibiotic to the ribosomal A-site RNA (Wright, 2003). A large number of aminoglycoside-modifying genes originate from various aminoglycoside-producing bacteria in the environment (Davies, 1994). These genes are located either on transposons or other mobile genetic elements. This therefore explains the reason for

their wide dissemination among Gram-positive and Gram-negative bacteria in the environment (Davies and Davies., 2010). The bi-functional enzyme aminoglycoside-6'-N-acetyltransferase-2''-O-phosphoryltransferase *aac(6')-aph (2'')* is the most frequently detected resistance determinant in methicillin resistant staphylococci that also exhibit resistance to aminoglycoside antibiotics (Hegstad *et al.*, 2010). In addition, this resistance determinant is largely responsible for reducing the efficacy of most aminoglycosides, especially Gentamicin (Hegstad *et al.*, 2010). Moreover, mutations in the active site or promoter regions have also been reported to account for the genetic basis for alterations in the substrate profile or regulation of the activity of aminoglycoside-modifying enzymes. Staphylococci resistance to aminoglycosides may occur due to reduced buildup of the antimicrobial drug in bacterial cell as a result of a multidrug efflux mechanism that pumps the antimicrobial out of the cell (Durante-Mangoni *et al.*, 2009).

2.8.5 Rifamycins

Resistance to rifamycins occurs through the modification of the drug target, *rpoB* and by mutation (Heep *et al.*, 2000). Rifamycins are the only class of clinically important antimicrobial agents whose mode of action occurs through inhibition of DNA dependent-RNA polymerase. However, a number of bacterial strains have already developed resistance against these drugs (Parenti *et al.*, 1997). Some rifamycins such as rifampin may confer cross-resistance to other antimicrobial agents and therefore, it is imperative to establish such a finding before investigating whether resistance patterns observed are as a result of the presence of other RNA polymerase inhibitors (Iwakura *et al.*, 1973; Jin and Gross, 1988; Heisler *et al.*, 1993; Irschik *et al.*, 1995). Most mutations associated with resistance to Rifamycins are point mutations which result in the substitutions of a single amino acid, but with a few occurring through deletions or

insertions (Jin *et al.*, 1988; Jin and Zhou, 1996). The ability of organisms to resist destruction when exposed to rifamycins leads to decreased sensitivity of DNA-dependent RNA polymerase and/or impedes cellular uptake of the antibiotic. This explains why different organisms display varying degree of susceptibility to these drugs (Floss and Yu, 2005).

2.8.6 Glycopeptides

Resistance to glycopeptide antibiotics typically occurs due to the alteration of glycopeptide recognition sites. This modification of target site occurs during the biosynthesis of peptidoglycan (Walsh *et al.*, 1996; Walsh, 2000). However, the mechanism of resistance to vancomycin often observed in staphylococci is due to *VanA* gene which plays a role in the thickening and release of bacterial cell wall segments in the presence of the antibiotic. This, in turn, prevents the antibiotic to enter the cell and reach the target site (Sieradzki *et al.*, 1999; Hiramatsu, 2001). Transpeptidation of peptidoglycan leads to excessive production of the antibiotic binding site, and decreases the concentration and effectiveness of the drug, enabling the bacterial cell to grow in the presence of vancomycin (Hiramatsu, 2001; Wright, 2005).

Table 2.1: Antibiotics, mechanisms of action and mechanisms through which bacteria evade destruction

Antibiotic group	Examples	Target	Active against G+	Resistance mechanism
B-Lactams ^a	Ampicillin	^a Cell wall synthesis. inhibitor – acts on penicillin binding proteins (PBP)	√	Penicillin-G impermeable to G ⁻ . Mutation in PBPs. Produce β-Lactamase
Aminoglycosides ^b	Gentamycin Kanamycin Streptomycin	^b Binds to 30S subunit of ribosomes – inhibits protein synthesis	√	Aminoglycosides modifying enzymes. Fluz mechanisms RNA modifications
Tetracyclines ^c	Tetracycline	^c Binds to 30S subunit of ribosomes- inhibits protein synthesis	√	Efflux mechanisms 16S mutations
Macrolides ^d	Erythromycin	^d Binds to the large ribosomal subunit (50 S)	√	Methylation of macrolide on the 23S rRNA Efflux mechanisms
Glycopeptides ^e	Vancomycin	^e Cell wall synthesis inhibitor	√	Binds to D-anil-D-alanine, inhibit transfer of linear glycan acceptor to the N-acetylmuramypentapeptide-N-acetylglucosamine
Rifamycin ^f	Rifampin	^f Inhibitors of bacterial DNA-dependent RNA polymerase interferes with the initiation phase of RNA synthesis	√	modification of the drug target, <i>rpoB</i> and by mutation

G⁺ = Gram positive; ^aHitchings, 1973; ^eVan Bambeke, 2010 ^dWeisblum, 1995; ^bMingeot-Leclercq *et al.*, 1999; ^aCapitano *et al.*, 2001; ^fKernodle, 2000; ^cConnell *et al.*, 2003.

Adopted from (Barlow and Hall, 2002)

2.9 VIRULENCE GENES ASSOCIATED WITH STAPHYLOCOCCI

Staphylococcus species are a group of microorganisms that are able to produce a variety of virulence factors associated with the occurrence of pathological conditions and infections in their hosts (Foster and Hook, 1998; Holmes *et al.*, 2005). These factors include microbial surface components that recognise adhesive matrix molecules (MSCRAMM), cytolytic toxins, exoenzymes, exotoxins, haemolysins, and leukocidins such as the Panton Valentine Leukocidins (PVL) and super antigens (Portillo *et al.*, 2013). The super antigens comprise of staphylococcal enterotoxins (SE), toxic shock syndrome toxin (TSST) and exfoliative toxins (Lina *et al.*, 2004). These exoenzymes and exotoxins produce a proteolytic activity and toxic or lytic effects in the cells, thereby facilitating local invasion and dissemination (Dinges *et al.*, 2000).

2.9.1 Enterotoxins

Staphylococcal enterotoxins are members of a large family of different toxins that are functionally related and with very close similarities (Pinchuk *et al.*, 2010). These proteins are similar in structure and toxicological properties and are mostly produced by *S. aureus* although they have been detected in other staphylococci such as *S. intermedius* and *S. hyicus* (Pinchuk *et al.*, 2010). These are bacterial proteins in nature produced by *Staphylococcus* strains in different environments, including food products and substrates. These toxins are highly associated with major complications in humans including food poisoning and toxic shock syndromes (Pinchuk *et al.*, 2010). Staphylococcal enterotoxins are virulence factors whose genes are located on mobile genetic elements such as plasmids, bacteriophages and pathogenicity islands and can be easily transferred horizontally between bacterial strains (Malachowa and DeLeo, 2010; Madhusoodanan *et al.*, 2011).

2.10 TREATMENT

Treatment for a wide variety of infections caused by bacterial species is usually achieved through administration of antimicrobial agents such as β -lactams and macrolides antibiotics (Roberts *et al.*, 1999; Leclercq, 2002; Davies and Davies, 2010). In the United Kingdom, guidelines for prophylaxis and treatment of staphylococci infections, especially when the strains are resistant to methicillin requires the use of glycopeptide (Gemmell *et al.*, 2006). In some instances, it is recommended that glycopeptide antibiotics such as teicoplanin be administered simultaneously with other antibiotics (Moran *et al.*, 2006; Byrne and Wilcox, 2011). However, the success rate in the use of antimicrobial agents depends largely on the antibiotic resistance profiles of the isolates. In 2008, it was suggested that the prudent use of glycopeptides could greatly reduce hospital stays in patients (Coia *et al.*, 2006; Dhillon and Clark, 2009). Unfortunately, the uncontrolled usage of antimicrobial agents usually results in the development and dissemination of resistant strains in the environment (Davies and Davies, 2010).

2.11 SCREENING AND PREVENTION

The main prevention strategy that is very useful in the elimination of staphylococci involves the robust screening of anterior nares, axilla, perineum, any breaks in the skin or any other sites that may harbour these organisms but with the objective of identifying virulent strains (Coia *et al.*, 2006). According to the Prophylactic Guidelines, patients who harbour MRSA strains must be subjected to decolonisation procedures to reduce shedding of MRSA and therefore, minimise the risk of autoinfection and transmission to other individuals (Coia *et al.*, 2006). Skin decolonisation involves the use of antibacterial shampoo and body washes (4% chlorhexidine or 7.5% povidone iodine), and antibacterial nasal creams such as 2% (w/v) Mupirocin. In hospital

settings, isolation of in-patients is highly recommended (Byrne and Wilcox, 2011). Cleaning of contaminated environments, proper sterilisation of hospital equipment and strict usage of single surgical equipment for particular patient is known to reduce cross-contamination (Coia *et al.*, 2006). Proper sanitisation of hands, decontamination of the environment and equipment, active surveillance and prevention of contact with contaminated surfaces through the utilisation of laboratory gowns and gloves are very important in reducing staphylococcal, especially MRSA contamination (Griffin, 2007). In addition, the extensive usage of alcohol-based hand hygiene and proper administration of antibiotics have played an important role in combating and reducing the prevalence of MRSA in humans (Lee *et al.*, 2013).

2.12 IDENTIFICATION OF STAPHYLOCOCCI

Identification of disease causing organisms is very important in both epidemiology and treatment of infections caused by bacterial pathogens (Chin, 2000). Culture-based methods are useful and cost-effective approaches for the recovery and identification of many bacterial pathogens including staphylococci (Gracias and McKillip, 2004). They are widely used in many microbiological laboratories. Identification of staphylococci is achieved through gram staining, catalase, sugar fermentation and coagulase tests.

Molecular typing methods commonly used include *Sma*I genomic DNA restriction fragments that are separated by pulse field gel electrophoresis (PFGE). This method provides a short-term epidemiological data of two genetically different populations (Deplano *et al.*, 2000), while MLST is widely used to study long-term epidemiological data globally by targeting seven

housekeeping genes of organisms (Thomas *et al.*, 2007). In addition, the SCC*mec* typing method is normally used to determine the *ccr* and *mec* gene complex on bacterial isolates.

2.12.1 Matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF-MS)

The technology for routine identification of bacteria in clinical microbiology has recently advanced with the advent of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). This technique offers precise bacterial identification and has proven to play an important role in the diagnosis and efficient treatment of the outbreak of infectious diseases (Croxatto *et al.*, 2012). MALDI-TOF-MS has a specific discriminative power against other techniques in that it has the capability to recognise a large spectrum of proteins that may be known to be specific to a certain organism. It also provides correct identification. For example, the technique is able to differentiate among closely related species and classify organisms not only at the species level but sometimes, at sub-species and strains levels, allowing the detection of epidemic lineages (Murray, 2010). In addition, MALDI-TOF-MS has prospective advantages over other techniques in terms of bacterial classification such as minimal sample preparation, rapid results and negligible cost of reagents (Seng *et al.*, 2010). Furthermore, information about bacterial toxins as well as associated virulence, antibiotic resistance profiles and potential resistance to certain antimicrobial agents might be discovered with the use of MALDI-TOF-MS (Seng *et al.*, 2010). Although MALDI-TOF-MS is a fast and economical technology for the identification of bacterial species known to work well for many bacterial species, it has, however, also been indicated that the technique has its disadvantages and does not

work very well for other species such as streptococci and staphylococci (Rees and Voorhees, 2005).

2.12.2 Molecular typing methods for *Staphylococcus* species

Microbial strain typing is very important for epidemiological investigations of infections during food-borne outbreaks or in the characterisation of staphylococci isolated from hospital - and community - acquired infections and the identification of antibiotic-resistant strains (Croxatto *et al.*, 2012). Data obtained from molecular typing may provide information that could be valuable in the development of effective control measures to reduce the dissemination of pathogens and also facilitate the effective detection of point source contaminations during outbreaks. There are several molecular typing methods used to type microbial species. To date, a number of typing techniques are commonly used for molecular typing of staphylococci as follows: Pulsed-field gel electrophoresis (PFGE); Multilocus sequence typing (MLST); *spa* typing; and SCC*mec* typing (Burnie *et al.*, 1997).

2.12.3 Pulsed-field gel electrophoresis

PFGE is one of the most discriminative typing methods for genotyping of staphylococci. It is considered the gold standard technique that is very effective for investigating MRSA outbreaks in hospitals as well as hospital-to-hospital transmission of MRSA (Bens *et al.*, 2006; Goering, 2010). PFGE for *S. aureus* and coagulase-negative staphylococci involves the digestion of chromosomal DNA with the restriction enzyme *Sma*I (Tenover *et al.*, 1995; Burnie *et al.*, 1997; Bens *et al.*, 2006), and the resulting DNA fragments are separated by agarose gel electrophoresis in an electric field with an alternating voltage gradient. The band patterns obtained for different

isolates are analysed through a special software package such as GelCompar II using Dice comparison and unweighted pair group matching analysis (UPGMA) (Tenover *et al.*, 1995; Goering, 2010).

2.12.4 Multilocus sequence typing

It has been proven that MLST is an excellent method to study the molecular evolution of staphylococci, especially *S. aureus* (Robison and Enright, 2004). This method is based on the sequence analysis of seven fragments of staphylococci housekeeping genes that include *arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL* (Robinson and Enright, 2004). In the analysis of data, differences in profiles result from differences in sequences and therefore, the differences in alleles present in the genome accounts for the genetic profiles observed. The gene combinations for these seven alleles present in a given isolate defines the lineage of the strain and therefore, the resulting allelic profile is designated as a sequence type (ST) (Verghese *et al.*, 2012). However, MLST is expensive, laborious and time consuming (Levesque *et al.*, 2011).

2.12.5 Typing of the *spa* locus

This single-locus sequence typing technique has become progressively popular during recent years. The method was developed in order to determine sequence variations of the polymorphic region of the *S. aureus* protein A (*spa*) locus (Hasman *et al.*, 2010). Sequence variations of the *spa* gene depends mainly on the number of repeats in the 24 bp genetic component and these variations are attributed to point mutations as well as to deletions and duplications (Shopsin *et al.*, 1999). *Spa* typing has a huge discriminative power over PFGE and MLST (Malachowa *et al.*, 2005). This explains why it is widely utilised in the study of both the molecular evolution as well

as the differentiation of hospital from community strains of MRSA during outbreaks (Koreen *et al.*, 2004). Despite this, results from some studies have shown that combination of *spa* typing with PFGE and MLST may provide more reliable epidemiological data (Strommenger *et al.*, 2006; Hallin *et al.*, 2009)

2.12.6 SCC*mec* typing

PCR-based methods have been developed to determine the structure of SCC*mec* in MRSA strains (Oliveira *et al.*, 2006). This technique is used to investigate the structure of the SCC*mec* element and characterise them into SCC*mec* types I to IV (Shore *et al.*, 2005; Zhang *et al.*, 2005). Recently, two studies have indicated that for the determination of SCC*mec* types I to IV and VI, sequencing of the *ccrB* locus can be used (Oliveira *et al.*, 2006). SCC*mec* types of staphylococci isolated from the same or different locations may vary. However, since a large proportion of CA-MRSA have been reported to predominantly harbour SCC*mec* type IV, this element and its variants have gained huge attention in recent years (Milheirico *et al.*, 2007). This is mainly due to the fact that CA-MRSA have been linked to a large proportion of staphylococci infections, especially in developing countries where proper hygiene is not practised (Marcel *et al.*, 2008).

CHAPTER THREE
MATERIALS AND METHODS

CHAPTER THREE

MATERIALS AND METHODS

3.0. MATERIALS AND METHODS

3.1 STUDY DESIGN

This cross-sectional study was conducted in order to ascertain the prevalence of multiple antibiotic resistant staphylococci among pregnant women who visited two clinics in the Mafikeng area for prenatal check-ups.

3.2 STUDY AREA

The study was conducted in the Mafikeng area, North West Province, South Africa. Pregnant women who visited the Montshioa and Stad clinics for pre-natal check-ups constituted the sample for the study.

3.3 ETHICAL CLEARANCE

Ethical clearance was obtained from both the North West University Ethics Committee (NWU-00023-13-A9) and the North West Provincial Department of Health. All participants were provided with a consent form and samples were collected only after they had given their consent and signed the form. Samples were collected by participants under the guidance of clinic staff. All the ethical approval documents are attached in Appendix -B.

3.4 SAMPLE COLLECTION

A total of 68 vaginal swabs were collected from women who were in their 35 to 37 week of pregnancy and reported for routine prenatal check-ups in the Montshiwa and Stad clinics. Cotton

tipped swabs were used to collect all vaginal swabs. The swabs were properly labeled and placed in transport media. The samples were transported on ice to the laboratory for analysis (Dowell *et al.*, 2004).

3.5 ISOLATION OF *STAPHYLOCOCCUS* SPECIES

The swabs were washed in 5mL of 2% (w/v) buffered peptone water. Tenfold serial dilutions were prepared using a homogenised mixture and sterile 2% (w/v) buffered peptone water. An aliquot of 100µL from each dilution was spread-plated on Mannitol Salt Agar (MSA) and plates incubated aerobically at 37°C for 18-24 hours. Presumptive isolates that were either yellow or cream white (able to ferment mannitol) and pink (unable to ferment mannitol) in colour were stored at room temperature and used for further bacterial identification tests (Kateete *et al.*, 2010).

3.6 BACTERIAL IDENTIFICATION TESTS

Isolates were identified using the following criteria:

3.6.1 Cellular morphology

Isolates were Gram stained using standard techniques (Cruikshank *et al.*, 1975). Bacterial smears were prepared on clean microscope slides using sterile water and bacterial culture. The smears were heat fixed by passing the slide briefly over the Bunsen burner flame. The slides were flooded with the primary stain (crystal violet) for 30 seconds and later washed with water. The slides were later flooded with the mordant (iodine) for 30 seconds and washed. The excess stain on the slides was removed by washing with 70% (v/v) ethanol. Lastly, the slides were flooded with the counter stain (Safranin O) for 30 seconds, washed with water, air dried and observed

under the light of a microscope to determine the gram status, shape and cell arrangements of a particular isolate. The gram-stained smears from selected isolates that showed gram- positive cocci occurring in bunched, grapelike irregular clusters were considered as presumptive *Staphylococcus* species and all other Gram positive cocci that occurred singly or in pairs were subjected to further biochemical identification tests.

3.6.2 Preliminary identification

3.6.2.1 Mannitol hydrolysis

Presumptive staphylococci were streaked on MSA plates and incubated at 37°C for 24 hours. Isolates were assessed individually for the potential to ferment mannitol that results in a change in the colour of the medium from red to yellow. *S. aureus* is known to ferment mannitol while some CoNS strains may not ferment the carbohydrate (Kateete *et al.*, 2010).

3.6.2.2 Catalase test

A catalase test was performed to test for the presence of catalase enzymes that breakdown hydrogen peroxide (H₂O₂) that is toxic to the bacterial cell to water and oxygen. This test is used to potentially differentiate staphylococci from streptococci that are catalase negative. The test was achieved by placing a single colony of the test organism on a clean microscope slide followed by the addition of a drop of 3% (v/v) H₂O₂. The two were mixed thoroughly using a sterile wire loop and results were read within 30 seconds. Positive results were characterised by the formation of bubbles and vice versa (Piepers *et al.*, 2007).

3.6.2.3 *Haemolysis test*

The haemolytic patterns of isolates were determined by sub-culturing on blood agar supplemented with 5% (w/v) ox blood. The plates were incubated aerobically at 37°C for 24 hours. After incubation, the plates were observed for haemolytic patterns and results recorded (Elek and Levy, 2005).

3.6.2.4 *Tube coagulase test*

The ability of *Staphylococcus* species to produce the coagulase enzyme was determined using the tube coagulase test assay. This was achieved by placing a colony of the selected test isolate in a glass test tube containing 0.5mL of citrated rabbit plasma (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). After mixing gently, the tubes were then incubated aerobically at 37°C for 24 hours together with a negative control tube that containing a mixture of 0.5mL of citrated rabbit plasma and 0.5mL of sterile water. The isolates were assessed for the ability to form a cloudy jelly-like clot after 24 hours of incubation. The formation of a clot results when soluble fibrinogen in plasma is converted to insoluble fibrin. The reaction was considered positive if the solid clot was immovable when the tubes were tilted and vice versa. Results were recorded (Aslanzadeh, 2006).

3.6.2.5 *Latex agglutination test*

The test was performed using a commercial latex agglutination test assay (Prolex Staphylococcal Grouping Latex Kit – (Lot No: 2G2543) obtained from Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada. The test was performed according to the manufacturer's instructions. This was achieved by growing fresh isolates on blood agar. The reagents of the test kit were kept briefly at

room temperature and the latex reagent was resuspended by inverting the dropper bottle several times. One drop of Staph test latex reagent was dispensed into a circle on the test card. Presumptive colonies were transferred into the test circle using a sterile loop and mixed with the test latex reagent to cover the complete area of the circle. The test card was gently rocked to allow the mixture to flow slowly over the entire test ring area. The results were considered positive if agglutinations were observed.

3.6.2.6 Determination of identities of isolates based on total protein profiles using the Matrix-Assisted Laser Desorption / Ionisation – Time of Flight Mass Spectrometry (MALDI-TOF MS)

Pure isolates were sub-cultured on MSA agar and plates incubated aerobically at 37°C for 24 hours. The plates were transported to the Microbiology Laboratory of the University of Pretoria (South Africa) for MALDI-TOF MS analysis by the equipment specialist using standard protocols and procedures. The MALDI Biotyper identifies organisms using the Matrix-Assisted Laser Desorption / Ionisation - Time of Flight Mass Spectrometry by determining the unique protein fingerprint of highly abundant proteins specific to an organism. The characteristic patterns of these proteins were used to reliably identify the particular microorganism by matching the respective patterns with an extensive database. In addition, both positive and negative control strains were included in each run. Analysis was performed and isolates identified to the level of species (Giebel *et al.*, 2010).

3.7 MOLECULAR IDENTIFICATION OF STAPHYLOCOCCI

3.7.1 Genomic DNA extraction

Genomic DNA was extracted from isolates using the ZR Bacteria DNA MiniPrep™ (Lot No: ZRC178482; Zymo Research, the Epigenetics Company, USA) following the manufacturer's

instructions (Lee *et al.*, 2013). An aliquot of 500 μ L of beta-mercaptoethanol was added to 100mL of the DNA binding buffer before using it to ensure optimal performance. Overnight cultures were prepared by inoculating 10mL of nutrient broth with test isolates. Bacterial cells or a pellet containing the cells were obtained by centrifuging cultures in 1.5 mL micro centrifuge tubes. This was followed by the addition of 750 μ L of lysis buffer to the tube. The pellet and the lysis solution were thoroughly mixed and the contents transferred into a ZR BashingbeadTM lysis tube. The tubes were vortexed vigorously using a disruptor gene vortex (model SI-D257) obtained from Scientific industries Inc, USA. The content of the tube was centrifuged at 10,000rpm for 1 minute and 400 μ L of the supernatant was transferred to a Zymo-SpinTM IV Spin Filter in a collection tube and centrifuged at 7,000 rpm for 1 minute. An aliquot of 1,200 μ L of the resuspended Bacterial DNA Binding Buffer was added to the filtrate and 800 μ L of the mixture transferred to a Zymo-SpinTM IIC Column in a collection tube. The tube was centrifuged at 10,000 rpm for 1 minute, the flow through from the collection tube was discarded and the process repeated. The Zymo-SpinTM IIC Columns were transferred into a new collection tube and 200 μ L of DNA Pre-Wash Buffer was added. The tubes were centrifuged at 10,000 rpm for 1 minute followed by the addition of Bacterial DNA Wash Buffer to the Zymo-SpinTM IIC Column and the tubes further centrifuged at 10,000 rpm for 1 minute. The Zymo-SpinTM IIC columns were transferred into new, clean 1.5 mL micro centrifuge tubes and 100 μ L of DNA Elution Buffer added directly to the centre of each column matrix. The tubes were then centrifuged at 10,000 rpm for 30 seconds to elute DNA. The extracted DNA was stored at -80 °C for further assays.

3.7.2 Quantification of genomic DNA extracted

The DNA extracted from presumptive staphylococci were quantified by measuring the optical density at 260nm and 280nm using UV Visible Spectrophotometer (model S-22, Boeco, Germany). Concentration and purity were determined as previously reported (Sambrook *et al.*, 1989). The DNA was quantified in solution by measuring the absorbance at 260 nm in a spectrophotometer.

3.7.3 Polymerase Chain Reaction (PCR)

3.7.3.1 Amplification of the *nuc* gene

The identities of the isolates were determined using the *nuc* specific PCR assay as previously indicated (Becker *et al.*, 2005). Specific oligonucleotide primer combinations indicated in Table 3.1 were used to amplify target genes. Polymerase chain reactions were performed in final volumes of 25µL, with a reaction mixture containing 0.2 µg/µL of the prepared template DNA, 12.5µL of master mix, 1U of *Taq* polymerase and 50 μ mol of each oligonucleotide primer and 9.5µL of DNase-RNase free distilled water. All the reagents were Fermentas, USA products obtained from Inqaba Biotec Ltd, Pretoria, South Africa. Amplifications were performed using a TouchTM Thermal cycler (Model- C1000- TOUCHTM Thermal cycler, Singapore). PCR was performed using the following conditions: an initial heat denaturation at 94°C for 10 minutes; 22 cycles of 94°C for 10 minutes; 52°C for 1 minute; and 72°C for 2 minutes. A final elongation step performed at 72°C for 5 minutes and the PCR products were held at 4°C until they were resolved by electrophoresis (Ali *et al.*, 2014).

Table 3.1: Primer sequences used for *nuc* PCR analysis

Primer	Sequence (5' – 3')	Target gene	Amplicon size
nucF	GCG ATT GAT GGT GAT ACG GTT	<i>nuc</i>	270bp
nucR	AGC CAA GCC TTG ACG AAC TAA AGC		

3.7.3.2 Multiplex PCR for detecting virulence gene combinations *sea-to-see* in isolates

Multiplex PCR was performed to detect the presence of five virulence genes *sea-to-see* using oligonucleotide sequences presented in Table 3.2. The PCR reactions were prepared in standard 25µL volumes and consisted of 0.2 µg/µL of the prepared template DNA, 12.5µL of master mix, 1U of *Taq* polymerase and 50 *pmol* of each oligonucleotide primer and 9.5µL of DNase-RNase free distilled water. All the reagents were Fermentas, USA products obtained from Inqaba Biotec Ltd, Pretoria, South Africa. Amplifications were performed using a Touch™ Thermal cycler (model- C1000- TOUCH™ Thermal cycler, Singapore). PCR was performed using the following conditions: an initial heat denaturation at 94°C for 4 minutes; 35 cycles of 94°C for 30 seconds; 57°C for 30 seconds; and 72°C for 1 minute. A final elongation step performed at 72°C for 4 minutes and the PCR products were held at 4°C until they were resolved by electrophoresis (Rosec and Gigaud, 2002).

3.7.3.3 ERIC and RAPD PCR analysis

ERIC – PCR analysis was performed on all isolates using the primers ERIC-2 (Vázquez-Sánchez *et al.*, 2012) while RAPD-PCR was performed using the AP7 primer (Kurlenda *et al.*, 2007). In addition, a combination of ERIC-2 and AP7 primer sequences (this study) were used in order to increase the degree of polymorphisms. PCR was performed at an initial denaturation at 94°C for

3 minutes, 34 cycles of 94°C for 60 seconds, 40°C for 60 seconds, 72°C for 2 minutes, 72°C for 2 minutes and 72°C for 10 minutes.

Table 3.2: Oligonucleotide primer sequences used for ERIC and RAPD-PCR typing of staphylococci

Primer	Sequence (5'-3')	Typing procedure
AP7 ^a	GAGGGTGGCGTTCT	RAPD-PCR
ERIC-2 ^b	AAGTAAGTGACTGGGGTGAGCG	ERIC-PCR

^a Kurlenda *et al.*, 2007 ^bVázquez-Sánchez *et al.*, 2012

Table 3.3: Oligonucleotide primers used for multiplex detection of virulence genes

Primer	Sequence	Target gene	Amplicon size (bp)	References
Sea F	GGTTATCAATGTGCGGGTGG	<i>Sea</i>	102	Peles <i>et al.</i> (2007)
Sea R	CGGCACTTTTTTCTCTTCGG			
Seb F	GTATGGTGGTGTAAGTGAAGC	<i>Seb</i>	164	Peles <i>et al.</i> (2007)
Seb R	CCAAATAGTGACGAGTTAGG			
Sec F	AGATGAAGTAGTTGATGTGTATGG	<i>Sec</i>	451	Peles <i>et al.</i> (2007)
Sec R	CACACTTTTAGAATCAACCG			
Sed F	CCAATAATAGGAGAAAATAAAAG	<i>Sed</i>	278	Peles <i>et al.</i> (2007)
Sed R	ATTGGTATTTTTTTTCGTTC			
See F	AGGTTTTTTCACAGGTCATCC	<i>See</i>	209	Peles <i>et al.</i> (2007)
See R	CTTTTTTTCTTCGGTCAATC			

3.8 ANTIMICROBIAL SUSCEPTIBILITY TESTING

The antimicrobial susceptibility profile of isolates was determined using the disc diffusion method on Mueller- Hinton Agar as described by the Clinical and Laboratory Standards Institute (CLSI, 2014). The antimicrobial agents used are presented in Table 3.4 and obtained from Mast Diagnostics (UK) and used for the assay. Aliquots of 100µL of bacteria suspensions were spread plated on Mueller-Hinton agar and the antibiotic discs placed at reasonable distances apart on the agar surface and the plates incubated at 37°C for 24 hours. The susceptibility of isolates to each antimicrobial agent was determined by measuring the zones of growth inhibition and results interpreted according to (CLSI 2014) guidelines for staphylococci. The inhibition zone diameter data obtained was used to classify isolates as resistant, intermediate resistant or susceptible to a particular antibiotic.

3.8.1 Multiple antibiotic resistance phenotypes of staphylococci

The multiple antibiotic resistance phenotypes, defined as resistance to three or more antimicrobial agents of oxacillin resistant isolates, were determined for all confirmed staphylococci using the method of Rota *et al.* (1996).

3.9 ELECTROPHORESIS OF DNA AND PCR PRODUCTS

PCR products were separated by electrophoresis on a 2% (w/v) agarose (Seakme®, Rockland, USA) gel using Tris-acetate-EDTA (1X TAE) (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid; pH 8.0) on a horizontal agarose gel equipment (BiocomDirect, Bridge of Weir, UK). Electrophoresis was performed at 100V for the first 10 minutes and then at 80V for 60 minutes. A 100bp or 1 kb DNA ladder (Fermentas, Glen Burnie, USA) was included in all PCR and

fingerprinting gels as a molecular weight standard. Gels were stained in 0.1 µg/mL of ethidium bromide (Bio-Rad Laboratories, Canada). The ChemiDoc™ MP Imaging system (Bio-Rad, Hercules, USA) was used to visualise and capture images.

Table 3.4: List of antibiotics used in this study.

Class	Antibiotic	Disc code	Antibiotic conc	R	I	S
Macrolides	Erythromycin	ERY	15µg ^c	≤15	16-20	≥21
Beta- Lactams	Ampicillin	AP	10µg ^a	≤18	19-25	≥26
	Penicillin	PG	10µg	≤19	20-27	≥28
	Oxacillin	OX	5 µg	≤13	14-16	≥17
Aminoglycosides	Gentamycin	GM	10 µg	≤13	14-16	≥17
	Kanamycin	K	30µg	≤13	14-16	≥17
	Streptomycin	S	10 µg	≤13	14-16	≥17
Tetracyclines	Oxytetracycline	OT	30µg ^c	≤14	15-18	≥19
Glycopeptides	Vancomycin	VA	30µg	-	-	≥17
Rifamycin	Rifampin	RIF	30µg	≤24	25-26	≥27

The superscripts ^a and ^c indicate the generally accepted concentrations of the discs according to the standard method as stipulated by the manufacturer, Mast Diagnostics, Merseyside, United Kingdom.

3.10 STATISTICAL ANALYSIS

The genetic profiles generated from the ERIC-PCR were captured using GeneTools (version 3.00.22) software (SynGene, UK). The fingerprints were compared and analysed with the Total Lab Phoretix 1D Pro software (UK). The presence, absence and intensity of band data were obtained, exported to Microsoft Excel (Microsoft Office, 2003) and used to generate a data matrix. Unweighted pair group method with arithmetic mean (UPGMA) and complete linkage

algorithms were used to analyze percentage similarity and matrix data. Relationships between the various profiles and/or lanes were expressed as dendrograms (Sneath and Sokal,, 1973)

CHAPTER FOUR
RESULTS AND INTERPRETATION

CHAPTER FOUR

RESULTS AND INTERPRETATION

4.0 RESULTS AND INTERPRETATION

4.1 OCCURRENCE OF STAPHYLOCOCCI IN VAGINAL SWABS FROM PREGNANT WOMEN USING PRELIMINARY IDENTIFICATION TESTS

4.1.1 Cellular morphological analysis

Sixty eight (68) vaginal swabs were collected from pregnant women who visited clinics for prenatal check-ups in the Mafikeng area, North West Province, South Africa. Selective isolation was achieved with Mannitol Salt Agar (MSA). *Staphylococcus* species were presumptively identified by the presence of medium-sized yellow, cream white and pink colonies that either fermented or did not ferment mannitol incorporated into the media. All 68 isolates were Gram-positive spherical cocci that occurred singly, in pairs or in irregular clusters. In addition, the colonies were small and with entire margins.

4.1.2 Phenotypic characteristics

4.1.2.1 Catalase test

All sixty eight, 68 (100%) Gram positive cocci were catalase positive and produced bubbles when reacted with hydrogen peroxide (Table 4.1).

4.1.2.2 Proportion of *Staphylococcus* species that produced the coagulase enzyme

A total of sixty eight (68) isolates were screened to detect the ability to produce the coagulase enzyme using the tube coagulase test and a small proportion 32 (47%) produced the enzyme (Table 4.1).

4.1.2.3 Identification of Staphylococci using a Serological Latex Agglutination Test Assay

All sixty eight (68) isolates were subjected to the latex agglutination test assay for serological identification as staphylococci and 33 (49%) were positively identified based on the presence of the clumping factor (Table 4.1).

4.1.2.4 Haemolytic patterns on blood agar

All 68 isolates were cultured on 5% (w/v) ox-blood agar to determine their haemolytic patterns and a summary of the results obtained are shown in Table 4.1. Of these isolates, 36 (53%) were non-hemolytic while 32 (47%) were beta- hemolytic.

Table 4.1: Results of preliminary identification tests used for the detection of staphylococci

Sampling area	No. of isolates tested	Gram staining (+ve cocci)	Catalase test	Mannitol fermentation	Haemolysis tests		Coagulase test	Latex agglutination test
					Beta	Gamma		
Montshiwa Clinic								
NT	19	19	19	19	19	19	19	19
NP	19	19	19	4	9	10	10	10
Stad Clinic								
NT	49	49	49	49	49	49	49	49
NP	49	49	49	10	23	26	22	23
Total number	68	68	68	14	32	36	32	33
Total % positive	100%	100%	100%	41%	47%	53%	47%	49%

NT= Number tested; NP= Number positive

4.2 PERCENTAGE OF ISOLATES POSITIVELY IDENTIFIED BY THE MALDI-TOF-MS TECHNIQUE

A total of 68 isolates were subjected to the MALDI-TOF-MS technique and based upon protein spectra, only 33 (49%) were positively identified to the species level as belonging to staphylococci (Table 5). Of these 33 isolates, 21 were isolated from samples from Stad Clinic and 12 from Montshiwa Clinic. Among these isolates, *S. aureus* was the most frequently identified 14 (43%) followed by *S. vitulinus* 7 (21%), *S. epidermidis* 4 (12%), *S. cohnii* 3 (9%), *S. equorum* 3 (9%) and *S. succinus* 2 (6%) (Figure 4.2).

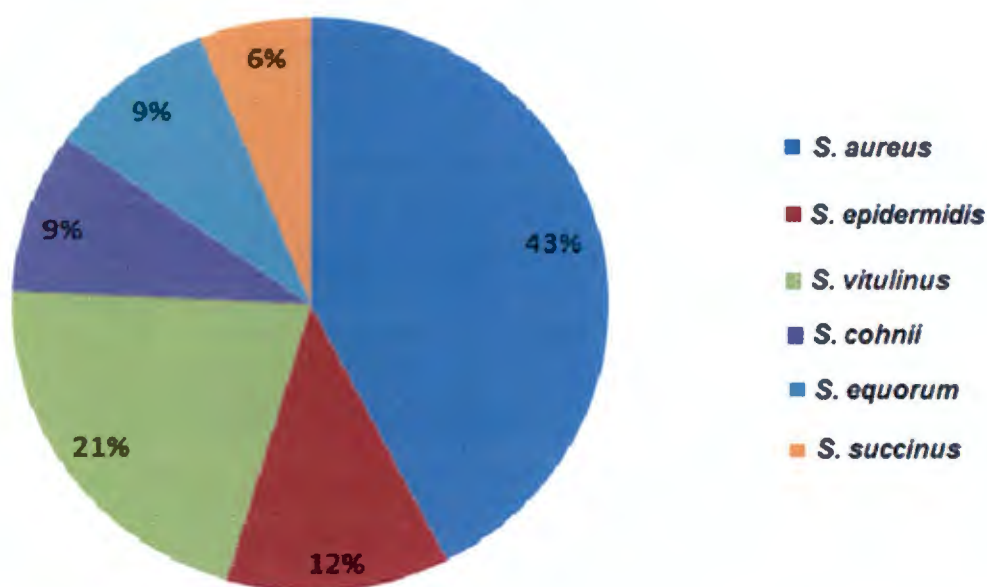


Figure 4.1: Percentage of *Staphylococcus* species isolated from vaginal swabs

Table 4.2: Number of staphylococci species positively identified using the MALDI-TOF-MS technique

Isolate Number	Score Value	Identity based on MALDI-TOF-MS analysis	Score Value	Identity based on MALDI-TOF-MS analysis
		Best Match		Second Best Match
S14	1.913	<i>Staphylococcus epidermidis</i>	1.85	<i>Staphylococcus epidermidis</i>
S16	1.758	<i>Staphylococcus vitulinus</i>	1.736	<i>Staphylococcus vitulinus</i>
S17	1.82	<i>Staphylococcus cohnii</i>	1.778	<i>Staphylococcus cohnii</i>
S18	2.045	<i>Staphylococcus equorum</i>	1.735	<i>Staphylococcus equorum</i>
S20	1.83	<i>Staphylococcus cohnii</i>	1.779	<i>Staphylococcus cohnii</i>
S24	1.759	<i>Staphylococcus equorum</i>	1.736	<i>Staphylococcus equorum</i>
S25	2.24	<i>Staphylococcus aureus</i>	2.167	<i>Staphylococcus aureus</i>
S29	2.142	<i>Staphylococcus aureus</i>	2.04	<i>Staphylococcus aureus</i>
S30	2.078	<i>Staphylococcus aureus</i>	2.076	<i>Staphylococcus aureus</i>
S31	2.121	<i>Staphylococcus aureus</i>	2.029	<i>Staphylococcus aureus</i>
S32	1.839	<i>Staphylococcus succinus</i>	1.756	<i>Staphylococcus succinus</i>
S35	2.169	<i>Staphylococcus aureus</i>	2.146	<i>Staphylococcus aureus</i>
M1	1.931	<i>Staphylococcus succinus</i>	1.707	<i>Staphylococcus succinus</i>
M3	2.291	<i>Staphylococcus aureus</i>	2.25	<i>Staphylococcus aureus</i>
M6	1.938	<i>Staphylococcus epidermidis</i>	1.921	<i>Staphylococcus epidermidis</i>
M7	1.757	<i>Staphylococcus vitulinus</i>	1.735	<i>Staphylococcus vitulinus</i>
M8	2.234	<i>Staphylococcus aureus</i>	2.054	<i>Staphylococcus aureus</i>
M9	1.873	<i>Staphylococcus vitulinus</i>	1.797	<i>Staphylococcus vitulinus</i>
M10	2.127	<i>Staphylococcus aureus</i>	2.079	<i>Staphylococcus aureus</i>
M14	1.768	<i>Staphylococcus vitulinus</i>	1.665	<i>Staphylococcus vitulinus</i>
M15	2.074	<i>Staphylococcus epidermidis</i>	2.026	<i>Staphylococcus epidermidis</i>
S1	1.924	<i>Staphylococcus epidermidis</i>	1.834	<i>Staphylococcus epidermidis</i>
S2	1.87	<i>Staphylococcus cohnii</i>	1.768	<i>Staphylococcus cohnii</i>
S3	1.848	<i>Staphylococcus aureus</i>	1.746	<i>Staphylococcus aureus</i>
S5	1.771	<i>Staphylococcus vitulinus</i>	1.753	<i>Staphylococcus vitulinus</i>
S8	1.758	<i>Staphylococcus vitulinus</i>	1.753	<i>Staphylococcus vitulinus</i>
S10	2.126	<i>Staphylococcus aureus</i>	1.905	<i>Staphylococcus aureus</i>
S11	2.143	<i>Staphylococcus aureus</i>	1.969	<i>Staphylococcus aureus</i>
S12	2.056	<i>Staphylococcus aureus</i>	1.891	<i>Staphylococcus aureus</i>
S13	1.915	<i>Staphylococcus aureus</i>	1.741	<i>Staphylococcus aureus</i>
M16	1.780	<i>Staphylococcus vitulinus</i>	1.624	<i>Staphylococcus vitulinus</i>
M18	1.896	<i>Staphylococcus equorum</i>	1.842	<i>Staphylococcus equorum</i>
M24	2.264	<i>Staphylococcus aureus</i>	2.25	<i>Staphylococcus aureus</i>

4.3 PCR FOR THE AMPLIFICATION OF *NUC* GENE FRAGMENTS FROM SUSPECTED STAPHYLOCOCCI ISOLATES

A total of 33 isolates that were positive for MALDI-TOF-MS were subjected to *nuc* specific PCR analysis for identification for staphylococci. Out of this number, 25 (76%) isolates were positive for the *nuc* gene. The amplicons generated were of the expected size (270 bp). Figure 4.2 shows a representative gel picture of the *nuc* gene fragments amplified from the isolates.

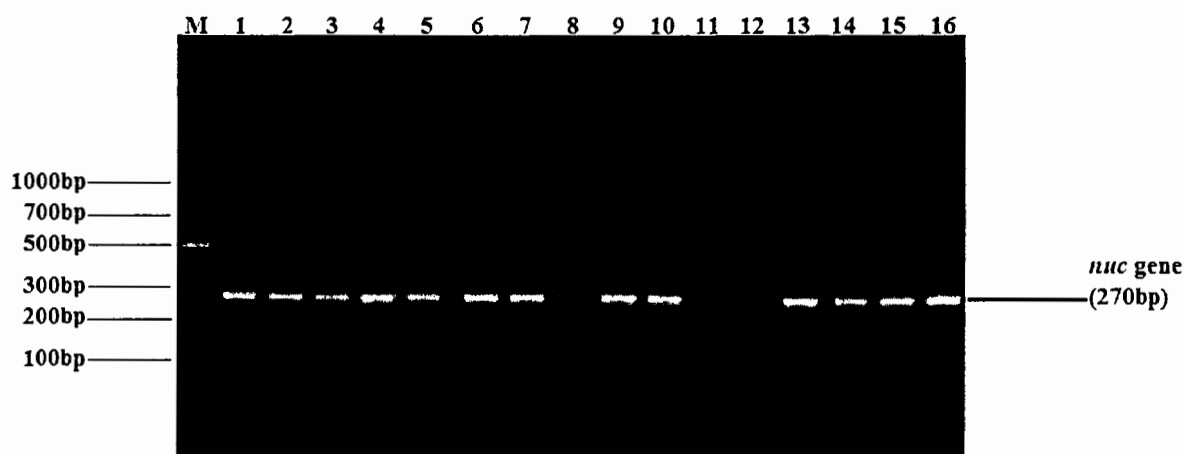


Figure 4.2: PCR amplification of the *nuc* gene from suspected Staphylococci isolates. Lane M: 100bp DNA ladder, Lane 1: M24, Lane 2: S31, Lane 3: S12, Lane 4: S13, Lane 5: M8, Lane 6: S29, Lane 7: S3, Lane 8: S14, Lane 9: M15, Lane 10: S10, Lane 11: S11, Lane 12: M3, Lane 13: S8 Lane 14: S35, Lane 15: S2, Lane 16: S16

4.4 PERCENTAGE ANTIMICROBIAL RESISTANCE OF ISOLATES

Antimicrobial susceptibility profiles of the isolates were evaluated by the disk diffusion assay and resistance patterns analysed according to the standards methods (CLSI, 2014). The data revealed that a large proportion (76% - 100%) of isolates were resistant to the antibiotics tested (Table 4.3). The isolates were most often resistant to penicillin (100%), ampicillin (100%), oxacillin (97%), oxytetracycline (97%), vancomycin (97%), rifampin (85%), erythromycin

(82%) and streptomycin (76%). In this study, a large proportion of isolates exhibited resistance to three (3) or more antibiotics and therefore, were used to compile multiple antibiotic resistant phenotype (MARP) pattern for the resistant isolates. Despite this, isolates from different sample sites portrayed slight variations in antibiotic resistant patterns.

Table 4.3: Percentage of antimicrobial resistance of isolates

Antibiotic group	Antibiotics	Percentage (%) of antimicrobial resistance
Beta-lactam	Penicillin	100
Beta-lactam	Ampicillin	100
Beta-lactam	Oxacillin	97
Aminoglycoside	Streptomycin	76
Aminoglycoside	Kanamycin	49
Aminoglycoside	Gentamycin	61
Glycopeptide	Vancomycin	94
Macrolides	Erythromycin	82
Rifamycins	Rifampin	85
Tetracycline	Oxytetracycline	97

4.4.1 Percentage of antimicrobial resistance of *S. aureus* isolates

Out of 14 *S. aureus* strains isolated from both clinics, resistance was most frequently observed against penicillin (100%), ampicillin (100%), oxytetracycline (100%), oxacillin (90%), vancomycin (90%) and rifampin (80%). On the contrary, isolates showed low resistance to streptomycin from Montshioa 0 (0%) and Stad (20%) clinics respectively. In addition, only a small proportion 2 (20%) of isolates from Stad clinic was resistant to Gentamycin. A cause for concern was the fact that isolates were not only multidrug resistant but also highly resistant to oxacillin. Overall, isolates from Montshioa Clinic showed more resistance to antibiotics compared to Stad Clinic.

Table 4.4: Percentage of antibiotic resistance of *S. aureus* from two clinics

<i>S. aureus</i> (NPI=14)	Antibiotics	Origin	
		Montshioa Clinic	Stad Clinic
	Penicillin	4 (100)	10 (100)
	NT= 14	4	10
	NR= 14	4	10
	Ampicillin	4 (100)	10 (100)
	NT=14	4	10
	NR=14	4	10
	Oxacillin	4 (100)	9 (90)
	NT= 14	4	9
	NR= 13	4	9
	Streptomycin	0 (0)	2 (20)
	NT= 14	4	10
	NR= 2	0	2
	Kanamycin	4(100)	6 (60)
	NT= 14	4	6
	NR=10	4	6
	Gentamycin	3 (75)	2 (20)
	NT= 14	4	10
	NR= 5	3	2
	Vancomycin	3 (75)	9 (90)
	NT= 14	4	9
	NR= 12	3	9
	Erythromycin	4 (100)	6 (60)
	NT=14	4	10
	NR= 10	4	6
	Rifampin	4 (100)	8 (80)
	NT= 14	4	8
	NR= 12	4	8
	Oxytetracycline	4 (100)	10 (100)
	NT= 14	4	10
	NR=14	4	10

NPI= Number positively identified, NT=Number tested, NR=Number resistant

4.4.2 Percentage of antimicrobial resistance of *S. vitulinus* isolates

A total of seven *S. vitulinus* strains were isolated, 4 (57%) from Montshioa Clinic and 3 (43%) from Stad Clinic. All the 7 *S. vitulinus* isolates were resistant to penicillin, ampicillin,

oxytetracycline, oxacillin, vancomycin, rifampin and erythromycin. However, these isolates were highly susceptible to kanamycin from Montshioa Clinic (25%) and from Stad Clinic (0%). Three of the 4 isolates from Montshioa Clinic and 1 out of 3 from Stad Clinic were resistant to gentamycin. However, none of the isolates was resistant to streptomycin. Isolates from Stad Clinic were less resistant than isolates from Montshioa Clinic. None of the *S. vitulinus* isolates was resistant to streptomycin (Table 4.4).

4.4.3 Percentage of antimicrobial resistance of *S. epidermidis* isolates

All the 4 *S. epidermidis* isolates 2 each from Montshioa and Stad Clinics were resistant to penicillin, ampicillin, oxytetracycline, oxacillin and vancomycin. On the contrary, only 2 (50%) of these isolates were resistant to rifampin and erythromycin. Despite the fact that 2 (50%) of the isolates from Montshioa were resistant to streptomycin and gentamycin, none of the isolates was resistant to these antibiotics (Table 4.5).

Table 4.5: Percentage of antibiotic resistance of *S. vitulinus* from two clinics

<i>S. vitulinus</i> (NPI=7)	Antibiotics	Origin	
		Montshioa Clinic	Stad Clinic
	Penicillin	4 (100)	3 (100)
	NT=7	4	3
	NR=7	4	3
	Ampicillin	4 (100)	3 (100)
	NT=7	4	3
	NR=7	4	3
	Oxacillin	4 (100)	3 (100)
	NT=7	4	3
	NR=7	4	3
	Streptomycin	0(0)	0 (0)
	NT=7	4	3
	NR=0	0	0
	Kanamycin	1 (25)	0 (0)
	NT=7	4	3
	NR=7	1	0
	Gentamycin	3 (75)	1 (33)
	NT=7	4	3
	NR=4	3	1
	Vancomycin	4 (100)	3 (100)
	NT=7	4	3
	NR=7	4	3
	Erythromycin	4 (100)	3 (100)
	NT=7	4	3
	NR=7	4	3
	Rifampin	4 (100)	3 (100)
	NT=7	4	3
	NR=7	4	3
	Oxytetracycline	4 (100)	3 (100)
	NT=7	4	3
	NR=7	4	3

NPI= Number positively identified, NT=Number tested, NR=Number resistant

Table 4.6: Percentage of antibiotic resistance of *S. epidermidis* strains from two clinics

<i>S. epidermidis</i> (NPI=4)	Antibiotics	Origin	
		Montshoa Clinic	Stad Clinic
	Penicillin	2 (100)	2 (100)
	NT= 4	2	2
	NR= 4	2	2
	Ampicillin	2 (100)	2 (100)
	NT= 4	2	2
	NR= 4	2	2
	Oxacillin	2 (100)	2 (100)
	NT= 4	2	2
	NR= 4	2	2
	Streptomycin	1 (50)	0 (0)
	NT= 4	2	0
	NR= 1	1	0
	Kanamycin	1 (50)	2 (100)
	NT= 4	2	2
	NR= 3	1	2
	Gentamycin	1 (50)	0 (0)
	NT= 4	2	2
	NR= 1	1	0
	Vancomycin	2 (100)	2 (100)
	NT= 4	2	2
	NR= 4	2	2
	Erythromycin	2 (100)	15 (50)
	NT= 4	2	2
	NR= 3	2	1
	Rifampin	2 (100)	1 (50)
	NT= 4	2	2
	NR= 3	2	1
	Oxytetracycline	2 (100)	2 (100)
	NT= 4	2	2
	NR= 4	2	2

NPI= Number positively identified, NT=Number tested, NR=Number resistant

4.4.4 Percentage of antimicrobial resistance of *S. cohnii* isolates

A total of three *S. cohnii* strains were isolated from Stad Clinic and all the 3 isolates were resistant to penicillin, ampicillin, oxytetracycline, oxacillin and vancomycin. Moreover, a relatively large proportion 2 (67%) was resistant to erythromycin and rifampin, while only 1

(33%) was resistant to kanamycin and gentamycin. All the isolates were found to be sensitive to streptomycin. (Table 4.6). However, the *S. cohnii* isolates resistance to 3 or more antibiotics including oxacillin (Table 4.6) is a cause for concern.

Table 4.7: Percentage of antibiotic resistance of *S. cohnii* strains from Stad Clinic

Antibiotics	Origin
<i>S. cohnii</i> (NPI=3)	Stad Clinic
Penicillin	3 (100)
NT= 3	3
NR=3	3
Ampicillin	3 (100)
NT= 3	3
NR= 3	3
Oxacillin	3 (100)
NT= 3	3
NR= 3	3
Streptomycin	0 (0)
NT= 3	3
NR= 0	0
Kanamycin	1 (33.33)
NT= 3	3
NR= 1	1
Gentamycin	1 (33.33)
NT= 3	3
NR= 1	1
Vancomycin	3 (100)
NT= 3	3
NR= 3	3
Erythromycin	2 (67)
NT= 3	3
NR= 2	2
Rifampin	2 (67)
NT= 3	3
NR= 2	2
Oxytetracycline	3 (100)
NT= 3	3
NR= 3	3

NPI= Number positively identified, NT=Number tested, NR=Number resistant

4.4.5 Percentage of antimicrobial resistance of *S. succinus* isolates

All *S. succinus* demonstrated resistance to penicillin, ampicillin, oxytetracycline, oxacillin, vancomycin, erythromycin and rifampin (Table 4.7). Despite the fact that only one *S. succinus* isolate was obtained from each clinic, the isolate from Montshiwa Clinic was found to be sensitive to streptomycin, kanamycin and gentamycin while the isolate from Stad Clinic was resistant to the tested antibiotics.

4.4.6 Percentage of antimicrobial resistance of *S. equorum* isolates

A total of three *S. equorum* isolates (1 from Montshioa Clinic and 2 from Stad Clinic) showed 100% resistance each to penicillin, ampicillin, oxacillin and erythromycin (Table 4.9). However, all the 3 isolates were sensitive to streptomycin, kanamycin and gentamycin. Also, the isolate from Montshioa Clinic was highly resistant to the antibiotics tested compared to isolates from Stad Clinic.

Table 4.8: Percentage of antibiotic resistance of *S. succinus* strains from two clinics

<i>S. succinus</i> (NPI=2)	Antibiotics	Origin	
		Montshioa Clinic	Stad Clinic
	Penicillin	1 (100)	1 (100)
	NT= 2	1	1
	NR=2	1	1
	Ampicillin	1 (100)	1 (100)
	NT= 2	1	1
	NR= 2	1	1
	Oxacillin	1 (100)	1 (100)
	NT= 2	1	1
	NR= 2	1	1
	Streptomycin	0	1 (100)
	NT= 2	1	1
	NR= 1	0	1
	Kanamycin	0	1 (100)
	NT= 2	1	1
	NR= 1	0	1
	Gentamycin	0	1 (100)
	NT= 2	1	1
	NR= 1	0	1
	Vancomycin	1 (100)	1 (100)
	NT= 2	1	1
	NR= 2	1	1
	Erythromycin	1 (100)	1 (100)
	NT= 2	1	1
	NR= 2	1	1
	Rifampin	1 (100)	1 (100)
	NT=2	1	1
	NR= 2	1	1
	Oxytetracycline	1 (100)	1 (100)
	NT= 2	1	1
	NR= 2	1	1

NPI= Number positively identified, NT=Number tested, NR=Number resistant

Table 4.9: Percentage of antibiotic resistance of *S. equorum* strains from two clinics

<i>S. equorum</i> (NPI=3)	Antibiotics	Origin	
		Montshioa Clinic	Stad Clinic
	Penicillin	1 (100)	2 (100)
	NT= 3	1	2
	NR= 3	1	2
	Ampicillin	1 (100)	2 (100)
	NT= 2	1	2
	NR= 3	1	2
	Oxacillin	1 (100)	2 (100)
	NT= 3	1	2
	NR= 3	1	2
	Streptomycin	0 (0)	0 (0)
	NT= 3	1	2
	NR= 0	0	0
	Kanamycin	0 (0)	0 (0)
	NT= 3	1	2
	NR= 0	0	0
	Gentamycin	0 (0)	0
	NT= 3	1	2
	NR= 1	1	0
	Vancomycin	0	2 (100)
	NT= 3	1	2
	NR= 2	0	2
	Erythromycin	1 (100)	2 (100)
	NT= 3	1	2
	NR= 3	1	2
	Rifampin	1 (100)	2 (76)
	NT= 3	1	2
	NR= 2	1	1
	Oxytetracycline	1 (100)	1 (50)
	NT= 3	1	2
	NR= 2	1	1

NPI= Number positively identified, NT=Number tested, NR=Number resistant

4.5 PBP2a TEST FOR PHENOTYPIC SCREENING OF *MecA*

A total of 33 (49%) isolates were subjected to PBP2a test in order to detect the presence of an altered penicillin binding protein and large proportion 25 (76%) of isolates possessed the PBP2a. These isolates are of clinical importance since they are able to destroy β -lactam antibiotics.

4.6 MULTIPLE ANTIBIOTIC RESISTANCE PHENOTYPES (MARP) OF THE DIFFERENT SPECIES

The predominant MARPs of staphylococci isolates belonging to the different species was evaluated and the detailed results are presented in Table 4.10. It was observed that all the isolates were often resistant to the antibiotics tested. The MARPs PG-AMP-OX-VA-GM-VA-ERY-RIP-OT, PG-AMP-OX-K-VA-ERY-RIP-OT and PG-AMP-OX-S-VA-ERY-RIP-OT were dominant among *S. aureus* while PG-AMP-OX-GM-VA-ERY-RIP-OT, PG-AMP-OX-VA-ERY-RIP-OT was common among *S. vitulinus*. In addition, PG-AP-OX-K-VA-OT and PG-AMP-OX-K-VA-ERY-RIP-OT were dominant among *S. cohnii* and *S. epidermidis* respectively. Surprisingly only one *S. succinus* strain possessed the PG-AP-OX-S-K-GM-VA-ERY-RIP-OT..

Table 4.10: Predominant multiple antibiotic resistant phenotypes of displayed by *Staphylococcus* species.

Species	Phenotype	No observed	Percentage observed
<i>S. aureus</i>	PG-AMP-OX-VA-GM-VA-ERY-RIP-OT	5	15
	PG-AMP-OX-K-VA-ERY-RIP-OT	1	3
	PG-AMP-OX-S-VA-ERY-RIP-OT	1	3
	PG-AMP-S-K-OT	1	3
	PG-AMP-OX-K-VA-OT	1	3
	PG-AMP-S-VA-RIP-OT	1	3
	PG-AMP-OX-S-VA-RIP-OT	1	3
	PG-AMP-S-K-OT	1	3
	PG-AMP-OX-K-VA-RIP-OT	1	3
<i>S. vitulinus</i>	PG-AMP-OX-GM-VA-ERY-RIP-OT	3	9
	PG-AMP-OX-VA-ERY-RIP-OT	3	9
	PG-AMP-OX-K-GM-VA-ERY-RIP-OT	1	3
<i>S. cohnii</i>	PG-AMP-OX-K-VA-OT	2	6
	PG-AMP-OX-GM-VA-ERY-RIP-OT	1	3
	PG-AMP-OX-VA-ERY-RIP-OT	1	3
<i>S. epidermidis</i>	PG-AMP-OX-K-VA-ERY-RIP-OT	2	9
	PG-AMP-OX-K-VA-ERY-RIP-OT	1	3
<i>S. succinus</i>	PG-AMP-OX-S-K-GM-VA-ERY-RIP-OT	1	3
	PG-AMP-OX-VA-ERY-RIP-OT	1	3
<i>S. equorum</i>	PG-AMP-OX-VA-ERY	1	3
	PG-AMP-OX-GM-VA-ERY-RIP-OT	1	3
	PG-AMP-OX-K-VA-ERY-RIP-OT	1	3

PG (Penicillin), AMP (Ampicillin), OX (Oxacillin), S (Streptomycin), K (Kanamycin), GM (Gentamycin), VA (Vancomycin), RIP (Rifampin) OT (Oxytetracycline) and ERY (Erythromycin)

4.7 CLUSTER ANALYSIS OF STAPHYLOCOCCI FOR MULTIPLE ANTIBIOTIC RESISTANCE (MAR) RELATIONSHIP ON DENDROGRAM

A total of 33 *Staphylococcus* species isolated from human vagina were subjected to cluster analysis to generate dendrogram using their antibiotic inhibition zone diameter data. This

analysis was used to determine the relationship between the MAR phenotypes of staphylococci isolates from different individuals in the two clinics. The dendrogram (Figure 4.3) indicates that two major clusters (I–II) were obtained at 57% linkage distance. These clusters were further analysed for patterns of associations of the different species present in a given cluster (Table 4.11). Of the 33 staphylococci isolated from vagina, 21 (64 %) were present in cluster II which was the larger cluster while a small proportion 12 (36 %) of the isolates was present in cluster I. Cluster I was divided into three sub-clusters designated A-C and sub-cluster IA contained 2 (16.6%) *S. cohnii* and only 1 (8.3%) *S. aureus*. Cluster IB contained only *S. aureus* isolates while cluster IC was represented by 2 (17%) each of *S. aureus* and *S. vitulinus* including 1 (8.3%) each isolate of *S. equorum* and *S. epidermidis*. Cluster II was subdivided into 5 clusters designated A to E. Sub-clusters IIC and IIE respectively contained the largest number of isolates. As shown in Figure 4.3, sub-cluster IIC contained only the five *S. aureus* isolates. On the contrary, sub-cluster IIE contained 2 (9.5%) isolates each of *S. aureus* and *S. vitulinus*. It also contained one *S. epidermidis*. In sub-cluster IIA, 2 (%) *S. succinus* isolates clustered with 1 each of *S. aureus* and *S. vitulinus*. With the exception of sub-clusters IB and IIC that contained only *S. aureus* isolates, all the other sub-clusters obtained in the analysis were considered to be mixed clusters. Results obtained in this study revealed that staphylococci isolated from vagina of pregnant women in the two clinics belonged to different species but their antibiotic resistance profiles were highly similar and resistant to multiple antibiotics.

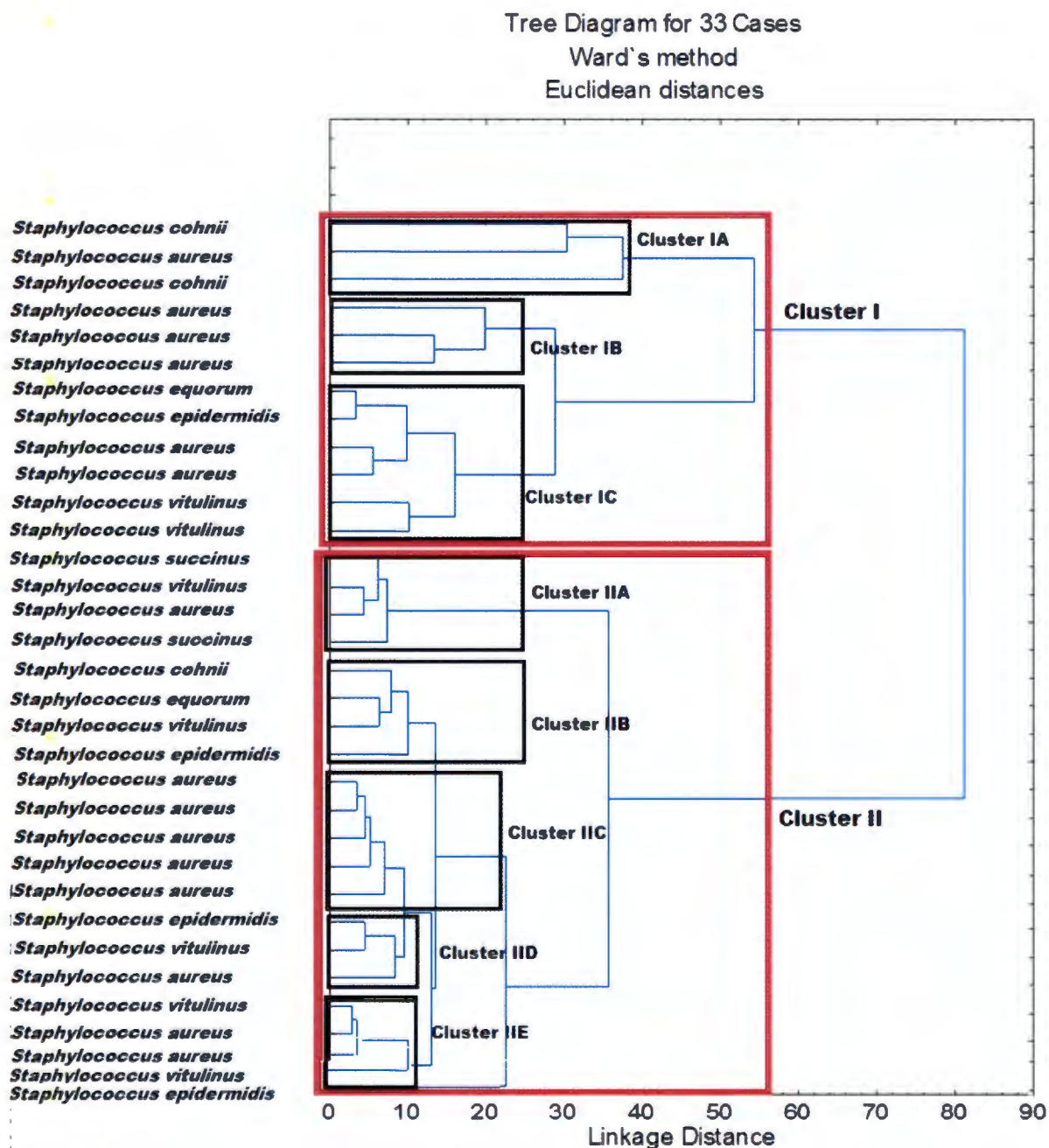


Figure 4.3: Dendrogram showing the relationship between staphylococci isolated from human clinical samples based on inhibition zone diameter (IZD) data. The isolates on the cluster diagram were identified to species level. The tree was constructed using Ward's method and Euclidean distances in Statistica, version 7 (Statsoft, US).

Table 4.11: Percentage representation of *Staphylococcus* species isolated from human clinical specimens within the various clusters

Species	Cluster I N=12					Cluster II N=21		
	Cluster IA	Cluster IB	Cluster IC	Cluster IIA	Cluster IIB	Cluster IIC	Cluster IID	Cluster IIE
<i>S. aureus</i>	1 (8.3%)	3 (25%)	2 (17%)	1 (4.8%)	0 (0%)	5 (23.8%)	1 (4.8%)	2 (9.5%)
<i>S. cohnii</i>	2 (16%)	0 (0%)	0 (0%)	0 (0%)	1 (4.8%)	0 (0%)	0 (0%)	0 (0%)
<i>S. epidermidis</i>	0 (0%)	0 (0%)	1 (8.3%)	0 (0%)	1 (4.8%)	0 (0%)	1 (4.8%)	1 (4.8%)
<i>S. equorum</i>	0 (0%)	0 (0%)	1 (8.3%)	0 (0%)	1 (4.8%)	0 (0%)	0 (0%)	0 (0%)
<i>S. vitulinus</i>	0 (0%)	0 (0%)	2 (17%)	1 (4.8%)	1 (4.8%)	0 (0%)	1 (4.8%)	1 (4.8%)
<i>S. succinus</i>	0 (0%)	0 (0%)	0 (0%)	3 (14%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total %	24.3	25	50.6	23.6	19.2	23.8	14.4	19.1

4.8 PCR AMPLIFICATION OF ENTEROTOXIN VIRULENCE GENES STAPHYLOCOCCI

A total of thirty three isolates were selected and characterised by multiplex-PCR analysis for the presence of staphylococcal enterotoxin genes. A total of 4 (12%) isolates were found positive for the *sec* gene and all belonged to *S. aureus*. The amplicons generated were of the expected size (439 bp). Figure 4.4 shows a 2% (w/v) agarose gel of the amplified *sec* gene fragments.

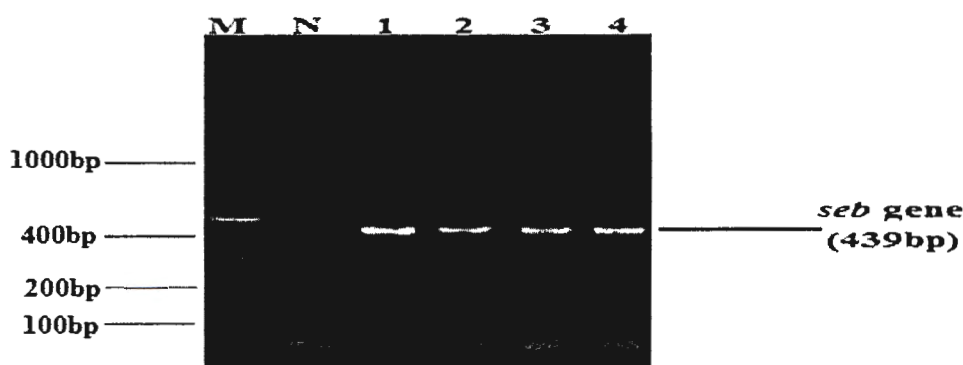


Figure 4.4: PCR amplicons of the *seb* gene from suspected *S. aureus* isolates. Lane M100 bp DNA ladder, Lane N: negative control, Lane 1: S13, Lane 2: S35, Lane 3: M10, Lane 4: S25

4.9 GENOTYPIC CHARACTERISATION OF STAPHYLOCOCCI BY RAPD-PCR ANALYSIS

A representative of 20 (60.6%) *Staphylococcus* isolates were subjected to non-specific PCR analysis using the primer M13 in order to determine their genetic relatedness. The band patterns of the isolates ranged from 1 to 6 (Figure 4.5). The PCR amplified product of isolates S35, S31, S10, S11, S13, S12 and M3 exhibited identical banding patterns (Figure 4.6). The other strains of

staphylococcal species had distinct banding patterns useful for differentiation from each other. Although some isolates belonging to different species possessed two identical bands with almost similar banding patterns, these were clearly distinguishable. Based on these band patterns, the genetic profiles of isolates were prepared as shown in Figure 4.6. A total of twenty isolates that produced reliable band patterns were subjected to cluster analysis to determine their genetic relatedness.

The clustering patterns generated with the RAPD-PCR protocol using the primer M13 revealed four main clusters (I-IV). Isolates belonging to different species and isolated from different locations mostly clustered together and were therefore considered to be mixed clusters. The largest cluster was cluster II with seven isolates (1 *S. epidermidis*, 2 *S. equorum* and 4 *S. vitulinus* which was the predominant species representing 57.1%). Within this cluster, a large proportion (71.4%) of isolates within this cluster was obtained from Montshioa Clinic. The second largest cluster with 6 isolates was cluster IV comprising 3 *S. aureus* isolates, 2 *S. epidermidis* and one *S. succinus*. The 5 isolates in this cluster were mostly obtained from Stad Clinic. Overall, there was random distribution of isolates irrespective of site of isolation. The remarkable similarities (50 to 83.3%) between the RAPD profiles of isolates of same or different species from the same site indicated that the ERIC-PCR protocol used in this study could be very effective in differentiating between the isolates.

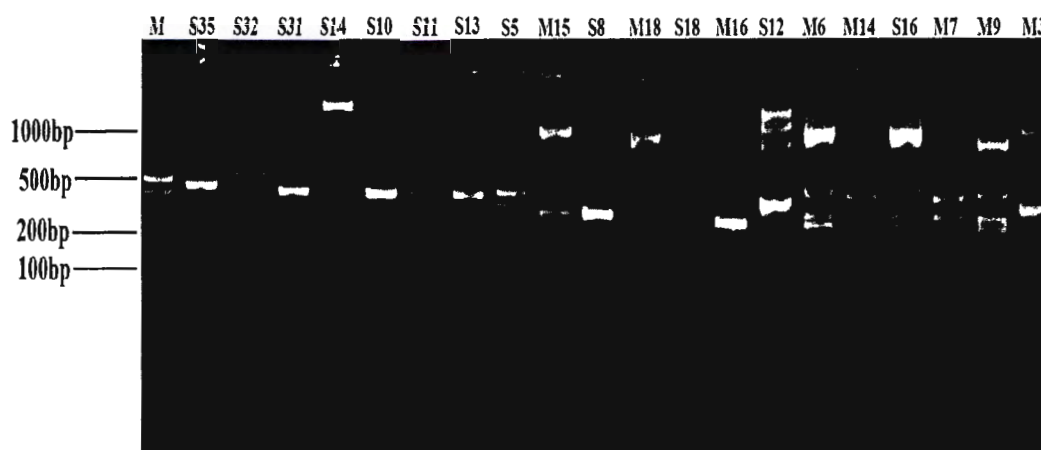


Figure 4.5: ERIC-PCR using (Primer AP7) PCR amplification of the ERIC gene from suspected staphylococci isolates Lane M:100 bp DNA ladder, Lane 1: S35, Lane 2: S32, Lane 3: S31, Lane 4: S14, Lane 5: S10, Lane 6: S11, Lane 7: S13, Lane 8: S5, Lane 9: M15, Lane 10: S8, Lane: 11 M18, Lane 12: S18, Lane 13: M16, Lane 14: S12, Lane 15: M6, Lane 16: M14, Lane 17: S16, Lane 18: M7, Lane 19: M9, Lane 20: M3.

4.10 ERIC PRIMER-2 PCR ANALYSIS

ERIC-PCR banding patterns displayed the highest similarity between isolates S16, S17, M7, M15, M6, M14, S5, M16 and S8, exhibited identical patterns with more than two distinct bands, whereas isolates S12, S13, S10, M3, S30 and S35 displayed the same banding pattern at approximately 500bp (Figure 4.6). The only difference was in the absence or presence of bands in the high molecular mass range. However, bands of high molecular masses (above 1200 bp) were less reproducible. The ERIC-PCR pattern of isolates differed in three bands of high

molecular masses. However, all *Staphylococcus* species investigated possessed four bands in common with sizes of approximately 400bp, 500bp, 550bp and 700bp.

A representative of 18 (55%) *Staphylococcus* isolates were subjected to a non-specific PCR analysis using the primer ERIC2 to determine genetic relatedness. The band patterns of the isolates ranged from 0 to 8 per isolate (Figure 4.6). A total of 18 isolates that produced reliable band patterns were subjected to cluster analysis. The genetic relationships of the isolates are shown in Figure 4.7. The clustering patterns generated with the ERIC-PCR protocol using the primer ERIC2 revealed three main clusters (I-III). Generally, isolates belonging to different species, and isolated from different locations clustered together and were considered mixed clusters. The largest cluster was cluster one (I) with eight isolates (44%) out of which *S. vitulinus* was the predominant species (37.5%) within this cluster. In addition, a large proportion of isolates were obtained from samples collected in Montshioa Clinic. Cluster II contained five isolates; *S. aureus* was the predominant isolate with only one isolate of *S. epidermidis* in this cluster. An interesting observation was that 75% of the isolates in this cluster were obtained from Stad Clinic. The species composition of isolates in cluster III was similar to that of cluster I since *S. vitulinus* was predominant. However, in this cluster, *S. cohnii* and *S. aureus* that were resistant to multiple antibiotics were also present. Overall, remarkable similarities (65 to 95%) between the ERIC profiles of some isolates from humans in the same clinic that also belonged to the same or different species revealed that the ERIC-PCR protocol used in this study could be very effective in differentiating between isolates.

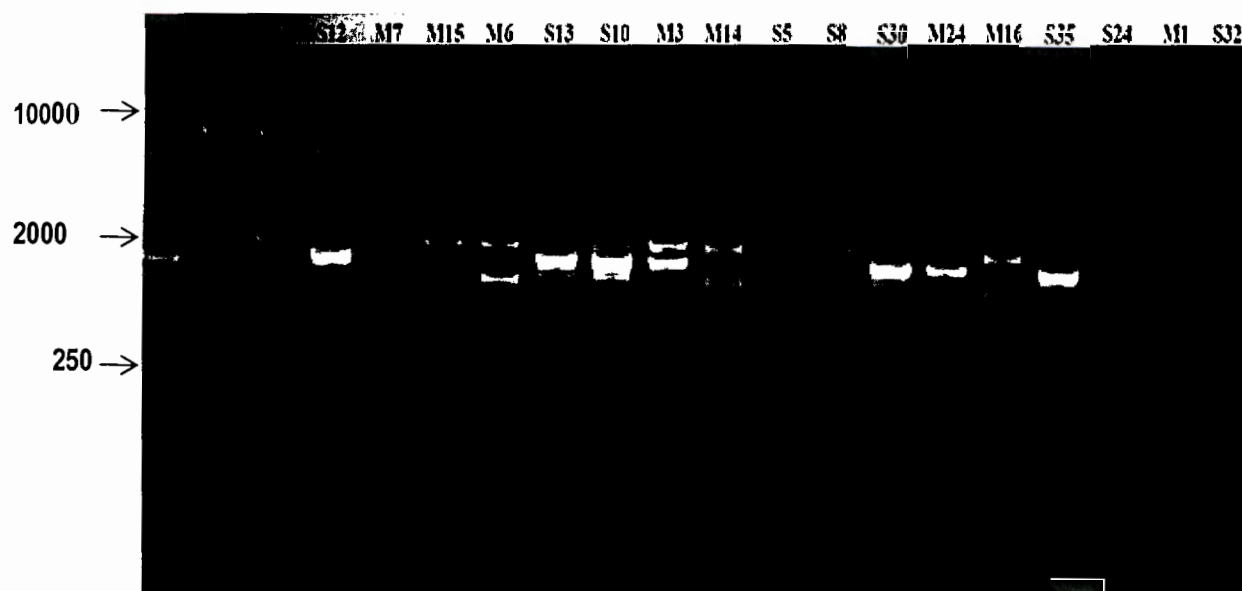


Figure 4.6: ERIC-PCR-generated DNA fingerprints of staphylococcal isolates. Lane M1000 bp DNA ladder, Lane 1: S16, Lane 2: S17, Lane 3: S12, Lane 4: M7, Lane 5: M15, Lane 6: M6, Lane 7: S13, Lane 8: S10, Lane 9: M3, Lane 10: M14, Lane 11: S5, Lane 12: S8, Lane 13: S30, Lane 14: M24, Lane: 15: M16, Lane 16: S35, Lane 17: S24, Lane 18: M1, Lane 19: S32.

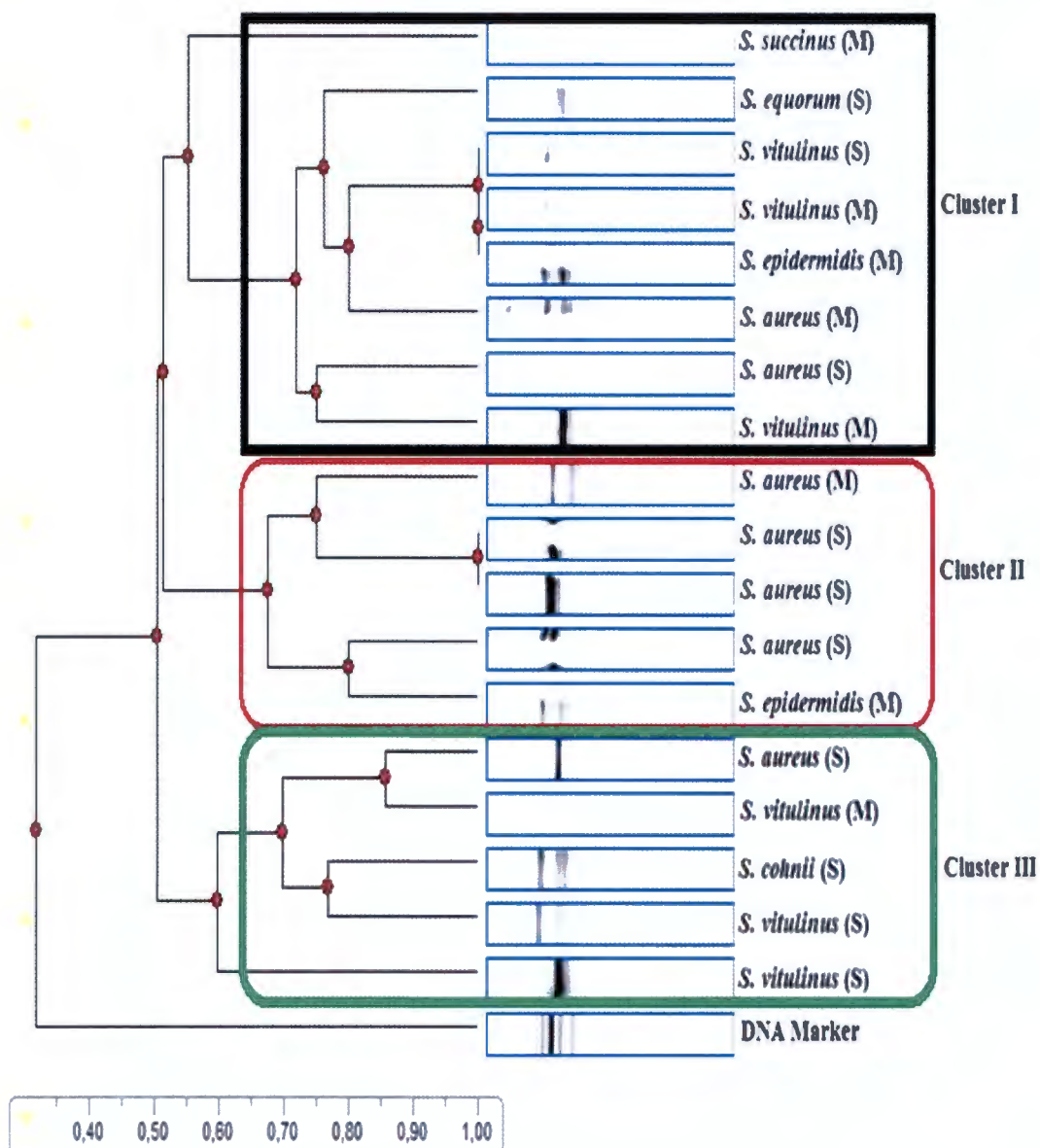


Figure 4.7: Dendrogram showing the genetic relationship of multiple antibiotic resistant *Staphylococcus* isolates obtained from human clinical samples using the ERIC –PCR combined primer sequences. The identities of the isolates and the clinics (S = Stad Clinic and M = Montshioa Clinic) where the isolates were obtained are indicated.

4.11 ANALYSIS OF GENETIC PROFILE OF STAPHYLOCOCCI BASED ON PCR AMPLIFICATIONS USING A COMBINATION OF PRIMERS M13 AND ERIC2

All the 33 *Staphylococcus* isolates were subjected to non-specific PCR analysis using the primer M13 and ERIC2 in order to determine their genetic relatedness. The band patterns of the isolates ranged from 1 to 11 per isolate (Figure 4.8). A total of 31 isolates that produced reliable band patterns were subjected to cluster analysis to determine their genetic relatedness (Figure 4.9). The clustering patterns generated with the ERIC-PCR protocol when the primers were combined revealed six main clusters (I-VI). Generally, clusters were mixed containing different isolates from different locations. The largest cluster was cluster one (I) with seven (22.6%) *Staphylococcus* isolates while *S. aureus* was the predominant species (42.9%) within this cluster. In addition, a large proportion of isolates was obtained from samples collected in Stad Clinic.

After cluster I, clusters III and VI were the next largest clusters with 6 isolates each. Despite the fact that *S. aureus* was also the predominant species in cluster III, it was observed that all isolates were from Montshioa Clinic. In addition, one isolate of each *S. cohnii*, *S. equorum* and *S. vitulinus* was present in this cluster. The species composition of isolates in cluster VI was similar to that of cluster III since *S. aureus* was the predominant species. However, in this cluster, *S. cohnii*, *S. epidermidis* and *S. vitulinus* that were resistant to multiple antibiotics were also present. Clusters IV and V contained 4 isolates each. Cluster IV contained most of the isolates from Montshioa Clinic while cluster V contained all the isolates from Stad Clinic only. The smallest cluster was cluster II with only two isolates (*S. equorum* and *S. epidermidis* both from Stad Clinic). The two species (*S. succinus* and *S. vitulinus*) did not fall under any cluster. *S. succinus* was isolated from Montshioa Clinic while *S. vitulinus* was obtained from Stad Clinic.



Figure 4.8: ERIC-PCR- using a combination of primers AP7, and ERIC2 generated DNA fingerprints of staphylococcal isolates. First gel: Lane M1000 bp DNA ladder, Lane 1: S14, Lane 2: M6, Lane 3: S16, Lane 4: S17, Lane 5: S10, Lane 6: S11, Lane 7: S3, Lane 8: S5, Lane 9: S29, Lane 9: S13, Lane 10: S31, Lane 11: S8, Lane 12: S32, Lane 13: M16, Lane 14: S12, Lane 15: S35, Lane 16: M14, Lane 17: M1. Second gel: Lane M1000 bp DNA ladder, Lane 1: M18, Lane 2: M24, Lane 3: SM3, Lane 4: M7, Lane 5: M15, Lane 6: S1, Lane 7: M10, Lane 8: M9, Lane 9: M8, Lane 9: S25, Lane 10: 24, Lane 11: S30, Lane 12: S18, Lane 13: S1.

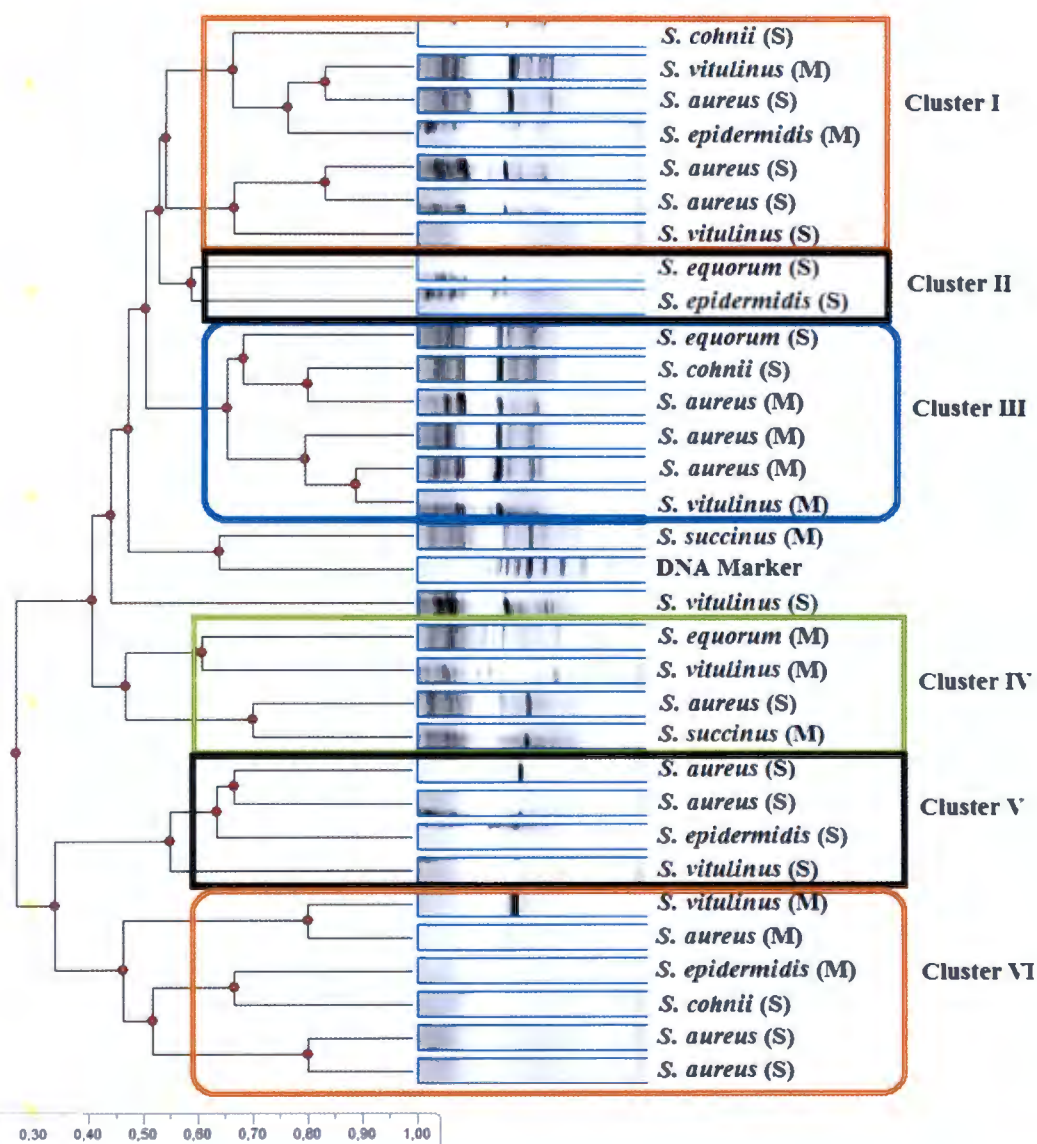


Figure 4.9: Dendrogram showing the genetic relationship of multiple antibiotic resistant *Staphylococcus* isolates obtained from human clinical samples using AP7 primer sequence. The identities of the isolates and the clinics (S = Stad Clinic and M = Montshioa Clinic) where the isolates were obtained are indicated.

CHAPTER FIVE
DISCUSSION

CHAPTER FIVE

DISCUSSION

5.0 DISCUSSION

Staphylococcus is one of the versatile pathogenic bacteria associated with hospital acquired infections and some strains have emerged in the community (Chambers, 2001; Tong *et al.*, 2015). These strains cause from mild infections of skin and soft tissue to more life-threatening sepsis, toxic syndromes and pneumonia (Stevens *et al.*, 2005). Besides being toxigenic, they also develop multiple antibiotic resistance to various antibiotics which further complicate the treatment process. There are increasing incidences of staphylococcal infection in pregnant, postpartum and neonatal women. The rate of vaginal colonisation with *Staphylococcus* in population ranges from 5-26% (Bourgeois-Nicolaos *et al.*, 2010).

In the North West Province of South Africa, the presence of multiple antibiotic resistant staphylococci in pregnant women has not been investigated. The primary objective of this study was to isolate antibiotic resistant *Staphylococcus* species from human vagina and to determine the extent of vaginal colonisation with these pathogens in pregnant women who attended antenatal clinic sessions in two clinics in Mafikeng, North West Province. Analysis and identification of *Staphylococcus* species of clinical importance from vaginal swabs were successfully confirmed using conventional methods. A number of the same biochemical assays have previously been used to detect the presence of *Staphylococcus* species (Chaieb *et al.*, 2005; Lange *et al.*, 2015).

In the present study, the ability to produce the coagulase enzyme, haemolytic pattern on 5% (w/w) blood agar and mannitol fermentation were used for preliminary identification of isolates.

The results revealed the presence of a large proportion of the isolates to be free from coagulase and varied potential in fermenting mannitol. Similar observations have previously been reported for the identification of staphylococci identification (Ishii *et al.*, 2006; Lange *et al.*, 2015). The coagulase test is generally used to differentiate coagulase negative from coagulase positive isolates (Ishii *et al.*, 2006). Despite the fact that CNS were previously considered to be non-pathogenic, recent studies have linked CNS strains as causative agents of human diseases (Chaieb *et al.*, 2005; Garza-Gonzalez *et al.*, 2011; Becker *et al.*, 2014).

To further confirm the identities of the isolates, they were subjected to molecular characterisation based on the presence of species-specific protein profiles using MALDI-TOF-MS. This technique is cheap, rapid and highly sensitive in identifying microorganisms (Singhal *et al.*, 2015). Results revealed the presence of seven *Staphylococcus* species as follows: *Staphylococcus aureus*, *S. vitulinus*, *S. epidermidis*, *S. cohnii*, *S. equorum* and *S. succinus*. In the present study, *S. aureus* was most frequently isolated (21%) among human samples compared to *S. vitulinus*, *S. epidermidis*, *S. cohnii*, *S. equorum* and *S. succinus*. This is consistent with previous studies which revealed that *S. aureus* is more prevalent in the vaginal tract of pregnant women (Laibl *et al.*, 2005; Andrews *et al.*, 2008). Moreover, among the CNS species isolated, *S. vitulinus* and *S. epidermidis* were frequently observed in the study and similar observations have also been reported in other studies (Dominguez *et al.*, 2002; Koksai *et al.*, 2009).

Of the six species, *S. aureus* was the only CPS while the other species were CNS strains. Coagulase positive *S. aureus* have a well-established pathogenic history (Tong *et al.*, 2015) while several CNS species such as *S. epidermidis*, *S. haemolyticus* and *S. hominis* are currently

considered as emerging pathogens, especially in hospitalised patients who have undergone surgical procedures including medical implants (Von Eiff *et al.*, 2002; Piette *et al.*, 2009). *S. epidermidis* is the most prevalent CNS species and accounts for approximately 60-70% of all infections caused by these strains (Favre *et al.*, 2005). Therefore, the presence of CNS and CPS in humans, particularly among pregnant women may pose severe risks such as opportunistic infections, sepsis, pneumonia and meningitis. These infections usually present more challenging consequences to neonates and immuno-compromised individuals (Wei *et al.*, 2005). Despite the fact that staphylococci are regarded as opportunistic pathogenic bacteria (Casadevall and Pirofsk, 1999), results of the present study revealed that vaginal colonisation with staphylococci may result in vertical transmission to infants during birth. Several studies have also suggested that maternal-to-infant transmission of staphylococci infection increases among pregnant women and neonates (Andrews *et al.*, 2009; Pinter *et al.*, 2009; Top *et al.*, 2012).

Due to selection pressure, there is a rapid shift in the microbiological and epidemiological characteristic of pathogenic strains and pathogens are becoming antibiotic resistant (Martinez, 2009). With increasing incidences of antimicrobial resistance, there are more chances of therapeutic failure (Gupta *et al.*, 2001). In the present study, in order to compare the antimicrobial resistance pattern of the screened isolates, an antibiotic susceptibility test was determined for ten antibiotics. The results revealed that all isolates were most often resistant to penicillin (100%), and ampicillin (100%), and variously resistant to xacillin (97%), oxytetracycline (97%), vancomycin (97%), rifampin (85%), erythromycin (82%) and streptomycin (76%). This shows an indiscriminate and excessive use these antibiotics in this region that produced antibiotic resistant strains, especially to the beta-lactam group. Similar

results have been reported in the past (Leclercq, 2002; Stanley *et al.*, 2013). Resistance to these different antibiotics is acquired either by intrinsic resistance or by acquired resistance (Martinez, 2009). There are different mechanisms reported through which pathogenic bacteria overcome the toxicity of antibiotics such as mutations (especially adaptive), horizontal antibiotic resistant gene transfer, antibiotic inactivation or modification by hydrolysis, group transfer and redox reactions (Lewis, 2013). Isolates of the present study could use one or a combination of the mechanisms mentioned above to overcome the toxic effect of antibiotics tested and become resistant to multiple antibiotics. Therefore, this multiple resistance phenotypes could pose a serious challenge in the treatment of clinical staphylococcal infections.

Based on antibiotic zone of inhibition, multiple antibiotic resistance (MAR) indices were determined and the results revealed that MAR indices of *Staphylococcus* species were above 0.2. More than 0.2 MAR indices showed that these isolates have been exposed to antibiotic misuse within the environment, indicating high risk of contamination (Karthi *et al.*, 2009). In the present study, levels of MAR observed among the isolates were very high and varying. The MAR phenotypes PG-AMP-OX--GM-VA-ERY-RIP-OT, PG-AMP-OX-K-VA-ERY-RIP-OT and PG-AMP-OX-S-VA-ERY-RIP-OT were dominant among *S. aureus* while PG-AMP-OX-GM-VA-ERY-RIP-OT, PG-AMP-OX-VA-ERY-RIP-OT were common among *S. vitulinus*. In addition, PG-AMP-OX-K-VA-OT and PG-AMP-OX-K-VA-ERY-RIP-OT were dominant among *S. cohnii* and *S. epidermidis* respectively. Despite the fact that only one *S. succinus* isolate possessed the PG-AMP-OX-S-K-GM-VA-ERY-RIP-OT, the fact that this isolate was resistant to all ten antimicrobial agents tested is a cause for concern (Subramani and Vignesh,

2012). The antibiogram data revealed that all the isolates were resistant to penicillin and ampicillin.

Staphylococcus species are known to carry or harbour various virulence determinants that help to facilitate disease causation (Foster, 2012; Priest *et al.*, 2012). In the present investigation, staphylococcal isolates were screened for the presence of staphylococcal virulence gene determinants by multiplex PCR analysis. Enterotoxin genes are known to be major virulence factors and play an important role in diseases caused by staphylococci and therefore, are responsible for most infections caused by staphylococci. Our findings show that isolates were only positive for the *sec*. However, similar observations have been reported where isolates showed a higher percentage to the presence of *sec* gene (Adesiyun *et al.*, 1992; Katsuda *et al.*, 2005; Oliveira *et al.*, 2011). Variation in the prevalence of enterotoxin genes are probably due to geographical variations and such variation is influenced by food, human, and animal sources of isolation of the strains (Fueyo *et al.*, 2001; Naffa *et al.*, 2006).

Nowadays, PCR-based techniques play an important role in the rapid identification of virulent bacterial isolates as these techniques are easy, fast and cheap (Emerson *et al.*, 2008). Among these techniques, RAPD and ERIC are important and sensitive tools used to differentiate bacteria at genus, species or strain level. For both techniques, no prior information of gene sequence is required (Tobes and Ramos, 2005; Shi *et al.*, 2010). In the light of this, the present study employed both techniques to differentiate clinical staphylococcal isolates. RAPD primer M13 produced a reproducible band ranging from 1 to 6. Dendogram based on these band patterns grouped isolates into 4 clusters. All the clusters were mixed containing different staphylococcal

species of isolates and contained isolates from both clinics. This showed that different species of staphylococcal isolates have emerged from the single respective species clone, undergone evolution and finally, spread through the consumption of contaminated water and food (plant or animal originated foods). Furthermore, amplification obtained by ERIC-2 primer resulted into distinguishable and reproducible bands ranging from 1 to 8 and the dendrogram constructed showed 3 clusters. Similar to RAPD, clusters formed by ERIC were mixed and for the same reasons.

The combination of RAPD M13 primer and ERIC-2 primer resulted into increased band numbers ranging from 1 to 11 compared to bands generated by individual M13 and ERIC-2 primers. Dendrogram based on fingerprint data showed 6 clusters and all clusters contained mixed staphylococcal isolates as seen in RAPD and ERIC. This showed that isolates had emerged from the same species clone and with the passage of time due to evolution diverted to different lineage or strains. Researchers have also used many primers for strain differentiation in the past (Byun *et al.*, 1997; Nema *et al.*, 2007). Also, these techniques have been used efficiently in studies yielding highly reproducible results capable of discriminating isolates (Morandi *et al.*, 2010; Vazquez-Sanchez *et al.*, 2012; Barbieri *et al.*, 2015). In the present study, it was observed that there was a high prevalence of vaginal colonisation with multiple antibiotic resistant staphylococci among pregnant women in the Mafikeng area, although participants did not manifest any disease symptom due to colonization (Andrews *et al.*, 2008). This may be a call for concern because the vertical transmission of these multiple antibiotic resistant *Staphylococcus* species to infants is possible, and even during labour. Early-onset sepsis due to acquisition of

these bacteria may occur in infants and treatment may also be difficult due to the increasing rate of multiple antibiotic resistant strains among staphylococci (Chan *et al.*, 2015).

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

To the best of our knowledge, this study is the first of its kind to be conducted in the North-West Province (Mafikeng) of South Africa. From the findings of this study, it can be concluded that the prevalence of multiple antibiotic resistant staphylococci and the level of colonisation among pregnant women were high and widespread. As a result, pregnant women visiting clinics for prenatal check-ups in the study area should be screened for the presence and vaginal colonisation of staphylococci. This may greatly minimise the degree of exposure of new born to these multiple antibiotic resistant bacteria strains.

In the present investigation, staphylococci species of clinical importance were isolated. Identities of *Staphylococcus* species were confirmed by both conventional and molecular techniques and the level of their occurrence calls for concern. Considering the high possibility of vertical transfer of multiple antibiotic resistant strains coupled with the ability to cause invasive infections in infants there is need to constantly screen pregnant women for these pathogens. Results for MALDI-TOF revealed the presence of some species such as *S. equorum* that are mostly isolated from animals but rarely from humans. It is therefore suggested that these strains may have transmitted to humans through contact with animal species. Moreover, PCR results revealed that the primers used were highly specific for the detection of *Staphylococcus* species and virulence genes from human samples. In addition, random amplification of polymorphic DNA using enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR revealed a high level of polymorphism among isolates and also showed that these species generated a species-specific banding pattern that is characteristic to staphylococci. The target *nuc*, *seb* gene

sequences were conserved among the isolates and control strains. Moreover, the target ERIC-like sequences among the isolates revealed that these sequences exist in the genomes of staphylococci.

Due to the increasing rate of infant mortality and morbidity, escalating rates of pregnancy and population growth and the limited data on antibiotic resistant staphylococci in human clinical samples in the North-West Province, the department of Health, Centre for Disease Control in South Africa, in collaboration with gynecological and obstetric departments, should put in place action plans that will healthy birth of infants and maintain them thereof.

Emphasis should be placed on the need to prevent the vertical microbial transmission of antimicrobial resistant bacteria from colonised mother to infants through the development and establishment of efficient strategies designed at eliminating possibilities of vertical transfer such as screening during prenatal checkups. Furthermore, an intensive health care education programme aimed at improving. A detailed microbiological investigation, routine screening and monitoring of vaginal colonisation among pregnant women are needed to provide sufficient data to assist in the management and elimination of multiple antibiotic resistant bacteria capable of being vertically transferred to infants not only in the environment but, the North-West Province the country at large.

It was difficult to successfully identify and amplify antibiotic resistant genes from suspected bacterial isolates that were phenotypically resistant to antimicrobial agents used in this study. In addition, the researcher was also unable to determine whether these organisms are really

transferred during child birth and to what extent will resistant bacteria cause invasive infections in infants. However, it is recommended that further research be conducted on the inspection and control of antibiotic resistant strains in hospitals and clinics.

APPENDICES

APPENDICES

Appendix1A: Results of Biochemical test for staphylococci isolated in the study

Isolate number	Gram stain reaction	Cell morphology	Catalase test	Latex agglutination	Tube coagulase	PBP2a	Beta haemolysis
M3	+	cocci	+	++	+	++	+
S18	+	cocci	+	+	+	+	-
S17	+	cocci	+	+	+	+	-
S3	+	cocci	+	++	++	++	+
M6	+	cocci	+	+	-	+	-
S30	+	cocci	+	+	+	++	+
S16	+	cocci	+	+	-	-	-
S20	+	cocci	+	+	-	-	-
S14	+	cocci	+	+	-	+	-
M15	+	cocci	+	+	++	++	+
S12	+	cocci	+	+	+	++	+
S5	+	cocci	+	-	-	-	-
S13	+	cocci	+	++	++	++	+
M14	+	cocci	+	+	+	+	-
M7	+	cocci	+	-	+	-	-
S11	+	cocci	+	+	++	++	+
M18	+	cocci	+	-	+	-	-
M8	+	cocci	+	-	+	++	+
S 35	+	cocci	+	++	++	+	+
S10	+	cocci	+	+	++	++	+
S2	+	cocci	+	-	+	-	-
S24	+	cocci	+	-	+	-	-
M1	+	cocci	+	++	++	++	+
M16	+	cocci	+	+	-	+	+
S29	+	cocci	+	+	+	+	+
S15	+	cocci	+	+	++	+	-
S1	+	cocci	+	V	-	++	-
M24	+	cocci	+	+	+	+	+
S8	+	cocci	+	+	-	+	-
S32	+	cocci	+	+	-	-	-
S25	+	cocci	+	+	+	++	+
M10	+	cocci	+	+	++	++	+
M9	+	cocci	+	+	-	+	+
S31	+	cocci	+	++	++	-	+

+=positive for the test; ++=strongly positive; V= result of variable test

Appendix 1B: Results of Biochemical test for staphylococci isolated in the study

Isolate number	Gram stain reaction	Cell morphology	Catalase test	Latex agglutination	Tube coagulase	PBPS 2a	Beta haemolysis
M2	+	cocci	+	+	+	NT	+
S9	+	cocci	+	+	V	NT	-
S26	+	cocci	+	V	+	NT	+
S27	+	cocci	+	-	+	NT	-
S22	+	cocci	+	+	+	NT	+
S23	+	cocci	+	+	+	NT	+
M20	+	cocci	+	+	+	NT	-
S7	+	cocci	+	-	-	NT	-
S37	+	cocci	+	++	-	NT	+
S34	+	cocci	+	-	-	NT	-
S4	+	cocci	+	-	-	NT	+
S2	+	cocci	+	V	-	NT	-
S33	+	cocci	+	-	+	NT	+
M17	+	cocci	+	V	-	NT	+
S40	+	cocci	+	-	-	NT	-
S41	+	cocci	+	V	-	NT	-
S44	+	cocci	+	-	-	NT	+
S15	+	cocci	+	-	+	NT	-
S42	+	cocci	+	-	-	NT	-
S36	+	cocci	+	-	-	NT	+
S39	+	cocci	+	-	-	NT	-
M13	+	cocci	+	-	+	NT	-
M11	+	cocci	+	-	-	NT	-
M5	+	cocci	+	-	-	NT	-
M19	+	cocci	+	-	-	NT	-
S6	+	cocci	+	-	-	NT	-
S38	+	cocci	+	V	-	NT	+
S43	+	cocci	+	-	-	NT	-
M4	+	cocci	+	-	-	NT	-
S21	+	cocci	+	V	-	NT	+
S19	+	cocci	+	-	-	NT	+
S28	+	cocci	+	-	+	NT	-
S45	+	cocci	+	-	-	NT	+
S34	+	cocci	+	-	-	NT	-

+=positive for the test; ++=strongly positive; V=result of variable test; NT=not tested

APPENDIX 2

Appendix 2A: Antibigram data of *Staphylococcus* isolates obtained from human clinical samples

Isolate No	PG	AMP	OX	S	K	GM	VA	ERY	RIP	OT
S14	Resistant	Resistant	Resistant	Sensitive	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant
S 16	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant
S 17	Resistant	Resistant	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Sensitive	Resistant
S 18	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Sensitive	Sensitive
S 20	Resistant	Resistant	Resistant	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant
S 24	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant
S 25	Resistant	Resistant	Resistant	Sensitive	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant
S 29	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant
S 30	Resistant	Resistant	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
S 31	Resistant	Resistant	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
S 32	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
S 35	Resistant	Resistant	Sensitive	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Sensitive	Resistant
M 1	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant
M 3	Resistant	Resistant	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
M 6	Resistant	Resistant	Resistant	Resistant	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant
M 7	Resistant	Resistant	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
M 8	Resistant	Resistant	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
M 9	Resistant	Resistant	Resistant	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant
M10	Resistant	Resistant	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
S1	Resistant	Resistant	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Sensitive	Resistant
M 14	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant
M 15	Resistant	Resistant	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
S 2	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant
S 3	Resistant	Resistant	Resistant	Resistant	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant
S 5	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant
S 8	Resistant	Resistant	Resistant	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant
S 10	Resistant	Resistant	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Sensitive	Resistant

S 11	Resistant	Resistant	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant	Resistant
S 12	Resistant	Resistant	Resistant	Resistant	Sensitive	Sensitive	Resistant	Sensitive	Resistant	Resistant
S 13	Resistant	Resistant	Resistant	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant

ERY: Erythromycin; K: Kanamycin; AMP: Ampicillin; S: Streptomycin; PG: Penicillin; OT: Oxytetracycline; OX: Oxacillin; VA: Vancomycin; GM: Gentamycin; RIF: Rifampin

Appendix 2B: Antibigram data of *Staphylococcus* isolates obtained from human clinical samples

Isolate No	PG	AMP	OX	S	K	GM	VA	ERY	RIP	T
M 16	Resistant	Resistant	Resistant	Sensitive	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant
M 18	Resistant	Resistant	Resistant	Sensitive	Sensitive	Resistant	Sensitive	Resistant	Resistant	Resistant
M 24	Resistant	Resistant	Resistant	Sensitive	Resistant	Sensitive	Resistant	Resistant	Resistant	Resistant

ERY: Erythromycin; K: Kanamycin; AMP: Ampicillin; S: Streptomycin; PG: Penicillin; OT: Oxytetracycline; OX: Oxacillin; VA: Vancomycin; GM: Gentamycin; RIF: Rifampin

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