Characterization of *Staphylococcus aureus* from Nigeria and South Africa

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Psalms 40:3

And he hath put a new song in my mouth, even praise unto our God: many shall see it, and fear, and shall trust in the LORD.

Psalms 40:8

I delight to do thy will, O my God: yea, thy law is within my heart.

Psalms 40:16

Let all those that seek thee rejoice and be glad in thee: let such as love thy salvation say continually, The LORD be magnified.

1 Samuel 2:8

He raiseth up the poor out of the dust, and lifteth up the beggar from the dunghill, to set them among princes, and to make them inherit the throne of glory: for the pillars of the earth are the LORD's, and he hath set the world upon them.

DEDICATION

This piece of academic work – thesis is dedicated to the Almighty God, Our Lord Jesus Christ and the Holy Spirit without whom I would not have been able to achieve this long-term dream.

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ABSTRACT

The detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in wastewater system from clinical sources has posed a public health challenge in many countries worldwide. Of a greater concern is the discharge of untreated or improperly treated hospital effluents into wastewater, which has been identified as possible hotspot for antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs). MRSA, a distinct form of *Staphylococcus aureus* (*S. aureus*) showing resistance to methicillin, is widely reported as a nosocomial pathogen. However, there is paucity of data and limited reporting about its occurrence in non-clinical environments, such as wastewater in Nigeria and South Africa. This present study is on the characterization of *S. aureus*, MRSA and other staphylococci from clinical and environmental sources in Ile-Ife, Nigeria and Potchefstroom, South Africa. The first part of the thesis emphasized the significance of the genus *Staphylococcus* and their importance as opportunistic pathogens, causing many diseases and infections in humans. The rationale, aim and objectives of the study were also highlighted. The second part of the study was a systematic review aimed to assess the prevalence of *mecA* gene in Staphylococcal isolates from clinical and environmental (wastewaters) sources. For this review, 50 peer reviewed full texts (35 clinical and 15 wastewaters) from 19 countries and 5 continents across the globe were selected based on pre-determined criteria. Specifically, there is insufficient information on *mecA* gene in *Staphylococcus* spp. from aquatic or environmental wastewaters (30.0%). Further, within the database search period (2000 – 2019), only very few studies (10) on *Staphylococcus* spp. with reference to *mecA* gene could be traced and retrieved from developed countries. Data extracted revealed that *S. aureus* and other *Staphylococcus* spp. are potential contributors of antibiotic resistance bacterial (ARB) infections from both clinical and environmental settings. In the third part

of the study, 76 *Staphylococcus* spp. were isolated and characterized from hospital and environmental sources in Ile-Ife, Nigeria. These comprised of 40.7% (31/76) coagulase-positive (*S. aureus*) and (45/76) 59.3% coagulase-negative staphylococci (CoNS) constituting *S. sciuri* and 3 other rare species - *S. xylosus, S. warneri and S. kloosi* which have not been widely reported. Furthermore, the study confirmed the presence of resistance and virulence genes of public health importance in the *Staph*. spp. Sixty-three (63%) of the isolates with these genes originated from clinical samples, while 37% were from environmental sources. Interestingly, the presence of *mecA* gene was also confirmed in coagulase-negative staphylococci (CoNS) which have been previously considered low-risk or non-pathogenic. This calls for concern since their possession of resistance and virulence genes signal that these species can endanger human health and life. The fourth part of the study also focused on *Staphylococcus* spp. specifically MRSA and *mecA* gene detection in a wastewater treatment plant (WWTP) in Potchefstroom, South Africa. In the WWTP, 35 *staphylococcal* isolates of 7 different species were isolated. These included 3 CoNS which are uncommon: *S. cohnii, S. nepalensis* and *S. arlettae*. The study confirmed the presence of MRSA and multidrug resistant (MDR) *Staphylococcus* spp. at the final effluent point which had undergone chemical treatment by chlorination. It was recommended that a more effective treatment plan or modification procedures should be adopted especially if the water is to be reused. The fifth and final part of the study, reported a high quality draft genomes of 8 CoNS isolates obtained from clinical and environmental settings in Nigeria and South Africa. The isolates were identified as *S. lentus* (1 from South Africa), *S. cohnii* (3 from Nigeria and 2 from South Africa) and *S. haemolyticus* (1 from Nigeria and 1 from South Africa). The isolates possessed an open pan-genome, with a few genetic barriers to horizontal gene transfer. Clustered regularly interspaced short palindromic repeats

(CRISPR) associated with Cas protein system (CRISPR/Cas) were identified in one of the 8 isolates. This study has generated readily available information on the local antimicrobial resistance patterns of potential bacterial pathogens which could assist in improved assessments of human health risks.

Keywords: Antibiotic resistance genes, CoNS, *mecA*, MRSA, *Staphylococcus aureus*, wastewater treatment plant, hospital effluents; receiving wastewater.

LIST OF ABBREVIATIONS

ANI: Average Nucleotide Identity

Atl: Autolysin

AMR: Antimicrobial Resistance

ARB: Antibiotic Resistance Bacteria

ARGs: Antibiotic Resistance Genes

CARD: Comprehensive Antibiotic Resistance Database

ClfA: Clumping Factor A

ClfB: Clumping Factor B

COG: Cluster of Orthologous Groups

CoNS: Coagualse-Negative Staphylococci

CoPS: Coagualse-Positive Staphylococci

DNA: Deoxyribonucleic Acid

GLASS: Global Antimicrobial Resistance Surveillance System

MLST: Multi-locus Sequence Typing

MRSA: Methicillin-Resistant *Staphylococcus aureus*

NCDC: Nigeria Centre for Disease Control

PCR: Polymerase Chain Reaction

PFGE: Pulsed-Field Gel Electrophoresis

PHASTER: PHAGE Search Tool Enhanced Release

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-analyses:

PVL: Panton Valentine-Leukocicdin

qPCR: Quantitative Polymerase Chain Reaction

RAPD-PCR: Random amplified polymorphic DNA-Polymerase Chain Reaction

REAP: Restriction Endonuclease Analysis of Plasmid DNA

WHA: World Health Assembly

WHO: World Health Organization

WWTP: Wastewater Treatment Plant

VFDB: Virulence Factors of Pathogenic Bacteria Database

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CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Background of the study

Antibiotic resistance (AR) is a major healthcare threat worldwide, particularly in developing countries, where there is an increasingly higher burden of pathogenic infections attributed to antibiotic resistant bacteria (ARB) (Laxminarayan et al. 2013; 2016). Although AR is common in hospital settings, of more recent concern are environmental wastewaters (Malassa et al. 2013; Goldstein et al. 2017; Said et al. 2017). Water bodies such as surface waters, effluents from hospital wastewaters and community wastewaters, have been identified as key reservoirs contributing to the spread of ARB such as *Staphylococcus* spp. and antibiotic resistance genes (ARGs) into surface waters (Marti et al. 2013; Czekalski et al. 2014; Ekwanzala et al. 2018).

Currently, among the *Staphylococcus* spp., the most common pathogen and the most virulent is *Staphylococcus aureus* (CDC, 2013). Clinical infections of *S. aureus* include pus-forming (pyogenic) infections leading to various diseases such as bacteraemia, pneumonia, and osteomyelitis (Nanoukona et al. 2017). The burden of *S. aureus* infections worldwide has increased owing to the advent of antibiotic resistance *Staphylococcus aureus* particularly, the occurrence of a distinct strain known as methicillin-resistant *Staphylococcus aureus* (MRSA).

Resistance fitness of MRSA strains is considered to be due to the acquisition of the *mecA* gene. This gene is present in the staphylococcal cassette chromosome mec (SCC*mec* types I–XIII) (Yamaguchi et al. 2020). In addition, resistance across different strains of *S. aureus* and other *Staphylococcus* spp. have been attributed to the presence of gene coding for antibiotic resistance and virulence via horizontal gene transfer (Robinson & Enright, 2004; Tormo et al. 2005). MRSA poses difficulty with regards to the treatment of patients thereby elongating their hospital stay which in turn imposes a huge financial burden (Kong et al. 2016). Although, the clinical and economic burden of MRSA infection in emerging nations is yet to be comprehensively determined, it may be comparable or even higher in developed countries.

In the 1960s MRSA was considered to be restricted to the hospital environment and was commonly referred to as hospital-acquired MRSA (HA-MRSA). However, by the 1990s, MRSA had begun to increase and spread rapidly into the communities and among people who were not at risk to acquire it (Tenover et al. 2006). This subsequently led to the naming of strains linked to the community known as community-acquired MRSA (CA-MRSA) (Uhlemann et al. 2014) and those associated with livestock to be named livestock-associated MRSA (LA-MRSA) (Grema et al. 2015). There is currently no environmental-associated MRSA, but perhaps such naming should be considered.

1.2 The genus *Staphylococcus* and taxonomy

Staphylococcus is a group of Gram-positive cocci belonging to the family Staphylococcaceae in the order Bacillales. They are normally non-spore forming and facultative anaerobic (Cheesbrough, 2006; Koneman et al. 2006) *Staphylococcus* sp. are innocuous and reside normally on the skin and mucous membranes of humans and other organisms (Wertheim et al. 2005). Among these species, *S. aureus* strains produce free or bound coagulase, depending on direct or indirect activation of a coagulase reacting factor present in plasma (Cheesbrough, 2006). Staphylococci consist of more than 40 species that are found to exist either as commensals or pathogens of humans and/or animals.

1.3 Genotypic methods for characterizing *Staphylococcus* species

Molecular characterization of *S. aureus* and other staphylococci isolates could be achieved using different genotypic methods. DNA typing methods produce clear-cut and transferable data, thereby allowing the comparison of data from different geographic locations (Ruppitsch et al. 2006). Examples include Pulsed-Field Gel Electrophoresis (PFGE), *spa* typing, Multi-locus Sequence Typing (MLST) etc. *Spa* typing is based on the sequencing of a single gene (*spa* coding for protein A) (Harmsen et al. 2003). MLST is based on the sequencing of internal fragments of seven housekeeping genes generating a sequence type (ST) (Enright et al. 2000). The evaluation of partial sequences from seven housekeeping genes are defined by a standardized MLST database and strains are defined by particular combinations of alleles (Enright et al. 2000). Further imaging of MLST sequence typing data sets is possible with the

development of eBURST (http://eburst.mlst.net). Next generation sequencing technique, such as Whole Genome Sequencing (WGS) is a sequence-based method to determine the genomic characterisation of organisms of interest (e. g. *S. aureus* isolates). For *spa* typing, sequences are submitted to an online database for assignment of *spa* types (http://*spa*.ridom.de/index.shtml).

1.4 Clinical significance of *Staphylococcus aureus* and other species

Staphylococcus aureus can cause opportunistic diseases such as boils, impetigo, pustules, wound infections, ulcers and burns, osteomyelitis, mastitis, septicaemia, meningitis, pneumonia and pleural empyema, when the immune system is weakened. Moreover, approximately 30-50% of human populations carry *S. aureus* in their skin or nares as part of the normal flora (Wertheim et al. 2005). *Staphylococcus aureus* are also associated with food poisoning and toxic shock syndrome (Frazee et al. 2005).

1.4.1 Hospital-acquired MRSA (HA-MRSA)

Hospital-acquired-methicillin-resistant *Staphylococcus aureus* is mostly isolated from those who have been in health care settings either as patients or health practitioners. According to Frazee et al. (2005), HA-MRSA is responsible for some hospital-acquired infections, including pneumonia, bacteraemia and bone infections. The strains also harbour a comparatively large staphylococcal chromosomal cassette mec (SCC*mec*) which is of type I, II, or III origin (Buck et al. 2005). SCC*mec* is a key player in antimicrobial resistance characteristics, molecular epidemiology and evolution of MRSA.

It is the defining feature of MRSA (Falagas et al. 2013). They tend to show resistance to many classes of antimicrobials (non-beta lactam) and do not commonly carry the Panton-Valentine leukocidin (PVL) genes (Lina et al. 1999; Jahamy et al. 2008).

1.4.2 Community-acquired MRSA (CA-MRSA)

Community-acquired MRSA (CA-MRSA**)** are linked with serious clinical conditions like sepsis and pneumonia accompanied by cell death (Lowy, 1998). According to Afroz et al. (2008), CA MRSA showed resistance to fewer non-beta lactam antibiotics compared to HA-MRSA. In addition, they also carry SCC*mec* elements types IV or V which is smaller. Furthermore, in CA-MRSA isolates, increased virulence coupled with frequent production of the Panton-Valentine leucocidin (PVL) genes is constantly noted. Moreover, expression of toxin-producing genes is higher in the CA MRSA than in HA MRSA (Gillen et al. 2015).

1.4.3 Livestock-associated MRSA (LA-MRSA)

Livestock-associated MRSA infection was firstly reported in Belgium in the 1970s (Devriese et al. 1972). This was an incident of bovine mastitis; however, various reports of MRSA infections in animals were cases of bovine mastitis in Belgium in the early 1970s. Subsequently, several studies have been conducted and documented as showing MRSA as an important veterinary and zoonotic pathogen for infections in animals (Devriese et al. 1972). Furthermore, genetic typing showed that some animal

lineages could be host specific while others are able to colonize or infect wide range of animals including humans (Silva et al. 2006).

1.5 Antibiotic resistance and virulence

Antibiotic resistance due to increasing antibiotic overuse is a serious global health threat with about 700,000 related deaths every year (CDC, 2013, World Bank, 2017). Furthermore, it is estimated that an unrestricted rise in antimicrobial resistance may lead to 10 million deaths per year by 2050 (CDC, 2013). The prevalence of antibiotic resistance (AR) in many *S. aureus* strains or across different *Staphylococcus* spp. has been credited to horizontal (parallel) transmission of genes encoding antibiotic resistance and virulence (Lindsay, 2014). This makes the spread of MRSA in the African region worrisome, since there might be relatively limited data and unavailability of effective modern antibiotics against it (Laxminarayan et al. 2013; Lindsay, 2014).

1.5.1 *MecA*

The gene (*mecA*) is responsible for certain forms of bacterial cells showing resistance to antibiotics such as methicillin. In addition, previous studies have associated the *mecA* gene with MRSA. The gene promotes resistance to beta lactam antibiotics such penicillin, methicillin, oxacillin and some cephalosporins. According to Wu et al. (1996), *mecA* gene is acquired and transmitted through a mobile genetic element, which inserts itself into the host genome. Several studies have associated the *mecA* gene only to methicillin-resistant *Staphylococcus aureus* (MRSA) (Deurenberg & Stobberingh, 2009; Yang et al. 2009; Igbinosa et al. 2016). However, some coagulase-negative

staphylococci such as *S. sciuiri* are also carrying *mecA* gene. Moreover, evolutionary history shows that the *mecA* gene developed from a harmless core gene (*mecA*1) from staphylococcal species of animal origin i.e., the *Staphylococcus sciuri* group since the first *mecA* gene homologue was encountered in the chromosome of *S. sciuiri* strains (Fuda et al. 2007). This suggests that *S. sciuiri* strain was a precursor for *mecA* gene found in *Staphylococcus aureus*.

MecA gene is a gene that prevents the lactam structure of beta-lactam drugs to attach to the enzymes that form the cell wall of the bacterium (transpeptidases). Furthermore, this activity prevents the antibiotics from hindering cell wall synthesis and, hence the bacteria are able to replicate as normal (Fogarty et al. 2015). Resistant strains (MRSA) are responsible for many infections originating in hospitals and have spread throughout the world (Fogarty et al. 2015).

1.5.2 Panton-Valentine leukocidin

Panton-Valentine leucocidin (PVL) is commonly used as an indicator for community CA-MRSA (Haddad & Moineau, 2013; Gillen et al. 2015). According to Meyer et al. (2009), PVL is a two-component *S. aureus* pore-forming protein encoded by two genes (*lukSlukF*-PV genes), originally described by Van de Velde in 1894 as associated with staphylococcal skin infections (Panton & Valentine, 1932). PVL induces lysis of leukocytes, particularly neutrophil (Meyer et al. 2009) and can lyse granulocytes associated with skin and soft tissue infection. Further reports have also linked travellers

returning from Africa to Europe with *S. aureus* (pneumonia) infections to be frequently associated with isolates producing Panton–Valentine leukocidin (PVL) (Gillen et al. 2015). This may thus be an important marker for epidemiological studies in developing countries.

1.5.3 Protein A (spa)

Protein A is one of the virulence factors on the surface of *S. aureus* that prevents the phagocytosis of the bacteria by the immune system and is expressed on the surface of nearly all *S. aureus* strains (Loomba et al. 2010). *Spa* typing of *S. aureus* provides useful insights into the nature of *S. aureus* species and their virulence potentials. According to Harmsen et al. (2003), *spa typing* would further support in the grouping of *S. aureus* isolates into clonal lineages which is important for the detection of transmission routes and monitoring of bacterial strains in circulation.

1.6 Global MRSA prevalence

MRSA is not restricted to any geographic region, as it is found worldwide and can be spread from person to person and from country to country (Tenover et al. 2006). According to the Centre for Disease Control (CDC) report in 2013, in the United States in 2011 over 80 000 invasive MRSA infections occurred with 12 000 associated deaths. In 2013, another close to about 2,000,000 people were infected and 23,000 deaths recorded with antibiotic-resistant bacteria in the United States (CDC, 2013).

In Europe, MRSA accounted for nearly 44% of hospital-acquired infections in 2008 (Köck et al. 2009). According to Foster (2016), over 400,000 patients experience ill effects due to infection by antibiotic-resistant microorganisms, with an associated mortality of 25,000 patients. Additionally, MRSA accounts for over 380 million Euros in extra in-hospital costs for EU healthcare systems (Köck et al. 2009; Kanerva et al. 2011).

Likewise, a high frequency of MRSA (90%) was reported in some parts of Latin America (Guzm´an-Blanco et al. 2009; Jimenez et al. 2012). In addition, Vega et al., (2017), recoreded 62.0% and 67.3 MRSA rates respecyively in Chile and Guatamela. In recent years, MRSA is now an important cause of community-acquired infections (CA-MRSA). USA300 is one of the main clones representing CA-MRSA and has disseminated throughout Latin America as well as some European countries (Otter & French, 2010). Emphasizing the potential threat MRSA poses to healthcare systems in Africa, the WHO reported that, in some parts of Africa, as high as 80% of *S. aureus* infections are resistant to methicillin, rendering treatment with standard antibiotics ineffective (WHO, 2014). On the African continent over the last few decades, countries such as Nigeria and South Africa (among others) have recorded increased data generation on the prevalence of methicillin resistance in clinical *S. aureus* isolates (Falagas et al. 2013). This is probably due to the implementation of monitoring and control policies. Nevertheless, the spread of MRSA in Africa must be taken into consideration in the global battle against antimicrobial resistance (Falagas et al. 2013).

1.6.1 MRSA in Nigeria

Nigeria, with a current population of about 200 million is one of the most populous countries in Africa (World Bank, 2018). In Nigeria, MRSA has been acknowledged as a serious therapeutic challenge both in the clinical and community settings (Shittu et al. 2012; Kolawole et al. 2013; Ayepola et al. 2015; Ayeni et al. 2018; Oladipo et al. 2019, Chapter 3 on this study). Studies have reported on the burden of hospital-acquired infections (HAI) and community-associated (CA) *S. aureus* infections in Nigeria (Taiwo et al. 2005; Nwankwo & Nasiru, 2011). Most of these studies have focused on *Staphylococcus* spp. being isolated from hospital sources and not from wastewater environments.

Various authors have reported on the prevalence of MRSA isolates from variety of clinical and surgical samples with recorded figures ranging from 20 to 45% (Taiwo et al. 2005; Nwankwo & Nasiru, 2011). Additional data from studies conducted by Shittu et al. (2012) and Alli et al. (2015) corroborate this. Conversely, a lower MRSA prevalence (9% to 28%), were recorded in the northern region of the country (Adesida et al. 2005), while Okon et al. (2014), reported lower levels (12.5% in 2007 to 8% in 2012) from the same region for subsequent periods. Little is known on the impact of *Staphylococcus* spp. or MRSA in aquatic environment such as wastewaters in Nigeria. There is therefore dearth of data in this regard. However, a very recent report by Oladipo et al. (2019, Chapter 3 on this study) has confirmed the prevalence of *Staphylococcus* species or MRSA from water bodies or water bodies receiving hospital wastewaters. These authors also described their antibiotic resistance patterns and associated resistance and virulence genes.

1.6.2 MRSA in South Africa

Clinical MRSA isolates outbreal had been reported in Johannesburg, South Africa in 1986 and 1987 (Park & Pearce, 1989). Retrospective studies later revealed the yearly prevalence of *S. aureus* bacteraemia are at a level of 3.28 cases/1000 hospital admissions in South Africa (Naidoo et al. 2013). Furthermore, in 2007–2011 the prevalence of MRSA decreased from 0.6 (36%) in 2006 to 0.4 (24%). During this period, surveillance S. *aureus* was confirmed as the most frequently cultured bacterium and one out of the four major outbreaks of antibiotic resistance bacteria ARB) at the National level (Naidoo et al. 2013). In addition, a study conducted on hospitalized children in Cape Town, reported that the proportion of MRSA had been increasing over the last few years, with 11.6% being bacteremia attributed to *S. aureus* (Naidoo et al. 2013). From clinical sources, according to a prospective study conducted in 2002 (Brink et al. 2007), an MRSA prevalence of 35% was reported. Furthermore, some other retrospective studies conducted (2006-2012) reported MRSA prevalence of 23% to 39% (Shittu and Lin, 2006; Groome et al. 2012; Perovic et al. 2015). Nevertheless, there is a dearth of information on antibiotic susceptibility patterns and epidemiology of MRSA isolated from environmental sources such as wastewaters in South Africa.

1.7 Problem statement

MRSA is a known threat of which new emerging clones are being discovered worldwide with specific patterns of spread and biological characteristics (Urbaniak et al. 2014). The possibility also exists of the spread of resistant MRSA strains (of different clones), across countries and to other African nations including Nigeria and South Africa. Over

the years, skilled healthcare workers such as medical doctors, nurses, biomedical scientists, academic lecturers and non-skilled workers such as artisans have immigrated to South Africa, either to work or for further studies. During their work and travels, some of these personnel might have been exposed to MRSA infections unknowingly and may act as carriers. According to Shittu et al. (2012), the opportunistic nature of MRSA strains in hospital environments enables it to be easily spread by healthcare workers unknowingly. Additionally, staphylococci have the capability to survive for months. This is because they are highly resistant to drying and can easily spread among humans, either directly or indirectly by contact with healthcare workers or a contaminated environment. According to Gillen et al. (2015), those that spread the infections without knowing are referred to as carriers. This class of people poses a high risk to persons with compromised immune systems. Furthermore, carriers can also put themselves in danger when their immune system is compromised during illness. Consequently, carriers could encourage the spread of MRSA strain if they do not seek treatment.

Furthermore, the presence of *Staphylococcus* spp. showing antibiotic resistance and clinically relevant virulence genes are of interest due to the potential environmental link of CA-MRSA infections and recreational activities. Studies have shown that human activities related to healthcare practices can influence the transfer and selection of resistant bacteria into the environment via hospital wastewater (Varela et al. 2013; Iweriebor et al. 2015). Meanwhile, water systems receiving wastewater effluents are being utilized for agricultural, recreational, industrial purposes, land application or

recycled as drinking water (Goodwin et al. 2012). Therefore, the presence of *mecA* gene and antibiotic resistance genes in environmental isolates might thus indicate contamination from clinical sources. According to Falagas et al. (2013), there is a significant geographical difference in the frequency of the MRSA within and between countries. However, data that describes these trends of MRSA isolates in clinical and environmental settings, most especially from wastewater sources in Nigeria and South Africa, are sparce.

Prevalence of *Staphylococcus aureus* and MRSA in non-clinical environments such as wastewater has therefore been barely studied since most wastewater studies have focused on microbial indicators of faecal contamination (Tran et al. 2014). Hence, it is important to characterize staphylococcal species isolated from clinical and environmental sources, to confirm their identities and the possibilities of clones or mutual genes between Nigeria and South Africa.

1.8 Research aim

To study the prevalence of *Staphylococcus* species from clinical and environmental sources (water bodies) and utilizing molecular methods to characterize and confirm the MRSA.

Specific objectives were to:

i. Provide a systematic overview of the literature and gaps in research on staphylococci in environmental aquatic sources
ii. Isolate and characterise MRSA from clinical and environmental sources (water bodies receiving hospital wastewater) in Ile-Ife, Nigeria
iii. Isolate and characterise MRSA from a WWTP and the receiving water.
iv. Investigate through whole genome sequencing the genetic characteristics of selected staphylococci from Nigeria and South Africa

1.9 Outline of the thesis

Chapter 1 – Introduction, statement of problem, rationale, aims and objectives

This chapter contains an introduction into the different aspects of the study as well as the rationale of the study. The aims and objectives and outline of the thesis are also presented.

Chapter 2 is a systematic review aimed to assess the prevalence of *mecA* gene in staphylococcal isolates from clinical and environmental (wastewaters) sources.

Title: *MecA* positive *Staphylococcus* spp. in environmental waters and clinical settings: A systematic review

Authors: Adegboyega O. Oladipo, Oluwatosin G. Oladipo and Cornelius C. Bezuidenhout

Journal: Environmental Pollution

Manuscript number: ENVPOL_2020_2637

Contribution of A.O Oladipo –Data collection, data synthesis and manuscript writing, in collaboration with other authors.

Chapter 3 focuses on the diversity, characteristics and significance of *Staphylococcus* species associated with clinical and environmental settings. It dealt particularly with the impact of hospital efluents on the environment (wastewaters).

Published:

Oladipo, A.O., Oladipo, O.G. and Bezuidenhout, C.C. 2019. Multi-drug resistance traits of methicillin-resistant *Staphylococcus aureus* and other staphylococcal species from clinical and environmental sources. Journal of Water and Health, 17: 930-943.

Contribution of A.O Oladipo – Sampling, field data collection, data analysis and manuscript writing.

Chapter 4 chapter focuses on the detection of resistance and virulence genes in methicillin-resistant *Staphylococcus aureus* (MRSA) from a wastewater treatment plant (WWTP) in South Africa. The potential of wastewater treatment plants (WWTPs)

receiving various types of wastewater streams as sources of MRSA into environmental water was dealt with.

Title: Antibiotic resistant *Staphylococcus* species from a wastewater treatment plant in South Africa

Authors: Adegboyega O. Oladipo, Oluwatosin G. Oladipo and Cornelius C. Bezuidenhout

Journal: International Journal of Environmental Health Research

Manuscript number: CIJE-2019-0409

Contribution of A.O Oladipo - Sampling, and data collection, experimental/laboratory work, data analysis and manuscript writing.

Chapter 5 addressed the genetic link between clinical and environmental isolates. Coagulase-negative staphylococci (CoNS) are important reservoir of ARGs as well as virulence determinants that can be easily transferred between staphylococcal species. Thus, an in-depth understanding of the genetic characteristics and variability of CoNS is fundamental in understanding the complexity associated with these species using whole genome sequencing (WGS) technique.

Title: Genomic characterization of staphylococcal isolates from clinical and environmental wastewaters in Nigeria and South Africa

Authors: Adegboyega O. Oladipo, Tawanda E. Maguvu and Cornelius C. Bezuidenhout

Journal: META GENE

Manuscript number: MGENE-D-20-0018

Contribution of A.O Oladipo- Providing the DNA of the staphylococci isolates for WGS analyses, mainly assisting with interpretation of data and manuscript writing.

Overlap in the study were unavoidable, particularly in Chapters 2, 3 and 4.

Chapter 6 provides significant conclusions to the present study and recommendations for future research are provided.

CHAPTER 2

MECA POSITIVE *STAPHYLOCOCCUS* SPP. IN ENVIRONMENTAL WATERS AND CLINICAL SETTINGS: A SYSTEMATIC REVIEW

2.1 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) are opportunistic pathogens of importance responsible for countless number of community and hospital acquired infections worldwide. Recently, of critical concern is the influx of coagulase-negative staphylococci (CoNS). These exhibit multi-drug resistance tendencies/traits in both clinical and non-clinical settings (Oladipo et al. 2019, Chapter 3 on this study). CoNS have been recognized as key reservoirs of antimicrobial resistance genes (ARGs), which may be transferable among staphylococci (Becker et al. 2017).

In the clinical settings, CoNS strains are often generally considered less or non-pathogenic when compared with coagulase-positive staphylococci (CoPS) strains (Becker et al. 2017). However, in the environmental settings, CoNS are known important reservoirs of resistant-associated mobile genetic elements and antimicrobial resistance genes (ARGs) (Adekanmbi et al. 2019; Nnadozie & Odume, 2019). These species - CoNS are threateningly becoming potentials for public health concerns especially with their possession of *mecA* gene, an ARG with strong affiliation/linkage with methicillin resistance (Börjesson et al. 2009; Wan & Chou, 2014). Globally, studies have revealed that, non-clinical settings such as environmental wastewaters could possibly serve as medium for MRSA strains between humans and the environment (Samie & Shavumbu, 2011; Singh-Moodley et al. 2015; Amoako et al. 2016). In addition, various authors have also established that CoNS strains, harbouring the *mecA*

gene, are now more frequently isolated from wastewater environments (Börjesson et al. 2009; Goldstein et al. 2012). Meanwhile, reclaimed water is mainly used for human domestic purposes, drinking water for animals as well as farm irrigation (Coertze & Bezuidenhout, 2019). Eventually, the occurrence of *mecA* positive *Staphylococcus* spp. (CoNS and CoPS) in water environments could lead to significant health safety concerns.

Studies on *mecA* positive detection in *S. aureus* or MRSA in hospital and community settings have been widely documented (Deurenberg & Stobberingh, 2009; Kong et al. 2016). However, the impact of *mecA* positive or methicillin-resistant CoNS species as important pathogens in the environmental settings (specifically wastewater), with potential threats to public health are yet to be fully explored. The objective of this review was therefore to assess the prevalence of *mecA* gene in *Staphylococcus* spp. from environmental waters and clinical settings.

2.2 Methods

2.2.1 Selection criteria

This review focused on the detection of *mecA* gene in *Staphylococcus* species. Specifically, MRSA, *mecA* positive-and/or methicillin-resistant CoNS isolates obtained from clinical and environmental wastewater origin/sources. Since no meta-analysis was conducted, data gathered were qualitative in nature. The detail of the selection process is shown in Figure 1.

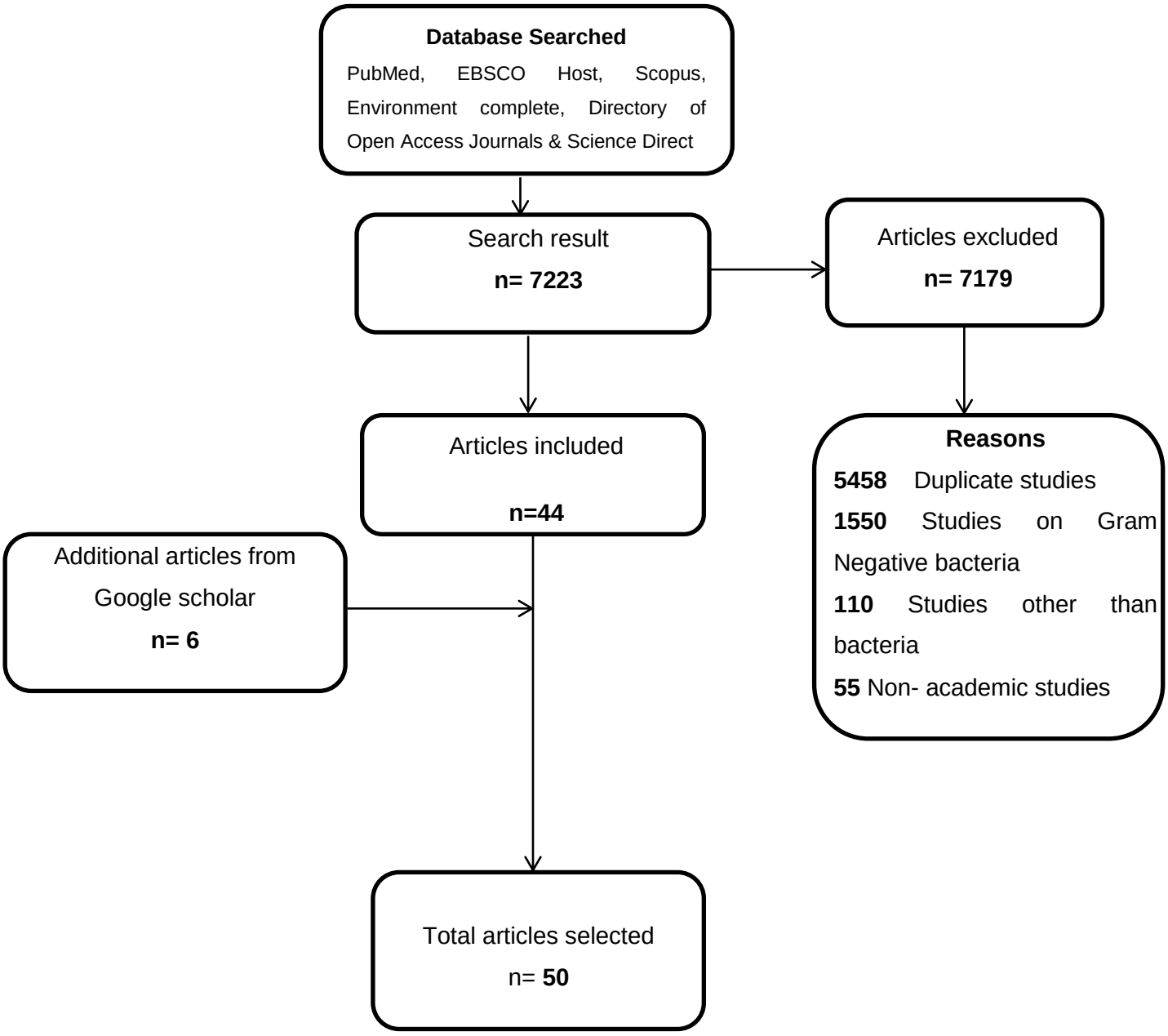


Figure 1: Flowchart illustrating the journal article selection process

2.2.2 Literature search and information sources strategy

Literature search for this review was performed using 7 online databases: PubMed, MEDLINE, Scopus, Environment Complete, Directory of Open Access Journals, Science Direct and Google scholar (Figure 1). Predefined terms such as “staphylococci in environmental waters” OR (“staphylococci in clinical waters”) OR (“staphylococci in clinical isolates”) OR (“MRSA in wastewaters”) “waste water” OR “wastewaters” OR “*mecA*” OR “genes” [All Fields] OR “gene” OR “hospital” OR “waste water” OR “wastewaters”) OR (‘’worldwide”) were used to retrieve relevant articles published between 1st January 2000 and 31st October 2019. Screening was done by applying filters to exclude literature in other languages apart from English. Pertinent data extracted from peer-reviewed articles included first author’s name, year of sample collection, country, isolated bacteria, settings (environmental or clinical), method used for molecular isolation, studied genes and final results obtained (Table 1).

2.2.3 Data synthesis

This systematic review was compiled using the PRISMA guidelines (Liberati et al. 2009).

2.3 Results and discussion

Seven (7) online database engines were searched and combined giving rise to 7223 search results. Screening for abstract and title excluded 7179 articles (Figure 1). Of the

7179 excluded from the full-text screening, 5458 were duplicates, 1556 were studies on gram negative bacteria, 110 were studies on organisms other than bacteria (viruses) and 55 were from non-academic journals. Thus, 50 articles were selected for full-text analysis. Based on the eligibility criteria, 44 articles were retained, an additional 6 publications were selected from Google scholar, making a total number of 50 articles included in the systematic review. Only experimental studies were considered and included; all review articles were excluded.

2.3.1 Global synopsis of reviewed literature

The case studies selected and examined originated from nineteen (19) different countries across 5 continents - North America, Europe, Africa, Asia and Oceania (Table 1). The countries are: United States of America (5), Australia (1), Germany (1), Sweden (1), Taiwan (1), Kenya (1), Slovakia (1), Iraq (1), Iran (1), Thailand (1) and Turkey (1). Others include, Morocco (1), Algeria, (1), Libya (1), Egypt (1), Ghana (1), Tunisia (2), Nigeria (17) and South Africa (11).

2.3.2 Overview of studies from clinical and environmental sources

For this review, 50 peer-reviewed articles of clinical and wastewater origin were selected. Of these, 70% (n=35) were from the clinical setting while 15 (30%) were based on environmental wastewaters (Table 1). From the clinical studies, only 1 article though a cross-continental study was from developed countries specifically the USA (Goering et al. 2008). The other 97% (n=34) of the articles were from developing

countries some of which included Nigeria (15) Taiwo et al., (2005), Shittu et al. (2012), O'Malley et al. (2015) etc., South Africa (9) Groome et al. (2012), Fortuin-de Smidt (2015) etc. and Ghana (Egyir et al. 2015). Others are: Tunisia (Karim et al. 2010; Raoudha et al. 2016), Thailand (Teeraputon et al. 2017), Turkey (Seyedmonir et al. 2015), Iraq (Hussein et al. 2019) etc. Clinical samples originated from specimens such as: pus, swabs, sputum, urine, aspirate, cerebrospinal fluid, blood cultures and samples from nose, ear and throat.

On the other hand, for the environmental articles, samples were collected from wastewaters of diverse sources such as domestic/municipal wastewaters, hospital effluents, slaughterhouse wastewater, sewage treatment plants etc. Nine (60%) of the 15 selected environmental studies were initiated from developed countries (Table 1). Four articles were from The United States (Levin-Edens et al. 2011; Goldstein et al. 2012; Fahrenfeld et al. 2013; Boopathy, 2017), Sweden (1) Börjesson et al. (2009) and Australia (1) Thompson et al. (2012). Others include Slovakia (1) Čuvalova et al. (2015), Turkey (1) Seyedmonir et al. (2015) and Germany (1) Schwartz et al. (2003). The other 6 articles originated from developing countries. These included: South Africa (3), Nigeria (2) and Tunisia (1). It is noteworthy that about 70% (4 of 6) of these articles worked on *Staph.* species from both environmental and clinical sources. These are: Adekanmbi et al. (2019) and Oladipo et al. (2019, chapter 3 on this thesis) from Nigeria, Samie and Shivambu (2011) – South Africa and Raoudha et al. (2016) from Tunisia.

Table 1: Literature used

First author/ Year	Country	Year of Study	Isolated bacteria	Genes involved in study	Method used	Settings	*mecA* gene	% MRSA
Developed Countries								
Boopathy, 2017	USA	Jan-Dec 2015	MRSA	*mecA*	PCR, Southern blot	Marine and fresh water, beaches	+	NA
Fahrenfeld et al. 2013	USA	NR	*S. aureus, S. warneri*	*sul1, sul2, tet(A), tet(O), vanA* and *mecA*	MALDI-TOF, PCR	Public distribution networks	+	10.0
Goering et al. 2008	USA	NR	MRSA	NA	PCR	Clinical	+	7.5
Goldstein et al. 2012	USA	October 2009 and October 2010	*S. aureus*, *Staph*. Spp.	*MecA*, *SCCmec*, PVL	PCR, PFGE,	Wastewater	+	6.13
Levin-Edens et al. 2011	USA	June-Aug 2010	MRSA	*mecA*	PCR, PB2a SDS-PAGE Analysis	Fresh water beach	+	22
Thompson et al. 2012	Australia	NR	MRSA	*mecA*	RAPD-PCR	WWTP	+	58.5
Schwartz et al. 2003	Germany	2002	*S. aureus*	*mecA*	HPC, Culture plate method	Sewage Treatment plant	+	NA

Börjesson et al. 2009	Sweden	NR	MRSA, MDR *S. aureus*	*tetA, tetB, aac mecA*	LUX real time assay	Wastewater municipal, swine slaughterhouse wastewaters	+	NA
Čuvalova et al. 2015	Slovakia	NR	*S. haemolyticus* *S. saprophyticus*	*mecA*	MLST, PFGE	Marine and freshwater, beaches	+	NA
Seyedmonir et al. 2015	Turkey	NR	*S. aureus*	*mecA*	MLD-TOR MS	Marine and freshwater, beaches	+	25
Developing Countries								
Adekanmbi et al. 2019	Nigeria	NR	*S. saprophyticus* *S. epidermidis*	*mecA, mecB, mecC*	PCR	Hospital wastewater	+	13.0
Alli et al. 2012	Nigeria	NR	MRSA	PVL, *mecA*	PCR	Clinical	+	41.4
Alli et al. 2015	Nigeria	2015	MRSA	*MecA,* PVL	PCR, SCCmec	Clinical	+	42.3
Ayepola et al. 2015	Nigeria	2010-11	*S. aureus*, MRSA	*MecA,* PVL, *Tst Eta*	PCR, *spa* typing	Clinical	+	2.41
Ghebremedhin et al. 2009	Nigeria	2006-2007	MRSA	*mecA*	PCR	Clinical	+	20.2
Ibadin et al. 2017	Nigeria	2017	MRSA	*mecA*	PCR	Clinical	+	41.8
Kolawole et al. 2013	Nigeria	Nov. 2008 – July 2010	MRSA, MSSA	*SCCmec, spa, agr, nuc, mecA, sea, sec, she*	SCCmec, *spa*, PCR, MLST	Clinical	+	11.5
Nwokah et al. 2016	Nigeria	Aug. 2012 - July 2013	*S. aureus,* MRSA	*mecA*	PCR	Clinical	+	12.2

Obianuju et al. 2015	Nigeria	2015				Clinical	+	40.2
Ogbolu et al. 2015	Nigeria	Jan. – Dec. 2010	MRSA	*mecA*	PCR	Clinical	+	47.4
Okon et al. 2014	Nigeria	Jan-Dec 2007	*S. aureus*	*mecA*		Clinical	+	12.5
Oladipo et al. 2019, Chapter 3 on this study	Nigeria	2017	*S. aureus*, CoNS	*mecA*	PCR	Clinical and environmental wastewaters	+	31.4
O'Malley et al. 2015	Nigeria	2014	MRSA	*MecA,* PVL	PCR	Clinical	+	42.1
Otarigho & Falade 2018	Nigeria	2018	MRSA, CoNS	*mecA*	WGS	Clinical	+	NA
Shittu et al. 2012	Nigeria	Jan-April, 2010	*S. haemolyticus* *S. scuiri*	*SCCmec, mecA*	PCR, SCCmec typing, MLST	Clinical	+	16.5
Taiwo et al. 2005	Nigeria	Jan-Dec 2001	MRSA	*mecA*	Restriction Enzyme Analysis of the Plasmid DNA (REAP)	Clinical	+	32
Torimiro & Torimiro 2012	Nigeria	NR	NR	NA	NA	Clinical	+	12.5
Amoako et al. 2016	South Africa	2015	*S. aureus*	*mecA, tetk, aa(6')-aph(2')*	PCR	Clinical	+	NA
Brink et al. 2007	South Africa	2006	*S. aureus*	*mecA*	PCR	Clinical	+	36.0
Fortuin-de Smidt, 2015	South Africa	Sept 2012-2013	*S, aureus* MRSA	*mecA, nuc*	Mulitplex-real time, PCR	Clinical	+	35.8

Groome et al. 2012	South Africa	2005-2006	MRSA	*mecA*	PCR	Clinical	+	39.0
Moodley et al. 2011	South Africa	2005/2006	MRSA	*NA*	PCR, *spa*, PFGE, MLST	Clinical	+	320
Nnadozie & Odume 2019	South Africa	2019	*S. aureus* MRSA	*mecA,* PVL,	qPCR	Freshwater Environment	+	7.34
Oosthuysen et al. 2014	South Africa	NA	MRSA, *S. aureus*	*mecA,* PVL	PFGE, MLST, *spa*	Clinical	NA	15.3
Perovic et al. 2015	South Africa	2010-2012	*S. aureus*	*mecA, nuc*	Real time PCR	Clinical	+	45.4
Ramessar & Olaniran 2019	South Africa	2019	MRSA	*mecA, luks P/V, sea, hla*	PCR	Treated effluent and receiving Water	+	23.0
Samie & Shivambu, 2011	South Africa	NR	*S. aureus*	*mecA*	PCR	Clinical and drinking water	+	12.9
Shittu & Lin, 2006	South Africa	2006	MRSA	*mecA, mupA*	PCR-RFLP	Clinical	+	26.9
Chaalal et al. 2018	Algeria	NR	MRSA	*mec A*	Real time PCR	Clinical	+	21.5
Ahmed et al. 2014	Egypt	Nov 2017-to Aug 2010	MRSA *S. aureus*	*mecA*	PCR	Clinical	+	23
Egyir et al. 2015	Ghana	Oct 20–0 - June 2012	*S. aureus,* MRSA	*spa, lukS/F-*PVL *mecA*	Multiplex PCR	Clinical	+	3.0
Mohajeri et al. 2016	Iran	Oct 2012 – August 2013	*S. aureus* MRSA	*mecA,* PVL	PCR	Clinical	NA	NA
Hussein et al. 2019	Iraq	NR	MRSA	*KPC, vanA, mecA*	NA	Hospital and municipal sewage	+	50.4

Kyanya et al. 2019	Kenya	2000-2007	*S. aureus*	*mecA*	MLST, PCR	Clinical	+	25
Buzaid et al. 2011	Libya	April–July 2007	*S. aureus*	ND	Oxacillin disk-diffusion	Clinical	NA	31
Elhamzaoui et al. 2009	Morocco	March 2006–March 2008	*S. aureus* MRSA	*mecA*	PCR	Clinical	+	19
Fang et al. 2014	Taiwan	June 25 to October 1 2012	MRSA	*mecA*	PCR, SCCmec	clinical	+	59.1
Teeraputon et al. 2017	Thailand	2014 and 2015	CoNS	*mecA*	PCR	Surface water	+	63.3
Karim et al. 2010	Tunisia	2008-2009	*S. aureus*, CoNS	*mecA*	PCR, MLST, PFGE	Reclaimed water		0.24
Raoudha et al. 2016	Tunisia	NR	CoNS *S. warneri, S. xylosus*	*tetk, tetM, aac(6')-Ie-aph(2")-Ia*	PCR qPCR	Reclaimed water	+	NA

Key: NA – Not available, NR – Not recorded

2.3.3 *MecA* and other genes detected in *Staphylococcus* species

On antimicrobial resistance genes (ARGs) present in *Staphylococcus* species, data gathered for this systematic review showed that 36% (n=18) of environmental and clinical studies worked on *mecA* alone (Table 1) while 64% (n=32) focused on *mecA* and other genes such as: *tetA, tetB*, *mecA*, *vanA, vanB, ampC aac*(6′)-*Ie-aph*(2″)-*Ia, sul1, sul2, tet(A), tet(O)* and PVL (Börjesson et al. 2009; Fahrenfeld et al. 2013; Raoudha et al. 2016). Antimicrobial resistance genes (ARGs) are well identified potential environmental contaminants. These are usually transferred to disease-causing from non-disease-causing bacterial strains and may eventually result in clinically significant antibiotic resistance (Becker et al. 2017). Characteristics that distinguish ARGs as environmental contaminants include their associated tendencies with mobile genetic elements, such as integrons, plasmids and transposons (Shoemaker et al. 2001). ARGs of notorious nature that pose threats to human health include *vanA*, exhibiting resistance to vancomycin and *mecA*, known genes which encode methicillin resistance.

The *mecA* gene is well known and identified as most associated with *Staphylococcus* species especially MRSA, being the most prominently known carrier (Deurenberg & Stobberingh, 2009). *MecA* is a gene found in bacterial cells that allows for resistance to beta-lactam antibiotics (methicillin, penicillin and penicillin-like antibiotics). *Staphylococcus aureus* plasmids carry diverse ARGs that encode an alternative penicillin binding protein (PBP) which confers resistance to all beta-lactams staphylococci (Fogarty et al. 2015).

2.3.4 Methods used for *mecA* gene characterization

On methods adopted for the detection of *mecA* gene (Table 1), 76% (n=38) of selected reviewed articles used only PCR method (qPCR, multiplex PCR) for sequencing the *mecA* gene (Ali et al. 2014; Ogbolu et al. 2015; Boopathy, 2017). The remaining 24% (n=12) of the studies used a combination of PCR and other methods (Taiwo et al. 2005; Moodley et al. 2011; Goldstein et al. 2012). In all 50 reviewed articles for *mecA* detection, 96% (n=48) utilized PCR method while Whole Genome Sequencing (WGS) was performed in only a single study (Otarigho & Falade 2018). Generally, for *mecA* gene detection, molecular methods employed in most of the studies included PCR, Deoxyribonucleic acid (DNA) Sequencing, Multi-locus sequence typing (MLST), Pulsed field gel electrophoresis (PFGE), Restriction Enzyme Analysis of the Plasmid DNA (REAP), Lux real time etc. (Börjesson et al. 2009). Across the sample origins (clinical or environmental), PCR appeared to be the most suitable method employed for detection of the *mecA* gene. This may be attributed to the effectiveness of this technique for easy detection of the *mecA* gene, access to PCR cyclers and reduced cost implication (Alli et al. 2012).

2.3.5 *Staphylococcus* species isolated from clinical and environmental sources

Across the 50 reviewed articles, data retrieved indicated that *S. aureus* and CoNS strains were isolated in 100% of the publications (Table 1). Of this, further data revealed that 76% were on *S. aureus* only (Taiwo et al. 2005; Shittu & Lin. 2006; Brink et al. 2007; Ghebremedhin et al. 2009; Levin-Edens et al. 2011; Thompson et al. 2012; Egyir

et al. 2015; Ogbolu et al. 2015; Perovic et al. 2015; Boopathy, 2017), 14% of the articles isolated *S. aureus* and CoNS (Goldstein et al. 2012; Raoudha et al. 2016) while only 10% of the studies worked on CoNS alone (Čuvalova et al. 2015; Teeraputon et al. 2017). Identified coagulase-negative staphylococci (CoNS) strains from clinical sources included: *S. haemolyticus* and *S. sciuri* while *S. warneri*, *S. haemolyticus, S. xylosus* and *S. saprophyticus* were recovered from wastewater sources and *S. lentus* and *S. sciuri* were isolated from both wastewaters and clinical origin. According to Oladipo et al. (2019, Chapter 3 on this study), *Staph.* species isolated from wastewaters and/ clinical sources are identified major incriminating bacteria accountable for many human diseases and infections.

2.4. *MecA* gene in *Staphylococcus aureus* from developed countries

During the study period (2000 to 2019), articles retrieved from the developed world accounted for 1500 clinically significant *S. aureus* (CoPS) strains. Of this, 150 (10%) were found resistant to methicillin. On the other hand, 1200 strains of *S. aureus* were isolated from environmental water sources viz: municipal wastewaters, freshwater marines and hospital effluents. Out of these, 20% were methicillin resistant.

2.4.1 *MecA* gene in *Staphylococcus aureus* from clinical setting in developed countries

Of the 10 articles retrieved from developed countries (the United States, Sweden, Slovakia, Australia, Turkey and Germany) for this systematic review, only the article by

Goering et al. (2008) from the United States originated from the clinical setting. The study conducted in 2004 and 2005, examined the epidemiology of MSSA (methicillin susceptible *Staphylococcus aureus*) and MRSA strains. The global study cut across 4 continents where isolates were obtained from clinical trials at investigator sites in North America, Europe, South Africa, Russia and the United States of America. Findings from the study confirmed *mecA* gene positive in 8% of about 1500 *S. aureus* isolates from skin specimens by a combination of molecular methods such as PFGE, MLST, *mecA* and *SCCmec* analyses.

2.4.2 *MecA* gene in *Staphylococcus aureus* from wastewaters in developed countries

Nine articles generated from developed nations detected the *mecA* gene in *Staphylococcus* spp. isolated from environmental wastewaters (Table 1). These included: Schwartz et al. (2003) who conducted a study on surface drinking water and wastewaters in Germany. From their study, prevalence of MRSA was 18% with detection of *mecA* gene being confirmed by PCR and southern blot technique. Another notable study conducted in Australia by Thompson et al. (2012) obtained 224 *S. aureus* strains isolated for 8 consecutive weeks period from untreated hospital effluents. The study recorded a reasonably high (68%) MRSA prevalence and *mecA* gene detection was confirmed by RAPD-PCR while cefoxitin resistance was determined using phene plate (PhP) typing.

Börjesson et al. (2009), confirmed *mecA* gene presence in *S. aureus* isolates obtained from a WWTP in Gothenburg, Sweden. Findings revealed a decrease in the number of

isolates with the *mecA* gene as treatment progressed. In Slovakia, Cuvalova et al. (2015), identified 8 CoNS species including *S. saprophyticus*, *S*. *haemolyticus* and *S. warneri* with the *mecA* gene. In addition, Seyedmonir et al. (2015) reported a prevalence of 25% *mecA* detection in CoNS out of 12 *S. aureus* isolates recovered from surface waters in Turkey**.**

In the United States of America (USA), 4 studies were conducted on wastewaters. One of these was conducted by Goldstein et al. (2012) and 360 *S. aureus* isolates were obtained between October 2009 and September 2010 from 4 WWTPs in California. Similar to Thompson et al. (2012), an MRSA prevalence as high as 67% was recorded. The *mecA* gene was detected in the study using both PFGE and PCR methods. The 2nd study was conducted by Levin-Edens et al. (2011). Samplings were drawn from marine and freshwater beaches environment. The study confirmed the identification of 93% of isolates as being MRSA. Fahrenfeld et al. (2013) in their study confirmed the presence of *mecA* in CoNS species specifically *S. saprophyticus* and *S. warneri* isolated from reclaimed wastewaters. In addition, 200 *S. aureus* strains with a 10% MRSA prevalence was recorded. The study also determined 2 genes - *mecA* and *Luk*-PVL by quantitative PCR (qPCR) method. This finding could have severe implications for direct WWTP effluent reclamation being used for potable purposes. Boopathy (2017) conducted the 4th study which established the prevalence of *S. aureus*, MRSA and the *mecA* gene isolated from raw and treated effluents of the Thibodaux sewage treatment plant in the USA. These studies all confirmed *mecA* gene positive in *S. aureus* and CoNS species isolated from various wastewater sources.

2.5 *MecA* gene in *Staphylococcus aureus* from developing countries

Among the developing nations, 85% of staphylococcal strains recovered were obtained from the clinical settings, while isolates from environmental waters such as: municipal wastewaters, hospital effluents and freshwater marines accounted for only 15% (Table 1). During the study period, about 20,000 clinically important *S. aureus* strains (CoPS) were isolated from the various developing countries for analysis, of which 6,000 (30.0%) were resistant to methicillin. From Africa and the Asian continents, a total of 40 studies were retrieved (2 from Tunisia, 1 each from Egypt, Ghana, Algeria, Kenya, Iran, Iraq, Morocco, Taiwan, Thailand and Libya. From Nigeria there were 17 (15 clinical and 2 wastewaters) and South Africa 11 (8 clinical and 3 wastewaters).

2.5.1 *MecA* gene in *Staphylococcus* species from clinical setting in developing countries

The study from Thailand focused on detection of antibiotic resistance genes in CoNS isolates obtained from clinical specimens in Thailand. Conducted between 2014 and 2015, Teeraputon et al. (2017) recovered 230 CoNS species. The methicillin-resistant CoNS (MRCoNS) species containing the *mecA* gene included: *S. saprophyticus*, *S. haemolyticus*, *S. epidermidis*, *S. capitis*, *S. hominis*, *S. sciuri, S. warneri* and *S. arlettae*. Prevalence of *mecA* gene among the CoNS species was up to 63%. In addition, Fang et al. (2014) carried out a community study involving humans and livestock in Taiwan. The study isolated *S. aureus* species with the confirmation of an MRSA rate of 14% and 13% in humans and pigs. Further, the study conducted in Egypt by Ahmed et al. (2014), isolated 241 staphylococcal isolates, of which 127 (61%) were strains of *S. aureus* with

>90% MRSA isolates being *mecA* positive. The study done in Algeria, isolated over 150 staphylococcal species, MRSA prevalence of >20% and 17% *mecA* gene detection was recorded (Chaalal et al. 2018).

Another study conducted by Egyir et al. (2015) in institutions across Ghana obtained about 300 *S. aureus* isolates of which MRSA and *mecA* positive strains were confirmed (Table 1). In Iran, a total of 260 *S. aureus* isolates and about 40% MRSA prevalence was confirmed (Mohajeri et al. 2016). In addition, Kyanya et al. (2019) recorded a 25% MRSA prevalence from isolates obtained from four Kenyan hospitals. Studies from clinical settings in Morocco (Elhamzaoui et al. 2009), Libya (Buzaid et al. 2011) and Iraq (Hussein et al. 2019) isolated 450, 200 and 110 *S. aureus* isolates with an MRSA prevalence rate of 19%, 31% and 50% respectively.

All these studies further establish the occurrence of CoNS, *Staph. aureus*, MRSA species and confirmation of the *mecA* gene from isolates of clinical sources. Since a greater number of retrieved articles on *mecA* and *Staphylococcus* spp. were generated from Nigeria and South Africa, the selected studies from these countries are discussed more extensively in the next sub-section of this review.

2.5.1.1 *MecA* gene in *Staphylococcus aureus* from clinical setting in Nigeria

In Nigeria, as earlier mentioned, 15 of the selected studies indicated that specimens were obtained from clinical settings (Table 2). One of the studies by Taiwo et al. (2005) included 141 *S. aureus* strains with 32% MRSA prevalence. Clinical isolates were sampled from soft tissue infections following surgery and skin. Specifically, in South-

West Nigeria, >90% of the studies confirmed the prevalence of MRSA and *mecA* positive isolates by molecular methods (Ghebremedhin et al. 2009; Shittu et al. 2011; 2012; Alli et al. 2012; Torimiro & Torimiro, 2012; Kolawole et al. 2013; Okon et al. 2014; Ayepola et al. 2015; Ogbolu et al. 2015; Nwokah et al. 2016). Furthermore, Alli et al. (2012) isolated about 120 clinical *S. aureus* strains from aspirates, wound, eye, blood samples and urine with an established MRSA prevalence rate of >40%.

Furthermore, Shittu et al. (2012) recorded a 16% MRSA prevalence and *mecA* gene detection in 91 *S. aureus* strains tested. Another study carried out in the same region of Nigeria obtained almost 50% MRSA prevalence among 116 clinical *S. aureus* isolates (Ghebremedhin et al. 2009). In addition, Ayepola et al. (2015) found 740 clinical *S. aureus* isolates with about 3% MRSA prevalence. Previous studies by Shittu et al. (2012); Kolawole et al. (2013) and O'Malley et al. (2015) had recorded higher clinical *S. aureus* strains of 91, 61 and 38 with MRSA rates of 42%, 12% and 42% respectively (Table 2). On a similar note, Obianuju et al. (2015), Alli et al. (2015) and Ibadin et al. (2017) confirmed the presence *mecA* gene and presence of multi-drug resistant *Staph.* species with about 40% MRSA prevalence rate each.

Table 2: Selected clinical studies on *mecA* and *Staphylococcus* species generated in Nigeria

Author, Year	Sampling period	No. of *S. aureus* isolates	No. of MRSA isolates	% MRSA
Taiwo et al. 2005	2001	141	45	32.0
Ghebremedhin et al. 2009	2006-2007	346	70	20.0
Shittu & Blanc, 2011	2009	68	11	16.0
Shittu et al. 2012	2010	91	15	16.0
Torimiro & Torimiro, 2012	NA	40	5	12.5
Kolawole et al. 2013	2008	61	7	11.5
Okon et al. 2014	2007	96	12	12.5
Alli et al. 2015	2015	156	66	42.0
Ayepola et al. 2015	2011	290	7	2.0
Obianuju et al. 2015	2015	246	41	40.0
Ogbolu et al. 2015	2010	116	55	47.0
O'Malley et al. 2015	2014	38	16	42.0
Nwokah et al. 2016	2013	205	25	12.0
Ibadin et al. 2017	2017	91	38	42.0
Total (15)		2100	460	22.0

Key: NA – Not Available

2.5.1.2 *MecA* gene in *Staphylococcus aureus* from a clinical setting in South Africa

The search results and analysis found 8 clinical studies conducted on *S. aureus* from South Africa (Table 3). Shittu & Lin (2006) obtained 227 *S. aureus* clinical isolates, 61 (26%) of this exhibited oxacillin resistance and detection of *mecA* gene was confirmed. Samie & Shivambu (2011) revealed 13% MRSA prevalence of the 140 *S. aureus* strains isolated in the Limpopo Province from both clinical specimens and drinking water. Further, another study sampled 13 academic centers between June 2010 and July 2012 across South Africa (Perovic et al. 2015). The study retrieved 2700 *S. aureus* clinical isolates. Of these, as many as 1160 (43%) isolates were methicillin-resistant and *mecA* gene detection was confirmed. In addition, another study done in academic hospitals, Gauteng province, South Africa between Sept. 2012 and Sept. 2013 on *S. aureus* bacteremia, an MRSA prevalence of 36% was confirmed using multiplex PCR (Fortuin-de Smidt, 2015).

The study by Oosthuysen et al. (2014) confirmed the isolation of 370 *S. aureus* strains from clinical specimens, of this an MRSA prevalence of 15% (56 isolates) was recorded. Brink et al. (2007), Moodley et al. (2011) and Groome et al. (2012) also confirmed the presence of >600, >300 and >150 clinical *S. aureus* species respectively with about 40% MRSA prevalence each. These studies further emphasize the presence of multi-drug resistant *S. aureus* species, MRSA prevalence and detection of *mecA* gene from clinical sources.

Table 3: Clinical studies on *mecA* and *Staphylococcus aureus* generated in South Africa

Author, Year	Sampling period	No. of *S. aureus* isolates	No. of MRSA isolates	% MRSA
Shittu & Lin, 2006	2002	227	61	27.0
Brink et al. 2007	2006	161	63	39.0
Samie & Shivambu, 2011	NA	140	18	13.0
Groome et al. 2012	2005-2006	629	226	36.0
Oosthuysen et al. 2014	NA	367	56	15.5
Fortuin-de Smidt, 2015	2012-2013	240	86	36.0
Perovic et al. 2015	2010-2014	2709	1231	33.0
van Rensburg et al. 2015	2007-2011	13746	3298	23.0
Amoako et al. 2016	2015	NA	NA	NA
Total (9)		18200	5040	28.0

Key: NA – Not Available

2.5.2 *MecA* gene in *Staphylococcus* species from wastewaters in developing countries

Forty - 40% (6 of 15) of articles on *mecA* gene in *Staphylococcus* spp. from the environmental setting originated from developing countries. From Africa, five studies were conducted on methicillin resistance CoNS in wastewaters in both Nigeria and South Africa (Table 4). One of the studies was conducted on hospital effluents/wastewater in Nigeria which included 56 CoNS isolates, such as: *S. saprophyticus* and *S. epidermidis* with detection of methicillin resistance in 7 (12.5%) of the isolates (Adekanmbi et al. 2019). The second study was conducted on both clinical and environmental wastewaters in Nigeria between May and October 2017 which involved 35 staphylococcal isolates, and 11 (31.4%) were *mecA* positive (Oladipo et al. 2019, Chapter 3 on this study). The third study from South Africa comprised 790 *S. aureus* isolates from freshwater environments with a prevalence of 7% for MRSA (Nnadozie & Odume, 2019). The fourth study also from South Africa determined virulence genes in MRSA species recovered from reclaimed wastewater effluents and receiving surface waters in Durban, KwaZulu-Natal. From the study, 80 *S. aureus* species were recovered with 23.0% being CoNS species (Ramessar and Olaniran 2019). The study from Tunisia by Raoudha et al. (2016), reported that *S. aureus* and MRSA isolates including *mecA* positive CoNS (*S. warneri* and *S. xylosus*) were found in reclaimed water.

These environmental studies further confirm the prevalence of multi-drug resistant *S. aureus* and CoNS species possessing the *mecA* gene from diverse wastewaters.

Table 4: Environmental studies on *mecA* and *Staphylococcus* species in developing countries

Author, Year	Country;	Sampling period	Environmental setting	No. of *Staph.* isolates	% *S. aureus*	% CoNS
*Adekanmbi et al. 2019	Nigeria	2019	Untreated Hospital effluent	56	45.0	55.0
*Oladipo et al. 2019	Nigeria	2017	Hospital effluent	41	46.0	54.0
*Samie & Shivambu 2011	South Africa	NR	Drinking water	17	88.0	12.0
Nnadozie & Odume 2019	South Africa	NR	Freshwater	790	93.0	7.0
Ramessar & Olaniran 2019	South Africa	NR	Treated effluent and receiving Water	80	77.0	23.0
*Raoudha et al. 2016	Tunisia	NR	Reclaimed water	200	58.0	42.0

Key: Author(s) with an (*) before their names conducted both environmental and clinical studies though only the details for environmental studies are indicated on this table; NR – Not Recorded

2.6 Antimicrobial resistant genes in CoNS

Staphylococcus spp. exhibit tendencies to develop antimicrobial resistance (AMR) which is a serious public and human health concern. Particularly, CoNS species have gained much attention since they serve as AMR reservoirs of importance (Becker et al. 2017). *Staphylococcus* spp. exhibit tendencies to develop antimicrobial resistance (AMR) which is a serious public and human health concern. Particularly, CoNS species have gained much attention since they serve as AMR reservoirs of importance (Otto, 2013; Vitali et al. 2014). Generally, the mechanism of antibiotic resistance in CoNS has been found to display much similarity with that of *S. aureus* species (Livermore, 2000).

For long the clinical significance of CoNS species had been overlooked and were regarded as mere contaminants when isolated from clinical specimens (Becker et al. 2014), however, CoNS are now identified as major implicated pathogens for numerous opportunistic infections in humans as well as animals (Otto, 2013; Loewen et al. 2017).

2.6.1 *MecA* positive coagulase-negative Staphylococci (CoNS)

Coagulase-negative staphylococci are species of much less occurrence in pathogens though are sometimes associated with prosthetic joint infections, wounds and endocarditis. Identified clinically significant CoNS species include: *S. epidermidis*, *S. lugdunensis, S. cohnii*, *S. saprophyticus*, *S. warneri*, *S. hominis* and *S. haemolyticus*. Čuvalova et al. (2015) confirmed the presence of CoNS from drinking water in Slovakia. CoNS species isolated included: *S. haemolyticus, S. saprophyticus* and *S. warneri*. The

authors concluded that CoNS represent an important opportunistic pathogen, with significant impact on human health and life.

Studies from Nigeria further buttress the occurrence of diverse strains of methicillin-resistant CoNS such as: *S. cohnii*, *S. saprophyticus*, *S. epidermidis* and *S. equorum* being recovered from both clinical and environmental settings (Adekanmbi et al. 2019; Oladipo et al. 2019, Chapter 3 on this thesis). Similarly, South African studies also documented the presence of methicillin-resistant CoNS from wastewaters such as: *S. haemolyticus*, *S. warneri*, *S. xylosus, S. kloosi, S. sciuri* and *S. arlettae* (Nnadozie & Odume 2019; Ramessar & Olaniran 2019).

Overall, this review found 380 CoNS strains isolated from environmental waters, of which (20%) were methicillin-resistant and *mecA* positive. Water environments such as streams, rivers, groundwater, lakes, municipal wastewaters and hospital effluents have been found to contribute significantly to the spread of antibiotic resistance gene and antibiotic resistance bacteria (ARB) (ARGs) (Loewen et al. 2017). The presence of ARB in the water environments is probably due to the strong affinity of bacteria for antimicrobial deposits of high concentrations in water, which stimulates the exchange of genetic materials (Lupo et al. 2012).

2.7 Human and public health implication of ARGs

Antimicrobial resistance poses a huge threat to human survival due to the unregulated/uncontrolled, misuse and/ excessive use of antibiotics (Thomas et al. 2016;

Tripathi & Tripathi, 2017). The effect of clinically relevant ARBs and ARGs released from anthropogenic sources into human and veterinary settings is currently one of the leading serious environmental and ecological challenges (Chen et al. 2018). Furthermore, associated links between ARGs of environmental sources have been established with human diseases (Vaz-Moreira et al. 2014). ARGs display transference across several bacterial species' boundaries, hence, transcending the host cells. These interactions of ARGs within the bacterial contaminants thus enhance proliferation/multiplication in their hosts, which is then transferred to other bacterial populations. These become potential subjects for further development, growth and transmission in the bacterial communities (Becker et al. 2017). Hence, ARGs are labelled undesired elements which negatively impacts human life and public health.

2.8. Conclusions

This review represented 19 nations and a total of 50 case studies were selected and examined on the prevalence of *mecA* positive *Staphylococcus* spp. from clinical and environmental waters. Retrieved articles for the search period (2000 – 2019) indicate that more studies on detection of *mecA* in staphylococcal species were conducted in developing countries (80%) compared to developed nations (20%). The *mecA* gene was detected in both *Staphylococcus aureus* and CoNS isolates from clinical and environmental sources. The PCR method (96%) was effective and widely used for the detection of *mecA* gene in *Staphylococcus* species. Generally, staphylococcal isolates particularly *Staphylococcus aureus* has been well studied and documented especially in the clinical settings with very little focus on CoNS. Overall, there is a paucity of

information on staphylococcal species isolated from wastewaters. This was especially the case with CoNS where only very few studies (6) could be traced on *mecA* gene detection in staphylococci from environmental sources. In addition, the potential of *S. aureus* and CoNS (sometimes multi-drug resistant) to exist and invariably serve as reservoirs of ARGs in wastewater environments (e.g. wastewaters) are yet to be fully explored. Furthermore, the human and public health implication and dissemination of the *mecA* gene particularly in wastewater environments are currently being understudied. The detection of the *mecA* gene in *Staphylococcus* species from the environmental and clinical settings is critical. Therefore, the impending global threat and impact of antibiotic resistance on human and public health may have a grave consequence if not studied and monitored more closely.

CHAPTER 3

MULTI-DRUG RESISTANCE TRAITS OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND OTHER STAPHYLOCOCCAL SPECIES FROM CLINICAL AND ENVIRONMENTAL SOURCES

3.1 Introduction

Currently in human medicine, antibiotics are the most effective antimicrobials used for combatting bacterial infections. However, the uncontrolled, excessive and misuse of antibiotics has played a key role in the selection of antibiotic resistance bacteria (ARB) (Finley et al. 2013; Tripathi & Tripathi 2017; Jensen et al. 2019).

Hospital effluents have been identified as a major reservoir for pollutants such as pharmaceutical wastes (antibiotics) and pathogenic microorganisms (Huang et al. 2019). Studies have reported that the discharge of hospital wastewaters is a highly selective route for the dissemination of ARB into natural environments (receiving water bodies) (Yilmaz et al. 2017; Kumar et al. 2019). However, the effect of antibiotics as environmental pollutants has been largely overlooked (Moges et al. 2014; Sabri et al. 2018). According to Finley et al. (2013) and Burgmann et al. (2018) there are no regulations guiding antibiotic residues in hospital effluents that are released into wastewaters. It is documented that only a few countries recommend the pre-treatment of hospital effluents before their discharge into receiving water bodies (Szekeres et al. 2017).

Staphylococcus species are among the most frequently encountered bacteria in hospital settings and has been associated with many infections in humans and animals such as skin and soft tissue infections, surgical site/wound infections, pneumonia, septicaemia and bone infections. (Nanoukona et al. 2017). The detection of *Staphylococcus* species in hospital wastewaters had been reported by many authors (Gómez et al. 2017; Tripathi & Tripathi 2017; Burgmann et al. 2018). Pathogenic *Staphylococcus* species have been confirmed to exhibit resistance to a wide array of antibiotics (WHO, 2017a). The *Staphylococcus* group comprises of the coagulase-positive staphylococci (CoPS) - *Staphylococcus aureus*, identified as the pathogenic species. The coagulase-negative staphylococci (CoNS) are regarded as non- pathogenic since they are considered part of the normal human flora (Casey et al. 2009). However, there is now increasing evidence that some of the CoNS species - *S. cohnii, S. epidermidis, S. haemolyticus, S. warneri* and *S. xylosus*) could be pathogenic and cause nosocomial infections (Tayyar et al. 2015; Hitzenbichler et al. 2017).

Staphylococcus aureus and specifically methicillin-resistant *S. aureus* (MRSA) have been isolated from wastewater effluent (Börjesson et al. 2009; Börjesson et al. 2010; Goldstein et al. 2012; Ekwanzala et al. 2018). In addition, the presence and multi-drug resistance of CoNS in reclaimed water had also been confirmed (Goldstein et al. 2017). Among the antibiotics that *Staphylococcus* species exhibit resistance to are ampicillin and methicillin (Moges et al. 2014; Delorme et al. 2017). Susceptibility/sensitivity to vancomycin and imipenem is still being recorded among

large numbers of isolates (Tayyar et al. 2015; Hitzenbichler et al. 2017; Premanadham et al. 2017).

Antibiotic resistance could be the result of mutations or acquisition of antibiotic resistance genes (ARG) due to horizontal gene transfer (HGT) (Calero-Caceres et al. 2017). Horizontal gene transfer thus facilitates the inter- and intraspecies transfer of antibiotic resistance and virulence genes (Tripathi & Tripathi 2017; Lerminiaux & Cameron 2018). The detection of *Staphylococcus* species in wastewaters poses an environmental risk which may result in the contamination of natural water reservoirs (groundwaters) and may eventually lead to public health challenges. In West Africa and particularly Nigeria, there is a paucity of data on the detection of *Staphylococcus* species in hospital effluents and the interplay with receiving wastewaters.

The aim of this study was therefore to investigate the prevalence of MRSA in clinical and environmental (hospital effluents and surrounding receiving wastewaters) sources, determine antibiotic resistance profiles and *mecA*, *nuc* and *luk*-PVL genes in *Staphylococcus* species in the Southwestern Nigeria.

3.2 Materials and Methods

3.2.1 Study location

This study was carried out at a hospital in Ile-Ife (7°16′48″N, 4°20′24″E), Osun State, South-Western, Nigeria. The medical institution provides health care services to about two million inhabitants in Ile-Ife and Southwest Nigeria.

3.2.2 Sample collection

3.2.2.1 Isolates from clinical samples

Isolates from various clinical samples were used and included wounds, skin, pus, urine, bone and blood. These were samples from different wards in the hospital submitted to the Microbiology Laboratory Unit for analysis. Clinical isolates were obtained from the Laboratory over a period of six months between May and October 2017. The clinical samples were aseptically cultured on appropriate MacConkay agar (Difco™ MacConkey Agar, New Brunswick, NJ, USA) and blood agar (Blood agar base, Oxoid Limited, Hampshire, UK) to select for growth of staphylococcal isolates. Purification and growth on Mannitol salt (Biotec Laboratories, Kentford, UK) and MRSA CHROMagar base (CHROMagar™ MRSA-ITK Diagnostics BV, Uithoorn, Netherlands) agar were to enhance the growth of *Staphylococcus aureus* and MRSA, respectively. Isolates were incubated on the two media at 37°C for 48 hours following standard protocols (Cheesbrough 2006).

3.2.2.2 Processing of water samples

Environmental samples were collected from wastewater generated within the hospital facility (hospital effluents). Water samples were also collected downstream at three receiving wastewater bodies within proximity (2 km) of the hospital. Each receiving wastewater body was approximately 750 m from each other. Sampling was done weekly over a period of six months for the purpose of collecting as many *Staphylococcus* isolates as possible. The water samples were collected in sterile bottles, preserved in cooler boxes and stored at 4°C until analysed (within 24 hours). On getting to the laboratory, the water samples were filtered using sterile 0.45 µm membrane filters. These filters were enriched within Bacto tryptic soy broth (soybean-casein digest medium) (Becton Dickinson, US) and were then placed on Mannitol salt agar (Biotec Laboratories, Kentford, UK) and incubated for 24 hours at 33°C. Resulting yellow colonies were presumptively considered as *S. aureus*. The yellow colonies were further plated on MRSA CHROMagar base (CHROMagar™ MRSA-ITK Diagnostics BV, Uithoorn, The Netherlands), to obtain pure isolates. Incubation was at 37°C for 48 hours.

3.2.2.3 Isolation of *Staphylococcus* species

Clinical and environmental isolates on mannitol salt agar and chromogenic agar that provided both the characteristic yellow and purple colours, respectively, were included in further analyses. These were subjected to Gram stain and identified as Gram-positive cocci with grape-like clusters under the microscope. *Staphylococcus*

aureus and other *Staphylococcus* species were confirmed with the coagulase test using standard protocols (Cheesbrough, 2006).

3.2.2.4 Antimicrobial susceptibility testing

Afterwards, all isolated and identified *Staphylococcus* species were then subjected to antibiotic susceptibility testing using the standard Kirby-Bauer's disk diffusion technique (CLSI, 2014).

3.3 Partial 16S rRNA gene-based identification of isolates

3.3.1 Extraction of genomic DNA

The Nucleospin® tissue extraction kit (Macherey-Nagel, Düren, Germany) was used to extract genomic DNA from the isolates following the manufacturer's instructions. Briefly, pure isolates were incubated overnight in Nutrient Broth (Biolab Diagnostics, RSA) at 37°C. Two (2) mL samples from the broth cultures were dispensed into 2 mL microfuge tube and centrifuged at 8000 rpm for 5 mins at room temperature. The supernatant discarded, and the pellet treated, following the Macherey-Nagel Nucleospin® tissue extraction kit, instructions. The quality and integrity of extracted DNA products were verified on 1% agarose gels and images were captured using a GeneGenius Bioimaging system (Syngene, Cambridge, UK) and GeneSnap software V. 2.2.2 (Syngene, Cambridge, UK). DNA purity and concentration were verified using NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, Delaware USA).

3.3.2 PCR amplification

PCR was performed in a C1000TM thermal cycler (Bio-Rad, Hercules, CA, USA) and amplification of the 16S rRNA gene was done using universal primer sets 27F and 1492R with PCR conditions (Table 5), as previously described (Lane, 1991). Each PCR reaction including positive and negative controls contained 12.5 µL of 2x PCR Master mix (Thermos Scientific Technologies, Waltham, MA, USA), 50 ng DNA template, 5 µM each of forward (27F) and reverse (1492R) primers and nuclease-free water to a final volume of 25 µL.

Table 5: Primers used for the identification of *Staphylococcus* species and the detection of marker genes

Primers	Primer sequence (5′–3′)	PCR Conditions	Size (bp)	References
27F 1492R	5′GAGTTTGATCATGGCTCAG3′ 5′GGTTACCTTGTTACGACTT3′	1 cycle of 2min at 95°C; 35 cycles of 30 sec at 94°C; 30sec at 53°C for, 1min at 72°C; 1 cycle 10 min at 72°C	1500	Lane,1991
mecA-F *mecA*-R	5′AACGATTGTGACACGATAGCC3′ 5′GGGATCATAGCGTCATTATC3′	1 cycle of 5min at 94°C; 30 cycles of 30s at 94°C; 30sec at 55^{o}C; 1min at 72°C; 1 cycle of 10min at 72°C	527	Kumar et al. 2016
nuc-1 *nuc*-2	5′TCAGCAAATGCATCACAAACAG3′ 5′CGTAAATGCACTTGCTTCAGG3′	1 cycle of 5min at 94°C; 35 cycles of 30s at 94°C; 30 sec at 55°C; 1 min at 72°C	255	Othman et al. 2014
luk-F luk-R	5′ATCATTAGGTAAATGTCTGGCA TGATCC3′ 5′AGCATCAAGTGTATTGGATAGC AAAAGC3′	1 cycle of 4min at 94°C; 30 cycles of 45s at 94°C; 1min at 72°C; 1 cycle of 2min at 72°C	433	McClure et al. 2006

3.3.3 Sequencing of 16S rRNA genes

Sequencing of purified PCR products was carried out using the Big Dye terminator V. 3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) on a 3130 Genetic analyzer (Applied Biosystems/Hitachi, Tokyo, Japan). Generated sequence electropherograms were then manually edited after inspection using Finch TV 1.4.0; http://www.geospiza.com/Products/finchtv.shtml). Edited sequences were aligned against other sequences for comparison with the Basic Local Alignment Search Tool (BLAST) program alignment tool of the GenBank on the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed using MUSCLE (Edgar, 2004) integrated into MEGA V. 7.0 (http://www.megasoftware.net/; Kumar et al. 2016). Phylogenetic sequence dendrograms with closely related sequences in GenBank were constructed with the Neighbor-Joining tree method using the substitution model. The partial 16S rRNA sequences from this study are available in the GenBank under the accession numbers: KY290702-KY290711; KY290719-KY290745 and MK208534- MK208572.

3.3.4 PCR amplification of *mecA, nuc* and *luk*-PVL genes

For MRSA differentiation, PCR amplification of the *mecA* gene (that encodes for methicillin-resistance), thermonuclease (*nuc*) gene and the *luk*-PVL gene that encodes for virulence in *S. aureus* species was performed. Each PCR reaction contained 12.5 µL 2x PCR Master mix (Thermos Scientific Technologies, Waltham, MA, USA), 50 ng DNA template, 5 µM each of forward and reverse primers and nuclease-free water to a final volume of 25 µL. Information on the primers and

conditions are detailed in Table 5. DNA amplification was performed in a C1000 thermocycler (Bio-Rad, Hercules, USA). Electrophoresis of the amplicons were performed with 1% w/v agarose gel and visualized with GelRed staining under an UV transilluminator.

3.3.5 Statistical analyses

Antimicrobial resistance data were analysed using WHONET 2017 software Version 5.6 (WHO; http://www.whonet.org/software.html). The multiple antibiotic resistance (MAR) index for individual isolates was calculated and interpreted according to Krumperman (1983) using the formula:

$$MAR\ index\ per\ isolate = \frac{the\ number\ of\ antibiotics\ to\ which\ the\ test\ isolate\ showed\ resistance\ to}{the\ total\ number\ of\ antibiotics\ tested}$$

MAR index values greater than 0.2 indicate high risk source of contamination where antibiotics are often used (Krumperman, 1983). The MAR index for each of the three compartments (clinical, hospital wastewater, environmental water) was determined using the method of Krumperman, (1983) using the formula:

$$MAR\ index\ per\ compartment = \frac{number\ of\ isolates\ in\ a\ specific\ sample\ population\ resistant\ to\ antibiotics}{(number\ of\ antibiotics\ tested)\ x\ (total\ number\ of\ organisms\ in\ sample)}$$

Multiple sequence alignment was performed using MUSCLE (Edgar, 2004) integrated into Molecular Evolutionary Genetics Analysis (MEGA) V. 7.0 (Kumar et al. 2016).

3.4 Results

3.4.1 Prevalence of *Staphylococcus* strains isolated from clinical and environmental samples

Over the sampling period, a total of 360 bacterial isolates were obtained, of which 140 isolates which were presumptively and phenotypically identified as Gram positive were investigated. Further biochemical testing confirmed 76 of the total number of samples collected both clinical and environmental isolates were *Staphylococcus* species.

3.4.2 Phenotypic and taxonomic identification of *Staphylococcus* strains

This study further confirmed the identities of the *Staphylococcus* species obtained from the clinical and environmental sources by 16S rRNA gene sequencing analysis (Figure 2). Of *Staphylococcus* species (n=76), 56.58% (43/76) were coagulase-negative (CoNS) and 43.42% (33/76) coagulase-positive (*Staphylococcus aureus*). The distribution of the 76 *Staph.* spp. was as follows: 27 from clinical samples, 14 from hospital effluents and 35 isolates originated from environmental (wastewater) sources. Of the 27 clinical isolates, 12 were from urine, 4 from wounds, 6 from skin 4 from blood and 1 isolate from pus. Environmental samples constituted 40% (n= 14)

from the hospital generated wastewaters (effluents) and 60% (n= 21) from surrounding wastewater bodies at proximity to the hospital.

3.4.3 Distribution of staphylococcal species

A total of 11 different *Staphylococcus* species were obtained in this study (Figure 2). Forty-three percent of the isolates were identified as *S. aureus*. Other species included *S. sciuri* (17.1%) and *S. cohnii* (11.8%) as well as *S. arlettae, S. epidermidis* and *S. saprophyticus (*6.5%). There were single representatives of *S. equorum, S. warneri, S. xylosus and S. kloosii*. In terms of species distribution, 78.87% of the *Staphylococcus* strains obtained were found in both clinical and environmental sources (Figure 2). These were: *S. aureus, S. cohnii, S. sciuri, S. haemolyticus, S. arlettae, S. saprophyticus and S. epidermidis*. On the other hand, *S. sciuri* and *S. haemolyticus* were isolated more frequently from environmental water sources. It was observed that 4 of the species were found either in clinical or environmental sources but not in both. Among these species were *S. equorum* (found only in clinical sources) and *S. warneri, S. kloosii and S. xylosus* occurring only in environmental samples. Furthermore, three *Staphylococcus* species exclusively found in environmental sources were isolated from the hospital effluents (Table 6). These observations are not absolute and could be biased since the isolation process was a culture-based selective process. None the less, it provided some insights into culturable staphylococci associated with hospital effluent, environmental water and a potential link to clinical staphylococci.

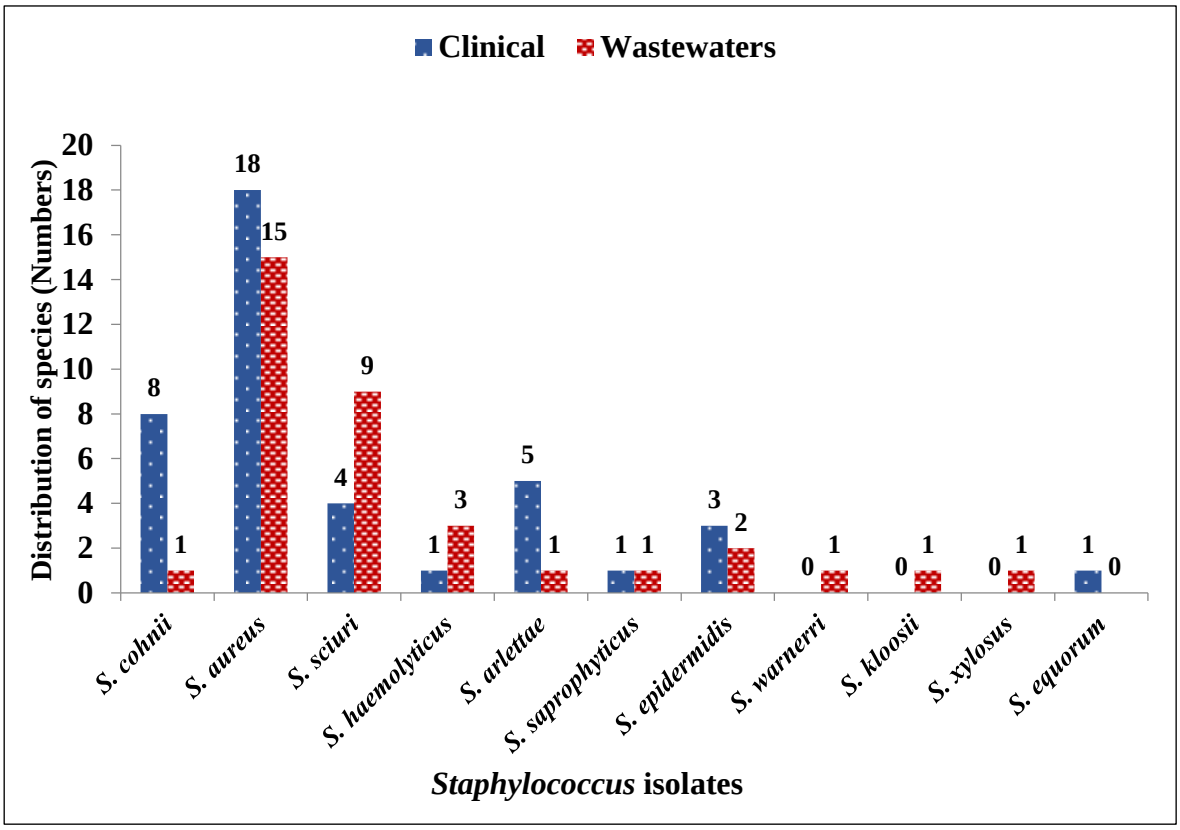


Figure 2: **Distribution of *Staphylococcus* isolates obtained from clinical and wastewater samples**

Table 6: Identification, source, antibiotic resistance pattern and MAR indices of *Staphylococcus* species isolated from clinical and environmental samples

S/N	Isolates	Accession Number	Sources	Sample type	Antibiotics														
					FOX	OFX	ERY	CLO	CRM	GEN	IPM	AZM	CAZ	AMC	AUG	CIP	VAN	R	MAR per isolate
1	*S. cohnii*	**KY290719**	Clinical	Wound	R	R	S	R	R	R	S	R	S	R	R	R	S	9	0.7
2	*S. arlettae*	**KY290720**	Clinical	Skin	S	R	R	R	R	R	S	S	S	R	R	R	S	8	0.6
3	*S. equorum*	**KY290721**	Clinical	Pus	R	R	S	R	S	S	S	R	R	R	R	S	R	8	0.6
4	*S. aureus*	**KY290722**	Clinical	Wound	R	R	S	R	S	R	S	R	R	R	S	S	S	7	0.5
5	*S. aureus*	**KY290723**	Clinical	Skin	R	S	S	R	S	S	S	R	S	R	S	R	S	5	0.4
6	*S. cohnii*	**KY290724**	Clinical	Wound	R	R	R	S	S	R	S	S	S	R	S	R	S	6	0.5
7	*S. arlettae*	**KY290725**	Clinical	Urine	S	S	R	R	S	R	S	S	R	S	S	S	S	4	0.3
8	*S. cohnii*	**KY290726**	Clinical	Wound	S	S	R	S	R	R	S	S	S	R	S	S	S	4	0.3
9	*S. epidermidis*	**KY290727**	Clinical	Skin	S	R	R	R	S	R	S	R	R	R	S	R	S	8	0.6
10	*S. sciuri*	**KY290728**	Clinical	Urine	S	S	S	R	R	R	S	R	R	R	S	R	R	8	0.6
11	*S. saprophyicus*	**KY290729**	Clinical	Urine	R	S	R	S	R	R	S	S	S	R	S	R	R	6	0.5
12	*S. aureus*	**KY290730**	Clinical	Urine	R	R	R	S	R	R	S	S	S	R	S	S	S	6	0.5
13	*S. cohnii*	**KY290731**	Clinical	Urine	S	S	R	S	R	S	S	S	S	R	R	R	R	6	0.5
14	*S. aureus*	**KY290732**	Clinical	Blood	R	R	S	R	S	S	S	S	S	S	S	S	S	3	0.2
15	*S. arlettae*	**KY290733**	Clinical	Urine	R	R	S	S	S	R	R	S	S	R	S	R	R	6	0.5
16	*S. cohnii*	**KY290734**	Clinical	Skin	S	R	R	R	R	R	R	R	R	R	R	R	S	11	0.8
17	*S. aureus*	**KY290735**	Clinical	Bone	S	S	R	R	S	S	S	S	S	R	R	S	S	3	0.2
18	*S. cohnii*	**KY290736**	Clinical	Blood	R	R	S	R	R	S	S	S	S	R	S	S	S	5	0.4
19.	*S. sciuri*	**KY290737**	Clinical	Urine	R	R	S	S	S	R	S	S	S	R	S	S	S	4	0.3
20.	*S. haemolyticus*	**KY290738**	Clinical	Skin	R	R	S	R	R	S	R	S	R	S	S	R	S	7	0.5
21.	*S. sciuri*	**KY290739**	Clinical	Urine	R	R	S	S	S	S	S	S	S	R	S	S	R	4	0.3
22.	*S. sciuri*	**KY290740**	Clinical	Urine	R	R	S	R	S	S	S	R	R	R	S	S	S	6	0.5
23.	*S. aureus*	**KY290741**	Clinical	Bone	R	R	S	R	S	R	S	R	R	R	S	R	R	9	0.7
24.	*S. cohnii*	**KY290742**	Clinical	Urine	R	R	R	R	R	R	R	R	R	R	R	R	S	12	0.9
25.	*S. cohnii*	**KY290743**	Clinical	Blood	S	S	S	R	R	S	S	R	R	R	S	S	S	5	0.4
26.	*S. epidermidis*	**KY290744**	Clinical	Skin	R	R	S	S	S	R	S	S	S	R	R	S	S	5	0.4
27.	*S. arlettae*	**KY290745**	Clinical	Blood	S	S	S	R	S	R	S	S	S	R	S	R	S	4	0.3
Multiple antibiotic resistance (MAR) index for clinical isolates = 0.482																			
28.	*S. aureus*	**KY290702**	Hosp.w/w	Effluent	R	R	S	S	R	R	S	S	R	R	S	S	S	6	0.5
29.	*S. sciuri*	**KY290703**	Hosp.w/w	Effluent	S	S	R	S	S	R	S	R	S	R	R	R	R	7	0.5
30.	*S. haemolyticus*	**KY290704**	Hosp.w/w	Effluent	S	S	R	S	S	S	S	R	S	R	R	R	R	6	0.5

31.	*S. arlettae*	**KY290705**	Hosp.w/w	Effluent	R	R	R	S	R	R	S	S	S	S	R	S	S	6	0.5
32.	*S. saprophyticus*	**KY290706**	Hosp.w/w	Effluent	R	R	S	R	R	S	S	S	S	R	R	S	R	7	0.6
33.	*S. epidermidis*	**KY290707**	Hosp.w/w	Effluent	S	R	S	R	S	R	S	R	R	R	S	R	S	7	0.6
34.	*S. epidermidis*	**KY290708**	Hosp.w/w	Effluent	S	S	R	S	S	S	S	R	R	R	R	R	S	6	0.5
35.	*S. warneri*	**KY290709**	Hosp.w/w	Effluent	S	S	R	R	S	S	S	R	S	R	R	S	S	5	0.4
36.	*S. Kloosi*	**KY290710**	Hosp.w/w	Effluent	R	R	S	S	S	S	S	S	R	R	S	S	S	4	0.3
37.	*S. xylosus*	**KY290711**	Hosp.w/w	Effluent	R	S	S	R	S	R	S	R	S	R	R	S	R	7	0.6
38.	*S. aureus*	**MK208534**	Hosp.w/w	Effluent	R	S	S	S	S	S	R	S	R	R	S	R	R	6	0.5
39.	*S. aureus*	**MK208535**	Hosp.w/w	Effluent	R	R	S	R	R	R	R	R	S	R	R	R	S	10	0.8
40.	*S. aureus*	**MK208536**	Hosp.w/w	Effluent	R	S	R	R	R	R	R	R	R	R	R	R	R	12	0.9
41.	*S. aureus*	**MK208537**	Hosp.w/w	Effluent	R	S	S	S	S	S	S	S	S	S	S	S	R	2	0.1
Multiple antibiotic resistance (MAR) index for hospital wastewater = 0.500																			
42.	*S. aureus*	**MK208538**	Environ.	Water	S	R	R	S	S	S	R	R	S	R	R	R	S	7	0.7
43.	*S. aureus*	**MK208539**	Environ.	Water	S	S	S	S	R	R	S	S	S	S	R	R	S	4	0.3
44.	*S. aureus*	**MK208540**	Environ.	Water	S	R	R	R	R	R	S	S	R	R	R	R	R	10	0.8
45.	*S. aureus*	**MK208568**	Environ.	Water	R	S	S	R	R	S	S	R	S	R	S	S	R	6	0.5
46.	*S. aureus*	**MK208568**	Environ.	Water	R	S	R	R	S	R	S	S	R	R	R	R	S	8	0.6
47.	*S. aureus*	**MK208568**	Environ.	Water	S	R	R	R	S	S	S	S	R	R	R	R	S	7	0.6
48.	*S. aureus*	**MK208568**	Environ.	Water	S	S	S	S	S	S	S	S	R	R	R	S	R	4	0.3
49.	*S. aureus*	**MK208545**	Environ.	Water	S	S	S	S	S	S	S	S	S	R	S	S	S	1	0.1
50.	*S. aureus*	**MK208546**	Environ.	Water	R	S	S	R	R	R	R	S	R	R	R	R	S	9	0.7
51.	*S. aureus*	**MK208547**	Environ.	Water	S	R	S	R	S	S	R	S	S	R	R	S	S	5	0.4
52.	*S. aureus*	**MK208548**	Environ.	Water	R	R	S	S	R	S	R	R	R	R	R	R	S	9	0.7
53.	*S. aureus*	**MK208549**	Environ.	Water	R	S	R	R	S	R	R	R	S	S	R	S	R	8	0.6
54.	*S. aureus*	**MK208550**	Environ.	Water	R	R	R	R	S	R	S	R	S	S	R	S	S	7	0.5
55.	*S. aureus*	**MK208551**	Environ.	Water	S	S	S	S	S	S	S	S	R	R	R	S	S	3	0.2
56.	*S. aureus*	**MK208552**	Environ.	Water	S	S	S	S	S	S	S	S	S	R	R	S	S	2	0.1
57.	*S. haemolyticus*	**MK208553**	Environ.	Water	S	S	S	R	S	S	R	S	S	R	R	s	S	4	0.3
58.	*S. aureus*	**MK208554**	Environ.	Water	S	S	S	S	S	S	R	S	S	R	S	S	S	2	0.1
59.	*S. aureus*	**MK208555**	Environ.	Water	S	S	S	S	S	S	S	S	S	S	R	S	R	2	0.1
60.	*S. cohnii*	**MK208556**	Environ.	Water	R	R	R	R	S	R	S	R	R	R	R	S	R	10	0.8
61.	*S. epidermidis*	**MK208557**	Environ.	Water	S	S	S	R	R	S	S	S	R	R	S	S	R	5	0.4
62.	*S. epidermidis*	**MK208558**	Environ.	Water	S	R	S	R	R	S	S	S	R	R	R	S	S	6	0.5
63.	*S. aureus*	**MK208559**	Environ.	Water	S	R	R	S	S	R	R	S	S	R	R	R	S	7	0.5
64.	*S. epidermidis*	**MK208560**	Environ.	Water	S	S	S	S	S	S	S	S	S	R	S	S	S	1	0.1
65.	*S. epidermidis*	**MK208561**	Environ.	Water	S	S	R	S	S	S	S	S	S	R	S	S	S	2	0.1
66.	*S. epidermidis*	**MK208562**	Environ.	Water	S	R	R	S	S	S	S	R	R	S	S	S	S	4	0.3
67.	*S. epidermidis*	**MK208563**	Environ.	Water	S	S	S	S	S	S	S	R	S	R	R	S	R	4	0.3
68.	*S. aureus*	**MK208564**	Environ.	Water	R	S	R	R	R	R	R	S	R	R	R	R	R	11	0.8
69.	*S. epidermidis*	**MK208565**	Environ.	Water	S	R	R	R	R	R	S	S	S	R	R	R	S	8	0.6

70.	*S. haemolyticus*	**MK208566**	Environ.	Water	R	R	S	R	R	R	S	S	R	R	S	R	S	8	0.6
71.	*S. epidermidis*	**MK208567**	Environ.	Water	S	R	R	S	S	R	S	R	R	S	S	S	S	5	0.4
72.	*S. aureus*	**MK208568**	Environ.	Water	S	R	S	S	S	S	S	R	R	R	R	S	R	6	0.5
73.	*S. aureus*	**MK208569**	Environ.	Water	S	R	S	R	S	S	R	S	S	S	R	S	S	4	0.3
74.	*S. epidermidis*	**MK208570**	Environ.	Water	S	S	S	S	R	R	S	R	S	R	R	S	S	5	0.4
75.	*S. haemolyticus*	**MK208571**	Environ.	Water	S	S	S	S	R	R	S	S	R	R	R	S	R	6	0.5
76.	*S. aureus*	**MK208572**	Environ.	Water	S	S	R	R	R	R	S	S	S	R	R	R	R	8	0.6
Multiple antibiotic resistance (MAR) index for environmental receiving water = 0.435																			

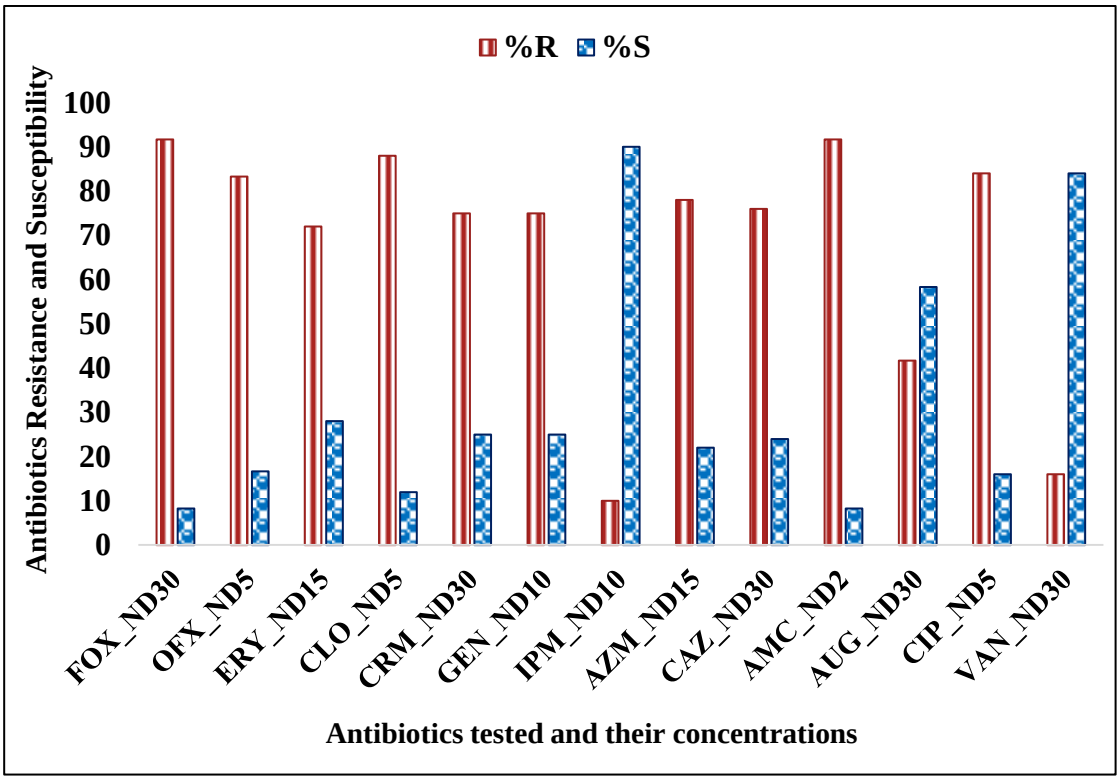
Notes: R = number of antibiotics an isolate was resistant to. FOX (Methicillin) (30 µg), OFX (Ofloxacin) (5 µg), ERY (Erythromycin) (15 µg), CLO (Cloxacillin) (5 µg), CRM (Cefuroxime) (30 µg), GEN (Gentamicin) (10 µg), IPM (Imipenem) (10 µg), AZM (Azithromycin) (15 µg), CAZ (Ceftazidine) (30 µg), AMC (Amoxycillin) (2 µg), AUG (Augmentin) (30 µg), CIP (Ciprofloxacin) (5 µg), and VAN (Vancomycin) (30 µg).

3.4.4 Antibiotic resistance patterns of *Staphylococcus* species

All the *Staphylococcus* species showed resistantance to several classes of antibiotics though at varying levels (Figure 3). The antibiotic(s) which the highest numbers of isolates were resistant to includes methicillin (antibiotic of interest and amoxycillin (91.7% each). More than 80% of the isolates were also resistant to cloxacillin, ciprofloxacin, ofloxacin while >70% of isolates showed resistance to azithromycin, ceftazidime, Gentamicin, cefuroxime and erythromycin (Figure 3). Forty-two percent were resistant to ceftriaxone. Amongst all these isolates, 90% and 84% were susceptible to imipenem and vancomycin, respectively. This implies that these antibiotics were still effective against the staphylococci from this region. About 60% of the isolates were resistant to at least 6 of the 13 antibiotics (Table 6). Over 50% of the *S. aureus* isolates were methicillin-resistant (thus MRSA) while the others were susceptible to methicillin.

3.4.5 Multiple antibiotic resistance (MAR)

In this study, nearly all the isolates (69 of 76; 90.8%) had a multiple antibiotic resistance (MAR) index higher than 0.2 while only 7 (9.2%) had a MAR index <0.2. The *Staphylococcus* isolates with MAR index greater than 0.2 were mainly clinical isolates (Table 6). Furthermore, the mean overall MAR values of clinical, hospital wastewater effluents and environmental water were calculated and compared. The overall MAR index values for *Staphylococcus* species isolated from the clinical, hospital effluent and environmental wastewaters were 0.482, 0.500 and 0.435, respectively (Table 6). These MAR index values were relatively similar.



Key: R indicates 'resistant and S 'susceptible'. FOX (Methicillin) (30 µg), OFX (Ofloxacin) (5 µg), ERY (Erythromycin) (15 µg), CLO (Cloxacillin) (5 µg), CRM (Cefuroxime) (30 µg), GEN (Gentamicin) (10 µg), IPM (Imipenem) (10 µg), AZM (Azithromycin) 15 µg), CAZ (Ceftazidine) (30 µg), AMC (Amoxycillin) (2 µg), AUG (Augmentin) (30 µg), CIP (Ciprofloxacin) (5 µg), and VAN (Vancomycin) (30 µg).

Figure 3: Antibiotic resistance profile of *Staphylococcus* isolates

3.4.6 Detection of resistance and virulence genes in *Staphylococcus* species

The detection of resistance and virulence genes among the *Staphylococcus* species varied. It was observed that of the 11-different species, six (54.5%) tested positive for the resistance gene - *mecA*. Of all 11 species, *S. aureus* was the only strain that was positive for all three genes*. S. sciuri* and *S. cohnii* (6 isolates each), *S. arlettae* and *S. epidermidis* (5 isolates each) and a single isolate of *S. saprophyticus* were positive for only *mecA* gene. Overall, approximately 55.3% (42 out of 76) of the isolates analysed in this study were positive for the antibiotic resistance gene – *mecA*. Neither resistance nor virulence genes were detected in *S. haemolyticus, S. equorum, S. warnerii, S. kloosii* and *S. xylosus* species.

The *mecA* gene was detected among 83.3% of the *Staphylococcus* species originated from clinical sources while it was detected in 16.7% environmental samples. These were mainly from the hospital wastewater. Similarly, for the *nuc* gene, 54.5% of the strains that were positive for this gene were isolated from clinical sources while 45.5% were isolated from wastewater sources. However, with the virulence gene all the isolates were *S. aureus* from clinical sources. Largely, 63% of the isolates which were positive for the resistance and virulence genes originated from clinical samples while 37% were from environmental sources.

3.5 Discussion

Studies have focused on *S. aureus* and CoNS from clinical sources in West Africa (Egyir et al. 2015; Nanoukona et al. 2017) and Nigeria (Shittu et al. 2012; Kolawole et al. 2013; Vitali et al. 2014; Ayepola et al. 2015; Nwokah et al. 2016; Ibadin et al.

2017). However, there is paucity of data regarding these species in environmental sources (wastewater effluents and water bodies). In the present study, we found that 43.42% of isolates were coagulase-positive (*Staphylococcus aureus*) and 56.58% coagulase-negative (CoNS). These isolates were from clinical, wastewater and environmental water samples. The 10 different CoNS species identified included – *S. cohnii, S. epidermidis, S. saprophyticus, S. haemolyticus, S. arlettae, S. scuiri, S. xylosus, S. warneri, S. equorum* and *S. kloosii*. Similar observations were made in previous studies. A study conducted by Gómez et al. (2017) on wastewaters in Spain demonstrated the occurrence of 16.67% *Staphylococcus aureus* and 83.33% CoNS species. These authors observed 12 different species. Furthermore, Shittu et al. (2012) and Tayyar et al. (2015) found *S. haemolyticus, S. epidermidis, S. saprophyticus* and *S. xylosus* in clinical samples. Similarly, Nanoukona et al. (2017) identified *S. cohnii, S. sciuri, S. arlettae, S. warneri and S. kloosii* also in clinical samples. Gómez et al. (2017) found *S. epidermidis, S. sciuri, S. xylosus, S. equorum and S. warneri* in wastewater samples.

The present study demonstrated the occurrence of *S. cohnii* and *S. equorum* in clinical samples and *S. xylosus, S. warneri* and *S. kloosii* in wastewater sources. These results are corroborated by previous studies. Both Chen et al. (2015) and Gómez et al. (2016) suggested that the reason few studies have reported *S. cohnii* in wastewater, is that they are mainly associated with nosocomial infections. Finding them in wastewater may suggest the origin is from a clinical setting (Vitali et al. 2014; Hitzenbichler et al. 2017; Nanoukona et al. 2017). A study by Gómez et al. (2017) demonstrated that *S. xylosus* and *S. warneri* to occur in wastewaters. This is

consistent with the findings from the present study. These observations confirmed that the *S. warneri* and *S. kloosii* could be present in hospital effluents. This may possibly suggest that they are likely to originate from clinical sources and then could be transported to environmental water bodies where they could be a potential health threat. *Staphylococcus equorum*, a very rare species could be present in wastewaters (Gómez et al. 2017). It had earlier been reported to also be associated with clinical scenarios (Nováková et al. 2006). The *S. kloosii* isolated from wastewaters in this study was also previously isolated from clinical sources (Nanoukona et al. 2017). In the present study various *Staphylococcus* spp. have been isolated from clinical samples, hospital effluent and receiving environmental waters. These findings were supported by previous studies showing similar trends. The prevalence of the nine different species found in these aquatic environmental habitats may be ascribed to their capability to survive under the existing conditions for extended periods (Šolić & Krustulović 1994; Tolba et al. 2008; Abulreesh, 2011).

With respect to antimicrobial resistance, almost all the *Staphylococcus* species were resistant to several classes of antibiotics. Isolates were defined as multi-resistant based on resistance to methicillin and at least two other classes of antibiotics (Magiorakos et al. 2012). Specifically, over 50% of the *S. aureus* isolates from both clinical and environmental sources were methicillin-resistant (MRSA) while the others were susceptible to methicillin (MSSA). *S. saprophyticus* isolates were all resistant to methicillin. Furthermore, a strain of *S. cohnii* was resistant to 12 of the 13 antibiotics tested. This finding is supported by Delorme et al. (2017) and Nanoukona et al. (2017) that found *S. cohnii* resistant to a large number of antibiotics. In the present

study it was demonstrated that most of the isolates (91.7%) showed resistance to methicillin and amoxicillin. This trend had also been confirmed by previous studies (Moges et al. 2014; Tayyar et al. 2015; Premanadham et al. 2017). On the contrary, most of the *Staphylococcus* species were susceptible to vancomycin (84.0%) and imipenem (90.0%) demonstrating that these antibiotics are still useful in clinical settings. A similar trend of antibiotic susceptibility of *Staphylococcus* spp. had been documented in previous studies (Thomas et al. 2016; Hitzenbichler et al. 2017). The high susceptibility rate recorded for vancomycin and imipenem had been ascribed to the fact these drugs are usually not commonly prescribed in the hospital settings (Thomas et al. 2016). This demonstrates that antibiotic stewardship and regulation may still retard the onset of antibiotic resistance, even in developing regions of the world. Vancomycin is a glycopeptide antibiotic that is seen as a drug of choice and a good therapeutic option for treating severe bacterial infections, especially multi-drug resistance MRSA strains (Hitzenbichler et al. 2017). Although, the efficacy of vancomycin may be reduced when administered intravenously as a single dose, its combination with other antibacterial agents such as imipenem has given better treatment outcomes (Erjavec et al. 1994, Courvalin, 2006).

Overall, all the species from *Staphylococcus* spp. displayed multiple resistance traits towards antibiotics. The CoNS in this study were resistant to a higher number of antibiotics compared to *S. aureus*. This result confirms the multi-resistance potential of *Staphylococcus aureus* and CoNS to antibiotics used in clinical settings. Our finding is corroborated by Center et al. (2003) who reported that CoNS species generally have decreased susceptibilities to various antibiotics.

It was confirmed that strains of the same *Staphylococcus* species demonstrated similar antibiotic resistance/susceptibility patterns regardless of their isolation sources (clinical or environmental). Of note is the very high number of *Staphylococcus* species that had a multiple antibiotic resistance (MAR) index greater than 0.2. Furthermore, the overall MAR values of clinical, hospital wastewater effluents and environmental water numerically relatively quite similar. These similar MAR indices may indirectly suggest that all the isolates were from the sources where antibiotics are often used. This also implies that the strains from the clinical/hospital wastewater effluent and environmental water might have a common origin (Krumperman, 1983; Riaz et al. 2011). Furthermore, this index demonstrates frequent exposure of the bacteria to antibiotics of various classes (Krumperman, 1983; Riaz et al. 2011) but also may indicate that antibiotics resistance genes (ARGs) which may be well distributed among the bacteria in environmental wastewaters where there is the interphase of the clinical and environmental strains (Goldstein et al. 2012; Gómez et al. 2017). Multiple antibiotic resistance (MAR) in bacteria is most commonly associated with the presence of plasmids or other mobile elements which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype. (Riaz et al. 2011).

The detection of multi-drug resistance phenotype, with *mecA* (54.5%) and *nuc* (100%) resistance and virulence genes respectively were confirmed in *Staphylococcus aureus* isolates. Only *mecA* was confirmed in *S. arlettae, S. scuiri, S. cohnii, S. epidermidis and S. saprophyticus.* The detection of the *mecA* resistance gene in CoNS species had been reported by previous studies (Shittu et al. 2012;

Vitali et al. 2014; Gómez et al. 2017). The *mecA* gene allows a bacterium to be resistant to antibiotics such as methicillin, MRSA is known as the most commonly known carrier of the gene (Igbinosa et al. 2016). In addition, the detection of the virulence gene, *luk*-PVL was confirmed in the six *S. aureus* representatives isolated from clinical samples only. Panton Valentine leukocidin (*PVL*) is considered one of the important virulence factors responsible for white cells destruction and as a marker of community acquired MRSA (Yang et al. 2009; Igbinosa et al. 2016).

All *Staphylococcus aureus* strains isolated from wastewater sources were negative for the *luk*-PVL virulence gene. This trend has been confirmed in a previous study (Gómez et al. 2017). The detection of *mecA* resistance gene in CoNS had been ascribed to possible horizontal gene transfer (HGT) by mobile genetic components (such as plasmids, integrons and transposons) from *S. aureus* species, which could facilitate the environmental dissemination of the antibiotic resistance genes to CoNS (Calero-Caceres et al. 2017; Tripathi & Tripathi 2017; Jensen et al. 2019). This scenario may be the case in the present study, where the hospital wastewater could be the source of *mecA* and HGT could be responsible for the dispersal of this gene to other staphylococci.

3.6 Conclusion

This study highlighted the prevalence and diversity of *Stapylococcus spp*. in both clinical and environmental sources. *Staphylococcus* spp. containing the *mecA* gene was detected in the water body receiving hospital wastewaters. Among the *mecA*

positive isolates were *S. cohnii*, a coagulase-negative staphylococci normally not considered as a pathogen in the clinical settings. All strains of the same *Staphylococcus* species displayed the same antibiotic resistance/susceptibility trend regardless of their isolation sources (clinical or environmental). This potentially shows that there may be a link between the various habitats. These findings on prevalence, diversity and multi-drug resistance staphylococci particularly CoNS in wastewater and receiving water sources is worrisome. Environmental water sources are used for agricultural, direct exposure (recreational) and drinking water production purposes. Finding these *Staphylococcus* species potentially from clinical origin in environmental water is cause for concern. These bacterial species are normally not tested for in microbiological water quality analyses. This raises the issue of regulations and testing regimes of environmental water that receives hospital wastewater. It calls for stringent regulations and proper treatment of hospital effluents prior disposal to receiving wastewaters. This would reduce the risks posed to human and animal health and safety and may as well also play an important role in mitigation of global antibiotic resistance, particularly of *mecA* associated resistance.

CHAPTER 4

ANTIBIOTIC RESISTANT *STAPHYLOCOCCUS* SPECIES FROM A WASTEWATER TREATMENT PLANT IN SOUTH AFRICA

4.1 Introduction

Due to increasing human population and urbanization as well as changing climatic conditions the challenge of water scarcity has heightened in many developed and developing countries (du Plessis 2019a). South Africa is no exception, and extended droughts in the catchment areas of reservoir dams in the Western and Eastern Cape, KwaZulu Natal and Northern Cape was responsible for major metropolitan cities to implement water restrictions (Botai et al. 2019; du Plessis 2019b). Alternative water sources, such as the reclamation of wastewater treatment plant (WWTP) effluent for various purposes become critical (Salgot & Folch 2018).

Treated wastewater effluents are usually discharged into receiving waters and reused for various purposes such as: preparing drinking water, agricultural irrigation and livestock water, recreation and industrial purposes (Angelakis et al. 2018). Effectively treated wastewaters are determined by their quality which are dependent on the physico-chemical properties (pH, temperature, electrical conductivity, various chemical constituents such as phosphates, nitrogen containing compounds, organic load metals etc.) and microbial indicators of faecal contamination, mostly *E. coli* (Jordaan & Bezuidenhout 2013). Indiscriminate discharge of poorly treated or untreated wastewater effluents are major contributors to surface water pollution

(Malassa et al. 2013). According to Börjesson et al. (2010) and Said et al. (2017), wastewaters are potential sources for the dissemination of antibiotic resistance bacteria (ARB) such as *Staphylococcus* species into natural water environments.

Studies have detected *Staphylococcus* species in wastewaters (Börjesson et al. 2009; Goldstein et al. 2012; Gómez et al. 2016). Porrero et al. (2016) reported the presence of *Staphylococcus aureus* in WWTP in Madrid, Spain while Faria et al. (2009) and Čuvalova et al. (2015) reported the survival of coagulase-negative *Staphylococcus* (CoNS) species in treated effluents and drinking water from Portugal and Slovak Republic, respectively. Specifically, Gómez et al. (2016) and Said et al. (2017) detected 5 coagulase-negative staphylococci (CoNS) - *S. lentus, S. cohnii, S. sciuri, S. haemolyticus* and *S. xylosus* in wastewaters. Börjesson et al. (2009) also identified *S. lentus, S. sciuri, S. cohnii* and *S. haemolyticus* in a municipal wastewater treatment plant. The coagulase test is an important distinguishing characteristic of staphylococci (Cheesbrough, 2006). Those that are coagulase-positive are generally regarded as *S. aureus* and are potential pathogens that are flagged for further diagnostic tests (Cheesbrough, 2006). The CoNS are generally regarded as non-pathogenic and are routinely disregarded in the clinical diagnostic sphere (Becker et al. 2014).

Staphylococcus species may exhibit resistance towards beta-lactam antibiotics such as ampicillin, methicillin, penicillin etc. (Thompson et al. 2012; Porrero et al. 2016; Said et al. 2017). The World Health Organization (WHO, 2017b) reported that in Africa, 80% of *Staphylococcus aureus* infections are methicillin resistant. Multi-drug

antibiotic resistance traits and antibiotic resistance genes (ARGs) such as in *S. aureus* and other CoNS species had been isolated from wastewaters (Börjesson et al. 2009; Thompson et al. 2012; Wan & Chou 2014; Said et al. 2017; Boopathy, 2017).

The confirmation of the *mecA* gene, has been the “golden standard” for detection of methicillin-resistant *S. aureus* (MRSA) worldwide (Yang et al. 2009; Igbinosa et al. 2016). The *nuc* gene detection is a confirmatory test for *S. aureus* strains while PVL is generally used as a marker for community acquired MRSA (Haddad & Moineau 2013; Gillen et al. 2015). The PVL gene is a virulence factor, which can enhance the ability of the bacterium to cause severe infections in human and animal hosts. *Spa typing is an investigation which could provide useful insight and information into the virulence potentials of S. aureus*. This test may further assist in the grouping of isolates into clonal lineages and *S. aureus* populations (Harmsen et al. 2003).

Occurrence of MRSA and genes in wastewater effluents discharged into water environments has therefore raised public health concerns due to likely threats posed to the human communities which could lead to community acquired MRSA (CA-MRSA) infections (Börjesson et al. 2009, 2010; Plano et al. 2011; Goldstein et al. 2012). In South Africa, there is limited data on the detection of *Staphylococcus* species in WWTPs and whether these make it into receiving water bodies as well as their persistence in these waters (Chidamba et al. 2016).

The aim of this study was to determine the prevalence of MRSA isolates and other staphylococci isolated from a South African wastewater treatment plant using standard protocols.

4.2 Materials and methods

4.2.1 Description and treatment processes at the wastewater treatment plant sampled

Water samples were collected from a wastewater treatment plant (WWTP) in the North-West Province of South Africa. From this plant, four sites were sampled, these were: inlet, primary effluent, secondary effluent and final effluent. The plant is a full scale-wastewater treatment plant which has a designed capacity of 45 000 m^3 per day with the potential of receiving wastewater streams from domestic, industrial, agricultural, abattoirs, hospital and storm water sources. The average flow to the works is 29 000 m^3 per day. The inlet receives raw sewage into the plant for treatment. The final effluent is chlorinated at 5 mg/L to reduce the *E. coli* levels to 0 cfu/100 ml and to reduce odour before discharge into receiving waters.

4.2.2 Sampling description

Samples were collected from the four sites in sterile 500 mL Schott glass bottles. Triplicate grab samples were collected. The wastewater samples were then transported and preserved under refrigeration conditions for microbiological analyses. The wastewater analyses were conducted within 12 h of collection.

4.3 Microbiological analysis of wastewater samples and preliminary identification of *Staphylococcus* species

Water samples collected were membrane filtered on sterile 0.45 µm filters. These were enriched in Bacto tryptic soy broth (soybean-casein digest medium; Becton Dickinson, US) and were later placed onto Mannitol salt agar (Biotec Laboratories, Kentford, UK). The resulting yellow colonies were presumed to be *S. aureus*. These were afterwards confirmed by culturing on MRSA CHROMagar base (CHROMagar™ MRSA-ITK Diagnostics BV, Uithoorn, The Netherlands). The putative MRSA produced characteristic purple color on the chromogenic agar. Gram staining was used to ensure that isolates were Gram-positive cocci and have characteristic cocci. *Staphylococcus aureus* and other *Staphylococcus* species were later confirmed by the coagulase and catalase tests using standard protocols (Cheesbrough, 2006; Igbinosa et al. 2016). All isolated and identified *Staphylococcus* spp. were subjected to antibiotic susceptibility testing using 13 antibiotics and the standard Kirby-Bauer's disk diffusion technique (CLSI, 2014).

4.3.1 16S rRNA gene-based identification of *Staphylococcus* isolates

The Nucleospin® tissue extraction kit (Macherey-Nagel, Düren, Germany) was used, according to the manufacturer's manual, to isolate genomic DNA. Briefly, 2 mL overnight broth cultures were centrifuged at 8000 rpm for 5 minutes at room temperature to harvest the cells. The supernatant was discarded, and pelleted cells were then resuspended in 100 µL T1 buffer. The Nucleospin® tissue extraction kit instructions were followed. Quality and integrity of extracted DNA products were

verified by micro-spectrophotometry and gel electrophoresis as described by Oladipo et al. (2018a).

4.3.2 PCR amplification

The C1000TM thermal cycler (Bio-Rad, Hercules, CA, USA) was used to perform PCR reactions. 16S rRNA gene amplification was conducted using primer sets and PCR conditions detailed in Table 7. Each PCR reaction included positive and negative controls as described by Oladipo et al. (2018b).

4.3.3 Sequencing of 16S rRNA genes

Purified PCR products were sequenced using the Big Dye terminator V. 3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) on a SeqStudio genetic analyzer and related software (Life Technologies, Holdings Pte Ltd, Singapore). Generated sequence electropherograms were inspected and then manually edited as described by Oladipo et al. (2018a). Edited sequences were aligned and compared against other sequences on the Basic Local Alignment Search Tool (BLAST) program alignment tool of the GenBank on the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The partial 16S rRNA sequences obtained from this study are available in the GenBank with assigned accession numbers: MF409347 - MF409381.

Table 7: Primers used for the identification of *Staphylococcus* species and the detection of marker genes

Primers	Primer sequence (5′–3′)	PCR Conditions	Size (bp)	References
27F 1492R	5′GAGTTTGATCATGGCTCAG3 5′GGTTACCTTGTTACGACTT3′ ′	1 cycle of 2min at 95°C; 35 cycles of 30 sec at 94°C; 30sec at 53°C for, 1min at 72°C; 1 cycle 10 min at 72°C	1500	Lane, 1991
spa 1095F new *spa* extend: f	5'-AGACGATCCWTCAGTGAGC-3' 5'-TAATCCACCAAATACAGTTGTACC-3'	1 cycle of 5mins at 94°C; 35 cycles of 45 sec at 94°C; 45 sec at 62; 90 sec at 72°C, 10 min at 72°C	200	Shopsin et al. 1999
mecA-F *mecA*-R	5′AACGATTGTGACACGATAGCC3′ 5′GGGATCATAGCGTCATTATC3′	1 cycle of 5min at 94°C; 35 cycles of 30s at 94°C; 30 sec at 55°C; 1 min at 72°C	527	Kumar et al. 2016
nuc-1 *nuc*-2	5′TCAGCAAATGCATCACAAACAG3′ 5′CGTAAATGCACTTGCTTCAGG3′	1 cycle of 5min at 94°C; 35 cycles of 30s at 94°C; 30 sec at 55°C; 1 min at 72°C	255	Othman et al. 2014
luk-F luk-R	5′ATCATTAGGTAAATGTCTGGCA TGATCC3′ 5′AGCATCAAGTGTATTGGATAGC AAAAGC3′	1 cycle of 4min at 94°C; 30 cycles of 45s at 94°C; 1min at 72°C; 1 cycle of 2min at 72°C	433	McClure et al. 2006

4.3.4 PCR amplification of *mecA*, *nuc a*nd *luk*-PVL genes in *Staphylococcus* spp.

To differentiate MRSA from other staphylococci the PCR amplification of the *mecA* gene (encoding for methicillin resistance), *nuc* gene that encodes for nuclease Staphylococcus species was conducted. Each of the PCR reaction contained 12.5 µL, 2x PCR Master mix (Thermos Scientific Technologies, Waltham, MA, USA), 50 ng DNA template, 5 µM each of the primers (forward and reverse) and nuclease-free water added to a final volume of 25 µL. Detailed information on the primers and conditions used are presented in Table 7. To determine if the PCRs worked, electrophoresis of the amplicons were performed using a 1% w/v agarose gel and conditions described in Oladipo et al. (2018a). Previously known positive genes of *mecA, nuc* and PVL and positive *Staphylococcus aureus* isolates were used as control strains.

4.3.5 DNA amplification and sequencing of the protein A (*spa*)

For amplification of the *Staphylococcus* repeat region, a PCR is performed in a total volume of 50 µl containing cleaned DNA, 200 µM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 10 pmol of each primer, 5 µl of 10-fold concentrated PCR Buffer II (Applied Biosystems), $MgCl_2$ 1.5 mM, and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems, Hitachi, Tokyo, Japan). Detailed information on the primers and conditions used are presented in Table 7. Sequencing of the protein A gene (*spa*) was carried out using the Big Dye terminator V. 3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) on a 3130 Genetic analyzer

(Applied Biosystems/Hitachi, Tokyo, Japan). The chromatograms obtained were analyzed with Ridom Staph Type software version 1.4 (RidomGmbH, Sedanstr, Germany; http://*spa*.ridom.de/index.shtml). *Spa* types were deduced by the differences in number and sequence of *spa* repeats with the BURP algorithm (Ridom GmbH, Sedanstr, Germany) and the Ridom *spa* Server database. *Spa* types with less than five or equal to 5 repeat units were excluded (Koreen et al. 2004; Montanaro et al. 2016).

4.3.6 Antimicrobial susceptibility testing

All isolated and identified *Staphylococcus* species were subjected to antibiotic susceptibility testing of 13 antibiotics using the standard Kirby-Bauer's disk diffusion technique (CLSI, 2014). The specific antibiotics selected are beta-lactam antibiotics, a class of antibiotic that contain a beta-lactam ring in their molecular structures, that usually acts by inhibiting the synthesis of bacterial cell walls. This includes (ampicillin, cloxacillin, amoxicillin, and methicillin). Others were macrolides (erythromycin, azithromycin), aminoglycosides (Gentamicin), carbapenems (imipenem), and glycopeptides (vancomycin). Methicillin was used to determine the antibiotic sensitivity of *Staphylococcus aureus* to other penicillins facing β-lactam resistance.

Antimicrobial resistance data were analyzed using WHONET 2017 software V 5.6 (WHO; http://www.whonet.org/software.html). Multi-resistant was determined based on resistance to at least two classes of antibiotics. The multiple antibiotic resistance

(MAR) index for the dominant isolates (*S. aureus, S. lentus* and other *Staphylococcus* spp.) at the influent and effluent compartments of the WWTP was calculated and interpreted according to Krumperman (1983) using the formula:

$$\boldsymbol{MAR\ index\ per\ compartment} = \frac{number\ of\ isolates\ in\ a\ specific\ sample\ population\ resistant\ to\ antibiotics}{(number\ of\ antibiotics\ tested)\ x\ (total\ number\ of\ organisms\ in\ sample)}$$

*MAR index values > 0.2 indicate high risk source of contamination.

4.3.7 Statistical analyses

A statistical difference of MAR index of the *Staphylococcus* spp. was done using one-way analysis of variance (ANOVA) at 5% level of significance using IBM SPSS Statistics 23 (IBM Corporation, Armonk, NY, USA). Multiple sequence alignment was performed using MUSCLE (Edgar, 2004) integrated into Molecular Evolutionary Genetics Analysis (MEGA) V. 7.0 (Http://www.megasoftware.net/; Kumar et al. 2016).

4.4 Results

4.4.1 Prevalence and distribution of *Staphylococcus* strains from the wastewater treatment plant

Thirty-five *Staphylococcus* isolates belonging to eight species were identified. The most prevalent were: *Staphylococcus aureus* (34.0%), *S. lentus* (29.0%), *S. cohnii*

(11.0%) and *S. sciuri* (9.0%). Other isolates were *S. haemolyticus* and *S. xylosus* (6.0%) each and *S. nepalensis* and *S. arlettae* with 3% each.

Twelve of the staphylococci isolates were from the influent, 8 (22.86%), primary effluent, 6 (17.14%), secondary effluent and 9 (25.71%) the final effluent (Figure 4a). Furthermore, the distribution of the species across the four sampling points showed that *S. aureus* and *S. lentus* were isolated from all the sampling compartments (Figure 4b). In addition, *S. arlettae* and *S. nepalensis* were exclusively isolated from the final effluent point while *S. xylosus* was isolated from the inlet and the final effluent.

4.4.2 Antibiotic resistance and susceptibility patterns of *Staphylococcus* species

All thirty-five *Staphylococcus* species isolated from the wastewater treatment plant were subjected to 13 antibiotics at recommended concentrations for prove of resistance or susceptibility (Table 8). All the isolates were resistant to several of the 13 antibiotics tested. Resistance to the various antibiotics were in the following order: methicillin (91%), ampicillin (89%), ciprofloxacin (86%), amoxycillin (80%), ceftazidine (74%) and cloxacillin (71%). Others to which isolates were also resistant were cefuroxime and azithromycin (43%), ofloxacin and vancomycin (40%), Gentamicin (37%), imipenem (29%) and erythromycin (23%). About 70% (24 out of 35) of the isolates were resistant to at least 7 of the 13 antibiotics tested with *S. aureus*, *S. lentus* and *S. scuiri* resistant to 10 of the 13 antibiotics (Table 8).

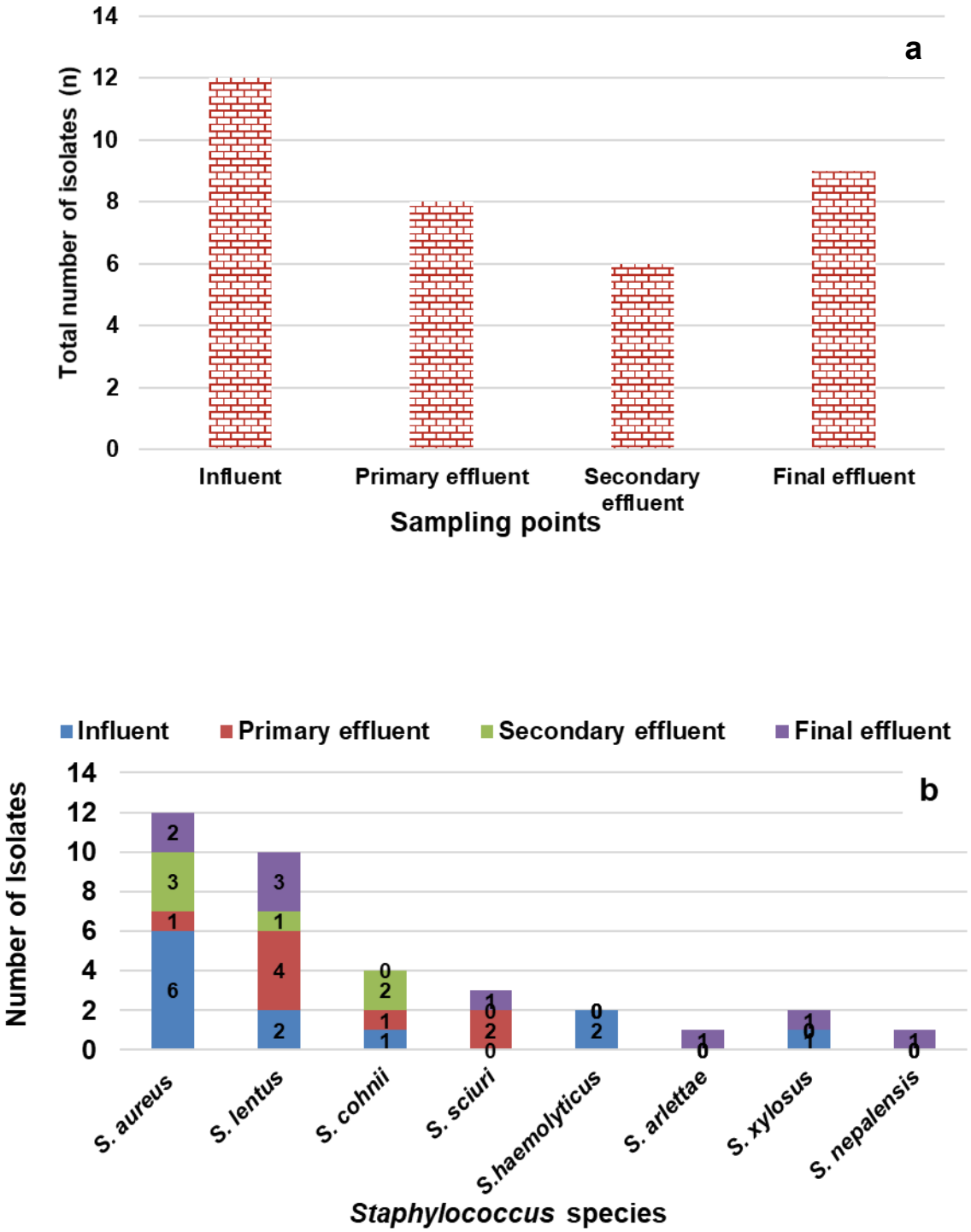


Figure 4: Distribution of *Staphylococcus* species according to (a) the sources of isolation and (b) number of isolates

Table 8: Identification, source, antibiotic resistance and susceptibility pattern of *Staphylococcus* species isolated from the wastewater treatment plant

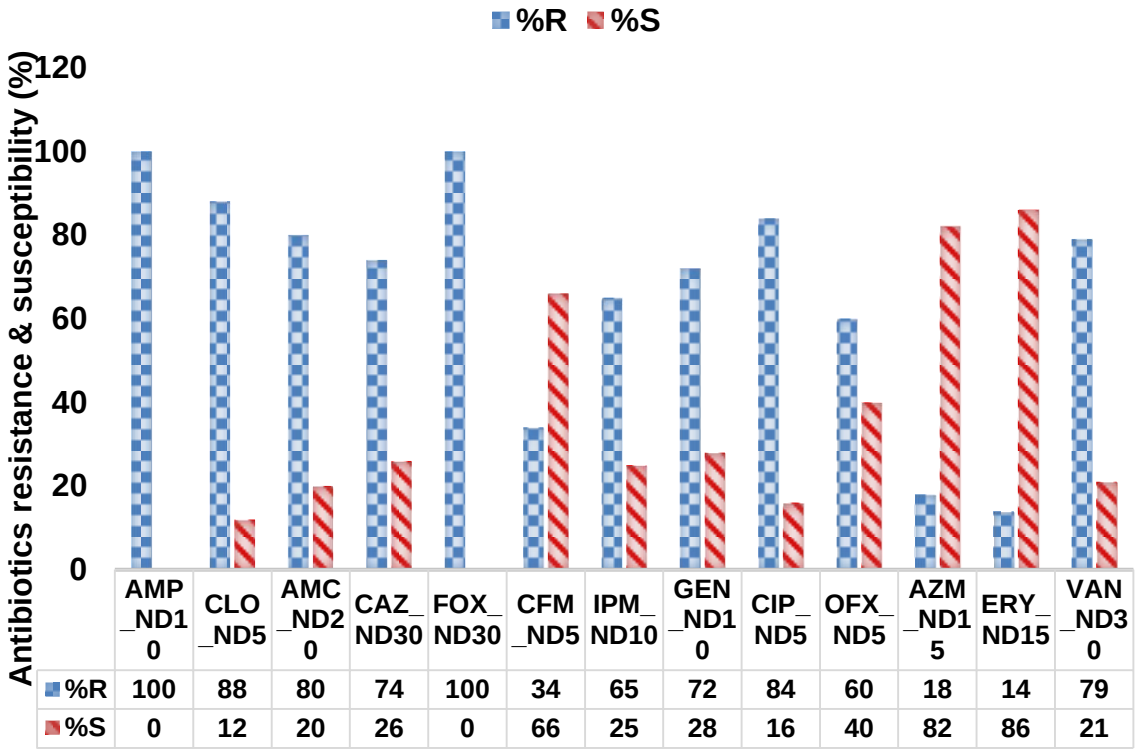
S/N	Isolates	Accession Number	Source of Isolation	Antibiotics Tested														Total	
				AMP	CLO	AMC	CAZ	FOX	CFM	IPM	GEN	CIP	OFX	AZM	ERY	VAN	R	S
	S. aureus	MF409348	Inlet	+	+		+	+	+		+	+	+	+		+	10	3
	S. aureus	MF409349	Inlet	+	+	+	+	+	+		+	+	+			+	10	3
	S. aureus	MF409350	Inlet	+	+	+	+	+						+	+		6	7
	S. aureus	MF409351	Inlet	+	+	+	+	+		+	+	+		+	+		10	2
	S. aureus	MF409352	Inlet	+	+	+		+		+		+	+	+	+		9	4
	S. aureus	MF409356	Inlet		+	+	+	+	+	+	+	+	+	+			10	3
	S. aureus	MF409359	Primary Effluent	+	+	+	+	+		+		+	+				8	5
	S. aureus	MF409367	Secondary Effluent	+		+	+	+		+		+	+			+	8	5
	S. aureus	MF409372	Secondary Effluent	+	+	+	+	+				+					6	7
	S. aureus	MF409370	Secondary Effluent	+		+	+	+	+		+	+			+		8	5
	S. aureus	MF409373	Final Effluent	+		+	+	+	+			+					6	7
	S. aureus	MF409381	Final Effluent	+	+	+	+	+	+			+	+			+	9	4
	S. arlettae	MF409378	Final Effluent	+		+	+	+				+			+	+	7	6
	S. cohnii	MF409347	Inlet	+	+	+			+		+	+		+		+	8	5
	S. cohnii	MF409364	Primary Effluent	+	+			+	+			+				+	6	7

S. cohnii	MF409368	Secondary Effluent	+	+	+	+	+	+					+				7	6
S. cohnii	MF409369	Secondary Effluent	+			+	+			+			+				5	8
S. haemolyticus	MF409353	Inlet	+	+						+	+						4	9
S. haemolyticus	MF409358	Inlet	+	+	+	+	+	+	+		+						8	5
S. lentus	MF409355	Inlet		+	+	+	+		+		+	+					7	6
S. lentus	MF409357	Inlet	+	+	+	+	+	+				+	+				8	5
S. lentus	MF409360	Primary Effluent	+	+	+	+	+		+		+		+				8	5
S. lentus	MF409361	Primary Effluent	+	+		+	+		+	+	+	+	+			+	10	3
S. lentus	MF409365	Primary Effluent	+		+		+	+			+			+			6	7
S. lentus	MF409366	Primary Effluent	+		+		+			+	+					+	6	7
S. lentus	MF409371	Secondary Effluent		+	+	+	+				+		+				6	7
S. lentus	MF409375	Final Effluent	+	+	+		+	+			+		+				7	6
S. lentus	MF409380	Final Effluent	+	+		+	+				+					+	6	7
S. lentus	MF409377	Final Effluent	+	+	+		+				+						5	8
S. nepalensis	MF409379	Final Effluent	+	+	+	+	+	+					+			+	8	5
S. sciuri	MF409362	Primary Effluent	+	+		+	+			+	+	+	+			+	8	5

S. sciuri	MF409363	Primary Effluent	+	+	+	+	+	+		+	+	+			+	10	3
S. sciuri	MF409374	Final Effluent	+		+		+	+			+	+			+	7	6
S. xylosus	MF409354	Inlet	+	+	+	+					+	+		+		7	6
S. xylosus	MF409376	Final Effluent			+	+	+		+	+	+			+		7	6
Total Resistance obtained per antibiotic			**31**	**25**	**28**	**26**	**32**	**16**	**10**	**13**	**30**	**14**	**15**	**8**	**14**		

Notes: R indicates 'resistant' and S 'susceptible'. AMP (Ampicillin) (10 µg), CLO (Cloxacillin) (5 µg), AMC (Amoxycillin) (20 µg), CAZ (Ceftazidine) (30 µg), FOX (Methicillin) (30 µg), CFM (Cefuroxime) (5 µg), IPM (Imipenem) (10 µg), GEN (Gentamicin) (10 µg), CIP (Ciprofloxacin) (5 µg), OFX (Ofloxacin) (5 µg), AZM (Azithromycin) (15 µg), ERY (Erythromycin) (15 µg) and VAN (Vancomycin) (10 µg).

It was observed that all the *S. aureus* strains isolated from the treatment plant regardless of their site of isolation were all resistant to methicillin and ampicillin (Table 8, Figure 5). *S. aureus* decreased from 50 to 17 % as treatment progressed from influent to final effluent point in the WWTP. Furthermore, it was observed that 50% of *S. aureus* were resistant to imipenem and erythromycin.



Key: R indicates 'resistant' and S 'susceptible'. AMP (Ampicillin) (10 µg), CLO (Cloxacillin) (5 µg), AMC (Amoxycillin) (20 µg), CAZ (Ceftazidine) (30 µg), FOX (Methicillin) (30 µg), CFM (Cefuroxime) (5 µg), IPM (Imipenem) (10 µg), GEN (Gentamicin) (10 µg), CIP (Cipoloxacin) (5 µg), OFX (Ofloxacin) (5 µg), AZM (Azithromycin) (15 µg), ERY (Erythromycin) (15 µg) and VAN (Vancomycin) (30 µg).

Figure 5: Antibiotic resistance profile of the *Staphylococcus aureus* strains isolated from a wastewater treatment plant

4.4.3 Multiple antibiotic resistance (MAR) index and MAR phenotypes

The MAR indices and phenotypes of the 2 dominant isolates (*S. aureus* and *S. lentus*) are presented in Table 9. For *S. aureus* strains, the MAR index at the influent and effluent were 0.705 and 0.615, while for *S. lentus*, 0.577 and 0.692 respectively. Furthermore, the MAR index of the other *Staphylococcus* spp. isolates comprising of *S. arlettae, S. haemolyticus, S. sciuiri* and *S. cohnii* (grouped as *Staphylococcus* spp.), were calculated as 0.519 for influents and 0.558 for effluents. There were no significant differences ($p > 0.05$) within the sampling sites or among the different species. Also, the dominant phenotypes exhibited diverse patterns (Table 9).

The MAR phenotype among *S. aureus* isolates from the influent showed diverse resistance to beta-lactam antibiotics and cephalosporins. The phenotype Amp-Clo-Amc-Caz-Fox and Amp-Clo-Amc-Caz-Fox-Cfm were present in all the *S. aureus*, *S. lentus* and *Staph* spp. isolated from the WWTP. These were observed in 16.7, 50.0% and 33.3% of the isolates from the influent chambers, respectively. However, at the effluent, the MAR phenotypes, Amp-Clo-Amc-Caz-Fox-Cfm-Cip-Ery-Van (*S. aureus*), Amp-Clo-Amc-Caz-Fox-Cip-Van (*S. lentus*) and Amp-Clo-Amc-Caz-Fox-Cfm-Azm (*Staph*. spp.) were observed in 50%, 25% and 33.3% of the isolates, respectively.

Table 9: MAR phenotypes and MAR index among the staphylococci species from the WWTP

Isolates	Source(s)	MAR phenotype	No Observed	%	Group MAR Index
S. aureus	Influent	AMP-CLO-CAZ-FOX-CFM-GEN-CIP-OFX-AZM-VAN	1	16.7	0.705
	n=6	AMP-CLO-AMC-CAZ-FOX-CFM-GEN-CIP-OFX-VAN	1	16.7	
		AMP-CLO-AMC-CAZ-FOX -AZM-ERY	1	16.7	
		AMP-CLO-AMC-CAZ-FOX-IMP--GEN-CIP-AZM-ERY	1	16.7	
		AMP-CLO-AMC-FOX-IPM-CIP-OFX-AZM-ERY	1	16.7	
		CLO-AMC-CAZ-FOX-CFM-IPM-GEN-CIP-OFX-AZM	1	16.7	
	Effluent	AMP-AMC-CAZ-FOX-CFM-CIP-ERY-VAN	1	50.0	0.615
	n=2	AMP-CLO-AMC-CAZ-FOX-CFM-CIP-ERY-VAN	1	50.0	
S. lentus	Influent	CLO-AMC-CAZ-FOX-IPM-CIP-OFX-AZM	1	50.0	0.577
	n=2	AMP-CLO-AMC-CAZ-FOX-CFM-OFX-AZM	1	50.0	
	Effluent	AMP-CLO-AMC-FOX-CFM-CIP-AZM	1	25.0	0.692
	n=4	AMP-CLO-AMC-CAZ-FOX-CIP-VAN	1	25.0	
		AMP-CLO-AMC-FOX-CIP	1	25.0	
		AMP-AMC-CLO-AMC-CAZ-CIP-OFX-ERY	1	25.0	
Other	Influent	AMP-CLO-GEN-CIP	1	33.3	0.519
Staph. species	n=3	AMP-CLO-AMC-CAZ-FOX-IPM-CIP	1	33.3	
		AMP-AMC-CLO-AMC-CAZ-CIP-OFX-ERY	1	33.3	
	Effluent	AMP-AMC-CAZ-FOX-CIP-ERY-VAN	1	33.3	0.558
	n=3	AMP-CLO-AMC-CAZ-FOX-CFM-AZM	1	33.3	
		AMC-CAZ-FOX-IPM-GEN-CIP	1	33.3	

4.4.4 Detection of resistance and virulence genes and in *Staphylococcus* species

Seventy-seven percent of the isolates (27 of 35) were *mecA* positive. Among the 12 MRSA isolates, 11 were *mecA* positive. Other *Staphylococcus* species that tested positive for *mecA* were: *S. lentus, S. sciuri, S. cohnii, S haemolyticus* and *S. xylosus*. Two (5.8%) *S. aureus* isolates were also positive for the *luk*-PVL gene, while the *nuc* gene was detected in all 12 *S. aureus* isolates (Table 10).

4.4.5 Detection of protein A (*spa*) types in *Staphylococcus aureus* strains

Seven different *spa* types were detected from the confirmed *S. aureus* strains recovered from the WWTP in this study (Table 11). *Spa* types t061, t6578 and t091 were detected from the inlet, t447 from primary effluent, t7835 from secondary effluents and *spa* types t091, t5126 final effluent. The most frequent *spa* type t091 (16.7%) was observed from the inlet and secondary effluent (Table 11).

4.5 Discussion

Several researchers in South Africa have investigated links between WWTP effluents and receiving waters. These have focused on physico-chemical properties (Agoro et al. 2018; Salvador-Oke et al. 2018) or microbial parameters (microorganisms) such as *Vibrio* (Okoh & Igbinosa 2010) and *Aeromonas* species (Igbinosa & Okoh 2012; Coetzee et al. 2017; Mann et al. 2019).

Table 10: Detection of resistance and virulence genes in *Staphylococcus* species from the wastewater treatment plant

Strains isolated	*mecA*	Site(s) with no *mecA* detected	*luk*-PVL	Site(s) with *luk*-PVL *nuc* detected	
S. aureus (12)	11	Final Effluent	2	Inlet and Secondary effluent	12
S. lentus (10)	7	Final Effluent (2) Secondary Effluent (1)	-	-	-
S. sciuri (3)	3	-	-	-	-
S. cohnii (4)	3	Secondary Effluent	-	-	-
S haemolyticus (2)	2	-	-	-	-
S. xylosus (2)	1	Final Effluent	-	-	-
S. arlettae (1)	0	Final Effluent	-	-	-
S. nepalensis (1)	0	Final Effluent	-	-	-
Total 35	**27**		**2**		**12**

Table 11: Detection of *mecA*, PVL and *nuc* genes and *spa* types of *S. aureus* isolates from the wastewater treatment plant

Source(s)	Isolates (n=12)	*mecA*	PVL	*nuc*	*spa* type	*spa* repeats	Sequence type (ST)
Inlet	*S. aureus*	+	-	+	*t061	09-02-16-13-34-17-34-16-34	ND
Inlet	*S. aureus*	+	-	+	UNK	23-21-17-34-12-23-02-12-23	ND
Inlet	*S. aureus*	+	-	+	*t6578	26-23-13-21-17-34-33-34	ST-398
Inlet	*S. aureus*	+	+	+	UNK	13-12-16-34-33-13	ND
Inlet	*S. aureus*	+	-	+	*t091	07-23-21-17-34-12-23-2-12-23	ND
Inlet	*S. aureus*	+	-	+	UNK	34-34-34-34-34-17-34-16-13	ND
P. effluent	*S. aureus*	+	-	+	*t447	26-23-34-17-20-17-12-17-16	ND
S. effluent	*S. aureus*	+	-	+	*t7835	7-82-21-17-34-34-16-34-33-13	ST-15
S. effluent	*S. aureus*	+	-	+	UNK	34-34-12-12-23-2-12-23	ND
S. effluent	*S. aureus*	+	+	+	*t657	23-13-21-17-34-33-34	ST-772
F. Effluent	*S. aureus*	+	-	+	*t091	07-23-21-17-34-12-23-2-12-23	ST-7
F. Effluent	*S. aureus*	-	-	+	*t5126	26-23-12-34-34-12-12-23-12-23	ND

Notes: The *spa* types in this study are asterisked (*). In some cases, the Clonal Complex was assumed according to the *spa* type in this case it is bolded, UNK denotes 'unknown' while means 'Not Done'. The isolate sources are indicated as: P. effluent (Primary effluent), S. effluent (Secondary effluent) and F. effluent (Final effluent) while the sequence type denoted as ND means "Not Detected"

The present study was designed to assess the prevalence of *Staphylococcus* species (*S. aureus* – CoPs and CoNS), their antibiotic resistance patterns and detection of resistance and virulence genes and *spa* types in the recovered *Staphylococcus* species from a WWTP in South Africa.

In the present study, eight *Staphylococcus* species (1 CoPS and 7 CoNS) were isolated and identified across the 4 sampling points of a wastewater treatment plant in South Africa. These are: *Staphylococcus aureus, S. arlettae, S. cohnii, S. haemolyticus, S. lentus, S. nepalensis, S. sciuri* and *S. xylosus*. The *Staphylococcus* spp. demonstrated multidrug resistance (MDR), high MAR indices (> 0.2), various MAR phenotypes, detection of *mecA* resistance gene and the *nuc* and *luk*-PVL virulence gene and *spa* types confirmed in *S. aureus* strains.

Similar results confirming the presence of *Staphylococcus* species in wastewater plant effluent have been demonstrated by previous studies. Gómez et al. (2016) detected multi-drug resistant *Staphylococcus* spp. (*S. aureus, S. lentus, S. cohnii, S. sciuri and S. haemolyticus*) in urban wastewater treatment plant in Spain. Furthermore, Goldstein et al. (2012) and Maimon et al. (2014) published the occurrence of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) in wastewater effluents from greywater, intended for reuse.

All *S. aureus* isolates in the present study were resistant to ampicillin and methicillin. This appears to be a constant observation amongst previous studies. Thapaliya et al. (2017) also recorded the prevalence of *S. aureus* and their antibiotic-resistance in wastewater treatment plant sites. Their study investigated the prevalence and molecular characteristics of *S. aureus* and MRSA in freshwater recreational beaches and water samples collected from 10 beaches in Northeast Ohio, USA. The results from their study revealed overall prevalence of *S. aureus* (22.8%) and PVL genes (21.4%), with 27 different *spa* types identified. In addition, 34.3% of the isolates showed oxacillin resistance while, all the isolates showed 100% resistance to penicillin. However, our present study revealed a higher prevalence of *S. aureus* (34.3%) with a prevalence of 5.71% for PVL genes and 7 *spa* types being identified among *S. aureus* isolates.

Results of Thompson et al. (2012) and Porrero et al. (2016) showed that 83% and 96% MRSA isolates, respectively, from urban effluents were resistant to ampicillin. The presence of MRSA and MSSA in river water and urban effluents was studied to analyze the *S. aureus* population and determine the genetic diversity. From their study, MRSA population in urban effluents and river water was 67.6% and 82.4% respectively, while *spa* type t067 was the predominant MRSA genotype detected. This differs from our study in that we only recorded an MRSA prevalence of 35%, in a WWTP with *spa* type t091 being dominant. Said et al. (2017) reported that most of the *S. aureus* in their study showed resistance to penicillin while Goldstein et al. (2012) also demonstrated that 93% of MRSA isolates recovered from wastewaters in United States were multidrug

resistant. The study by Goldstein et al. (2012), examined the occurrence of MRSA and methicillin-susceptible *S. aureus* (MSSA) at U.S. wastewater treatment plants. The study and findings were similar to ours since the presence of MRSA in a WWTP was investigated. Results from their study also showed 10 of 12 (83%) influent samples being MRSA-positive, while only one of 12 (8%) effluent samples was MRSA-positive.

In the present study it was shown that the *mecA* resistance gene was detected in 11 of the 12 *S. aureus* strains recovered from the WWTP sampled. This finding is corroborated by several previous studies (Wan & Chou, 2014; Boopathy, 2017). While Boopathy (2017) established the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) in a rural sewage treatment plant, Wan & Chou (2014) examined the spreading of β-lactam resistance gene (*mecA*) and methicillin-resistant *Staphylococcus aureus* through municipal and swine slaughterhouse wastewaters.

The *nuc* gene was detected in all *the S. aureus* strains isolated from this study. However, the PVL virulence gene was detected in very few isolates. A similar study of clinical isolates (Von-Eiff et al. 2004) examined the prevalence of genes encoding for members of the staphylococcal leukotoxin family of *Staphylococcus aureus*. Their findings revealed 0.9 to 1.4% detection of PVL virulence gene.

The occurrence of CoNS in WWTPs has also been well documented. Faria et al. (2009) reported the survival of CoNS in treated effluents. On the other hand, Čuvalova et al. (2015) demonstrated that CoNS also occurred in drinking water. Of the 7 CoNS species identified in this study, Gómez et al. (2016) and Said et al. (2017) detected 5 species in their studies that focused on wastewater samples. These species included: *S. cohnii, S. haemolyticus, S. lentus, S. sciuri, and S. xylosus* from wastewater samples. Börjesson et al. (2009) recovered *S. cohnii, S. haemolyticus S. lentus a*nd *S. sciuri* from a municipal wastewater treatment plant. Antibiotic resistance by CoNS had also been documented. This was the case in the present study and several previous studies. Said et al. (2017) reported that CoNS isolated from wastewaters in Tunisia were resistant to several classes of antibiotics including beta-lactam antibiotics. Previously, Schwartz et al. (2003), had reported the occurrence of methicillin-resistant CoNS from wastewater environments. The detection of *mecA* resistance gene in CoNS has also been reported in recreational waters, community and hospital wastewaters (Börjesson et al. 2009; Fogarty et al. 2015) and in other surface waters (Seyedmonir et al. 2015). Finding CoNS strains in the wastewater from the present North West Province of South Africa is thus not extraordinary.

The multiple antibiotic resistance (MAR) indices of all 35 (100%) *Staphylococcus* spp. in our study exceeded the 0.2 value associated with highly antibiotic resistance strains. In a previous study (Oladipo et al. 2019, Chapter 3 on this study), very high MAR indices (> 0.2) were also recorded for *S. aureus* isolates from clinical and environmental

sources. Our findings in the present study indicates that the presence of *Staphylococcus* spp. exhibiting antibiotic resistance and harbouring environmentally relevant virulence genes are of particular concern due to the possible link of community acquired MRSA and wastewater recycling for domestic, agricultural and industrial use. The frequencies of resistance of *S. aureus* to beta-lactams antibiotics (Amp-Clo-Amc-Caz-Fox) were high at all our sampling sites (inlets, primary and secondary effluents and final effluent). Studies have shown *S. aureus* resistance to antibiotics such as penicillins, amoxicillin and/or ampicillin have been isolated from both treated and untreated wastewater (Sahlstrom et al. 2004). In a previous study carried out in the United States, increase percentages of Ery-, Amp-, and Pen- resistant were also reported among *Staphylococcus spp.* isolated from a WWTP (Goldstein et al. 2012).

Seven distinct *spa* types were identified in this study with t091 being the most prevalent. Finding such a variety of *spa* types is a potential indication of diverse sources of isolation and that these could be from different geographical locations. This was also observed in a study in Nigeria (O'Malley et al. 2015; Ayeni et al. 2018) where *spa* type t091 was confirmed in nasal samples of clinical and poultry sources. In addition, Ilczyszyn et al. (2016) reported occurrence of *spa* type t091 amongst 5-year-old and younger patients in Poland. However, no data in searched databases could be found for a South African study on MRSA that had documented *spa* type t091 being associated with wastewaters.

The *spa* type t7835 had been affiliated with MRSA from clinical isolates in Nigeria (Kolawole et al. 2013), while *spa* type t447 had been reported in Netherlands and Spain. Also, *spa* type t6578 had been identified among swine (LA-MRSA) in Spain and United States as ST398 (CC398), and subsequently detected in several companion and food-chain animals and humans (de Boer et al. 2009). According to Smith et al. (2009), ST398 (CC398) has been well reported as a cause of livestock-associated (LA)-MRSA in Europe, while in Australia (Price et al. 2010) and the Americas (Grema et al. 2015) ST398 had been confirmed as a cause of LA-MRSA. *Spa* type t5126 had been identified in MRSA strains in Spain, USA, Germany and France (https://*spa*.ridom.de/*spa*-t5126.shtml) while *spa* type t061 had been associated with MRSA in the UK, Germany and United States (Von-Eiff et al. 2004). Notably, *spa* type t657, sequence type (ST) 772 was reported in this study among the PVL positive strains. This sequence type had been linked to community outbreak of CA-MRSA infections in some parts of the world e.g. India (D'Souza et al. 2010) and Ireland (Edmundson et al. 2011).

In this study, *S. aureus* constituted 34% of the total recovered *Staphylococcus* spp. which decreased as treatment progressed from influent to final effluent point. Similarly, studies conducted in Sweden, Spain and the USA respectively reported 50-55% prevalence of *S. aureus* in WWTPs with decreased prevalence as treatment progressed (Börjesson et al. 2009; Goldstein et al. 2012; Gómez et al. 2016). Although, this study reported a higher number of isolates being recovered in the final effluent, 75% of these

isolates did not carry the *mecA* resistance gene. This observation could potentially be similar to the findings of Mao et al. (2015) that also reported a reduction of antibiotics resistance genes (ARGs) and *mecA* gene from raw influent point to the effluent.

In this study three CoNS - *S. nepalensis, S. arlettae a*nd *S. cohnii* from the WWTP which have not been widely reported was detected. Of these, *S. cohnii* carried the *mecA* resistance gene. Nováková et al. (2006), had earlier isolated *S. nepalensis* from human urine, *S. arlettae* from textile effluents (Elisangela et al. 2008) while *S. cohnii* had been recovered from wastewater samples (Börjesson et al. 2009; Gómez et al. 2016; Said et al. 2017). *Staphylococcus cohnii* is known for its associations with nosocomial infections (Chen et al. 2015) and had also been confirmed in infections in animals (Sousa et al. 2014).

Previously, CoNS had been regarded as non-pathogenic since most of these species are established by association between humans and animals (Fitzgerald & Penades, 2008; Otto, 2010). However, their antibiotics resistance traits, possession of resistance and virulence genes reveal evidence that these species could have detrimental human health outcomes and should therefore be studied more closely.

This study confirmed the presence of staphylococci in the final effluent after chlorination. This may be due to staphylococci being resistant to chlorine or chlorine

disinfection is insufficient. WHO (2017) had reported chlorine resistance of staphylococci. Previous studies (Huang et al. 2011; Shi et al. 2013; Mao et al. 2015), reported on antibiotic resistance bacteria that are also resistant to chlorination. However, Tolba et al. (2008) documented that MRSA and other staphylococci could be eliminated by proper chlorination.

4.6 Conclusions

The present study demonstrates that environmental waters that receive WWTP effluent could be contaminated with MRSA and other potential pathogenic staphylococci. These findings indicate the possibility of treated wastewaters being a source for the dissemination of *Staphylococcus* species, their resistance and virulence genes to the environment which could have detrimental health impacts on the downstream users and consumers. The detection of a large proportion of MAR isolates in the present study is a cause for concern as this could pose health risks to humans and animals via resistant genetic elements that could be transferred from these isolates to other bacteria also of clinical importance. Therefore, a more effective treatment plan or treatment modification procedures of wastewaters may therefore be crucial, especially if the water is to be reused. The findings of the present study are aspects that the managers of wastewater treatment plants, policy formulators, down-stream users etc. should consider.

CHAPTER 5

GENOMIC CHARACTERIZATION OF ENVIRONMENTAL AND CLINICAL STAPHYLOCOCCI ISOLATES

5.1 Introduction

Coagulase-negative *Staphylococcus* species (CoNS) have traditionally been considered as commensals, however, many CoNS species are now being recognised as potential opportunistic human pathogens (Becker et al. 2017; Nnadozie & Odume, 2019). A number of studies have reported CoNS species being the most frequently isolated organism from bloodstream infections in intensive care units (Banerjee et al. 1991; Wisplinghoff et al. 2004). CoNS species are considered less virulent than *Staphylococcus aureus;* however, they have been shown to be an important reservoir of antibiotic resistance and virulence genes (Ekwanzala et al. 2018; Oladipo et al. 2019, Chapter 3 on this study). These could be transferred to closely related species such as *S. aureus*, augmenting the evolution and emergence of successfully clones of methicillin-resistant *S. aureus* (MRSA) (Otto, 2013). For example, evidence of extensive mobile genetic elements (MGE) sharing between *S. epidermidis* and *S. aureus* in particular SaPIn1 elements has been reported (Méric et al. 2015). MRSA are characterized by altered penicillin–binding protein (PBP2a) which has a low affinity for methicillin rendering the drug ineffective (Sabath & Finland, 1962; Eady & Cove, 2003; Turner et al. 2019). Moreover, MRSA are multidrug resistant and are resistant to the last resort ß-lactam, armamentarium (Turner et al. 2019). As a result, costs, length of hospital stay as well as mortality resulting from infections associated with MRSA are

unsurprisingly high (Lee et al. 2013; Gajdács, 2019). In addition to aiding through horizontal gene transfer, CoNS species have also evolved or acquired an array of virulence factors and antibiotic resistance genes (Becker et al. 2014). Outbreaks of multidrug antibiotic-resistant CoNS have been reported (Wang et al. 1999; Brito et al. 2009; Mazzariol et al. 2012), including methicillin resistance. Methicillin-resistant CoNS (MR-CoNS) could have high genetic diversity in genes conferring methicillin resistance (Miragaia et al. 2005; Zong et al. 2011).

For effective colonisation of the host, pathogens require an arsenal of strategies to enable adherence, persistence, aggression and evasion of innate and adaptive immunity. These strategies have been well studied for *S. aureus* (Becker et al. 2014). It has been demonstrated that *S. aureus* autolysin (Alt) mediate adherence of *S. aureus* to polystyrene surfaces. Adherence is an important role in initial colonization and biofilm formation (Heilmann et al. 1997; Biswas et al. 2006; Chandni et al. 2018). In addition, there are also a number of proteins such as fibronectin binding proteins (FnBPs), clumping factors (ClfA and ClfB) which also aid the initial surface adhesion and colonisation (ONeil et al. 2008; Foster, 2016). Homologous Atl protein as well as homologues of other surface adhesion proteins such as elastin-binding protein were reported for some CoNS (Hell et al. 1998; Allignet et al. 2001; Yokoi et al. 2008; Bourgeois et al. 2009; Gill et al. 2005), suggesting their ability to colonise hosts, an initial step of pathogenesis of staphylococci. Another factor essential for pathogenesis for *S. aureus* is internalisation by nonprofessional phagocytes and the ability for

persistence in host cells. These mechanisms have also been demonstrated in some CoNS (Khalil et al. 2007; Szabados et al. 2008; Hirschhausen et al. 2010).

Wastewater environments may play a significant role in the development and dissemination of antibiotic resistance (Martinez, 2006; Börjesson et al. 2010). This is mainly due to the release of antimicrobials including antibiotics and associated antibiotic resistant organisms into the environmental wastewaters. *mecA* a gene that encodes methicillin resistance in *Staphylococcus* species have been detected in hospital and environmental wastewaters (Volkmann et al. 2004; Börjesson et al. 2009; Oladipo et al. 2019, Chapter 3 on this study), thus the potential of wastewaters as a source for development and distribution of MRSA and MR-CoNS cannot be ignored.

Whole genome sequencing (WGS) has become a vital tool for comparative genomic studies, genomic characterisation of organisms of interest. WGS studies have been applied to characterise the virulence, antibiotic resistance, barriers to horizontal gene transfers including some CoNS (Zhang et al. 2003; Kuroda et al. 2005; Takeuchi et al. 2005, Gill et al. 2005; Heilbronner et al. 2011). The WGS was used to unveil the genomic characteristics of clinical and environmental CoNS isolates from Nigeria and environmental isolates from South Africa.

The main aim of this was to explore the virulent determinants and antibiotic resistance genes associated with the isolates. Distinct geographic locations improve the

comparative genomics of this study. In addition, we also aimed to compare our newly assembled genomes with some of the similar species available in public database including *S. aureus*.

5.2 Materials and methods

5.2.1 Sample collection

Clinical samples which comprised wound swabs and urine samples were collected from the Microbiology Laboratory Unit of an Hospital in Ile-Ife, Nigeria (West Africa). Environmental samples were collected from wastewater treatment plants within the same geographical location as the clinical samples. For comparative sake, environmental samples were also collected from a wastewater treatment plant in North-West, province, South Africa, a distinct geographical location (Southern Africa). Supplementary Table 1 provides information of the samples code, sample type (clinical/environmental) as well as the geographical location from which the samples were collected. Preparation and preliminary identification of the samples were done as described by Oladipo et al. 2019, (Chapters 3 & 4).

5.2.3 Genomic DNA extraction and sequencing library preparation

Bacterial DNA was extracted from overnight broth cultures using the Nucleospin® Tissue extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturers protocol (Oladipo et al. 2019, Chapters 3 & 4). Agarose gel electrophoresis and

NanoDrop spectrophotometer (ND-100, NanoDrop Technologies Inc, Wilmington, DE, USA) were used to determine the integrity and the purity of the resultant DNA, respectively. Paired end libraries were prepared from 1 ng bacterial DNA using Nextera XT DNA Sample Preparation Kit and Nextera XT Index kit (Illumina Inc., San Diego, California, USA) following the manufacturers protocol. Sequencing was performed using a MiSeq 2000 Illumina platform (250bp paired end reads). The raw sequencing data generated by Illumina MiSeq 2000 were deposited to the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with the accession numbers (SAMN14149990-SAMN14149997).

5.2.4 Genome assembly, functional annotation and downstream analysis

Raw sequence reads were quality filtered and trimmed using FastQC and Trimmomatic (Bolger et al. 2014) with the average quality set at Q15. De novo assembling of the quality reads was performed using Spades version 3.13.0 on the KBase server (Nurk et al. 2013; Arkin et al. 2018) with minimum contig length set to > 10 000 bp. CheckM (Parks et al. 2015) was used to assess the quality of the Spades assembled genomes. Spades assembled genomes were annotated by Prokka (Seemann, 2014) with default settings. The annotated genomes were inserted into a species tree using FastTree version 2.1.10 (Price et al. 2010). To determine the identity of the species, FastANI version 0.1.2 (Jain et al. 2017) was used to estimate average nucleotide identity (ANI) of the isolates using nearest genomes from the phylogenetic tree as reference

genomes. Kbase App (Arkin et al. 2018) was used to compute pan-genomes and generating pan-genome circle plots.

5.2.5 Identification of mobile genetic elements, virulence factors and antibiotic resistance genes

Integrated prophage regions were identified and annotated by using the online PHAge Search Tool Enhanced Release (PHASTER) (Arndt et al. 2016) with default parameters. Phaster provides the prophage region length, its GC content as well as its position on the contigs. In addition, it estimates the intactness (complete/incomplete) and the most common related phages. Plasmid identification was done using PlasmidFinder 2.1 (Carattoli et al. 2014) with default parameters. Virulence factors were identified using the Virulence factors of pathogenic bacteria database (VFDB) (Chen et al. 2016) using *S. aureus* subsp *aureus* as a genome for comparison. Non-*Staphylococcus* related factors were also identified. The comprehensive antibiotic resistance database (CARD) (Alcok et al. 2020) was used to find the antibiotic resistance genes from the isolates using the perfect and strict hits only criteria.

5.3 Results

5.3.1 Genomic features of the *Staphylococcus* isolates

Assembling of the genomes with Spades resulted in high quality draft genomes shown in Table 12. CheckM quality assessment of the assembled genomes showed that the

isolates had an average of 97.9 ± 2.4 % completeness, having an average contamination of 0.48 ± 0.43 % (Supplementary Table 2). Three of the isolates (T28, T27 and T28b) did not have any detectable contamination. We obtained on average 30.25 ± 17 contigs per genome which was corresponding to an average N50 of 289, 514.4 ± 220,373.6. The isolate L4 (*S. haemolyticus*) had the smallest genome size (2,135,828) corresponding to an N50 of 41,586, with the largest number of contigs 60.. In contrast, T28b (*S. cohnii*) had the largest genome size (2,690,294) corresponding to an N50 of 566,475, having a joint least number of contigs (12) with the isolate T28 (*S. cohnii*) Table 12. The G + C content were homogeneous in all isolates averaging 32.41 ± 0.27 %. The Prokka pipeline was used for functional annotation predicting on average 2,489.8 ± 163.7 genes. The L4 isolate had the least number of predicted genes 2,162 whereas the isolate T28 had the highest number of predicted genes 2,658. On average, the number of protein coding genes was 2,444.9 ± 173.7.

Table 12: General features of the isolates' genomes including the assembly metrics

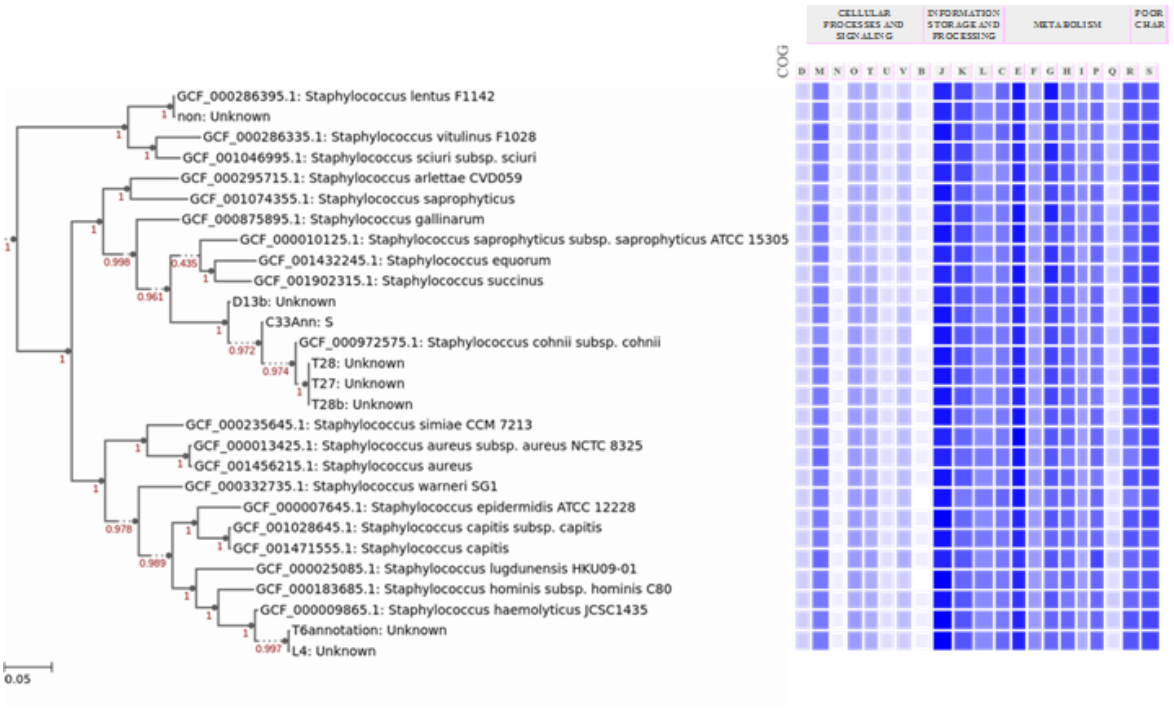
	L4	D13b	T6	T20	C33	T28	T28b	T27
Genome Size (bp)	2,135,828	2,685,820	2,299,081	2,648,007	2,576,421	2,643,319	2,690,294	2,648,416
G+C Content %	32.78	32.55	32.64	31.82	32.39	32.36	32.37	32.33
Number of Contigs	60	22	49	36	37	12	12	14
Largest Contig	247,342	984,404	278,121	200,372	434,148	1,296,694	1,298,284	1,296,793
N50	41,586	252,300	76,220	121,032	131,536	563,483	566,475	563,483
Genes	2,162	2,603	2,284	2,619	2,497	2,544	2,658	2,551
Protein coding gene	2,087	2,626	2,244	2,601	2,458	2,499	2,540	2,504
Plasmids	0	1	1	0	0	2	3	2

5.3.2 Phylogenetic relationship with other *Staphylococcus* isolates

Prokka annotated genomes were used to construct a phylogenetic tree with closely related genomes from the KBase list of species. Distribution of a selected subset of Cluster of Orthologous Groups (COGs) functional domains shown in Figure 7 was estimated from the phylogenetic tree. Core gene category repartition was highly similar among all the isolates (Figure 7). Sequenced genomes were identified as *S. lentus* (T20/non), *S. cohnii* (D13b, C33, T27, T28, T28b), and *S. haemolyticus* (T6 and L4) (Table 12). To validate the assigned species of the isolates, FastANI was used to compute Average Nucleotide Identity (ANI) of orthologous gene pairs between the query and reference genomes. Same species are considered to have an ANI of > = 95% (Jain et al. 2017). *Staphylococcus lentus* F1142 (GCF_000286395.1) when used as a reference genome had an estimated ANI of 99.15% with the query T20 (Table 13). They also had an approximately similar genome size of 2.5 Mb (Supplementary Figure. 1). Reference genome *S. cohnii* subsp. *cohnii* (GCF_000972575.1_assembly) had an estimated ANI of at least 98.7 % with (T28, T28b, T27, C33, and D13b) as a query (Table 13). Moreover, they had approximately the same genome size of 2.5 Mb (Supplementary Figure 1). Reference genome *S. haemolyticus* JCSC1435 (GCF_000009865.1_assembly) had an estimated ANI of at least 98.8% with (T6 and L4) as query. However, T6 and L4 had an estimated smaller genome size of 2 Mb in comparison with the reference genome size of 2.5 Mb (Supplementary Figure 1).

Table 13: Average nucleotide identity (ANI) of the isolates

Query Genome	Reference Genome	ANI estimate	Total orthologous sequence fragments matched	Total sequence fragments of query
T28	GCF_000972575.1_assembly (*S. cohnii* subsp. *cohnii*)	99.1921	816	874
T20/non	GCF_000286395.1_assembly (*S. lentus* F1142)	99.1546	743	863
T27	GCF_000972575.1_assembly (*S. cohnii* subsp. *cohnii*)	99.1311	812	874
T28b	GCF_000972575.1_assembly (*S. cohnii* subsp. *cohnii*)	99.0809	821	890
C33	GCF_000972575.1_assembly (*S. cohnii* subsp. *cohnii*)	98.9739	795	838
T6	GCF_000009865.1_assembly (*S. haemolyticus* JCSC1435)	98.8268	718	744
L4	GCF_000009865.1_assembly (*S. haemolyticus* JCSC1435)	98.7875	656	685
D13b	GCF_000972575.1_assembly (*S. cohnii* subsp. *cohnii*)	98.7468	805	884



KEY

CELLULAR PROCESS AND SIGNALING	D	Cell cycle control, cell division, chromosome-partitoning
	M	Cell wall/membrane/envlope biogenesis
	N	Cell motility
	O	Post translational modification, protein turnover and chaperones
	T	Signal transduction mechanisms
	U	Intracellular trafficking secretion and vesicular transport
	V	Defecnce mechanism
	W	Extracellular Structures
	Y	Nuclear Structure
	Z	Cytoskeleton
POOR CHAR	R	General function prediction only
	S	Function unknown
INFORMATION STORAGE AND PROCESSING	A	RNA processing and modification
	B	Chromatin structure and dynamics
	J	Translation, ribosomal structure and biogenesis
	K	Transcription
	L	Replication, recombination and repair
METABOLISM	C	Energy production and conversion
	E	Amino acid transport and metabolism
	F	Nucleotide transport and metabolism
	G	Cabohydrate transport and metabolism
	H	Coenzyme export and metabolism
	I	Lipid transport and metabolism
	P	Inorganic ion transport and metabolism
	Q	Secondary metabolites biosynthesis, transport, and catabolism

Figure 6: Distribution of the Cluster of Orthologous Groups (COGs) functions among the isolates and closely related species. The COGs were generated from the trees core genes annotation of the genomes was performed using Prokka

5.3.3 Pan-genome analysis

Isolates used in this study were identified as *S. lentus* (T20), *S. cohnii* (D13b, C33, T27, T28, and T28b) and *S. haemolyticus* (T6 and L4). Pan-genome analysis of the isolates was done using reference genomes of the same species. In addition, the pan-genome analyses for the five isolates identified as *S. cohnii* confirm similarities or differences in the clinical (D13b and C33) and environmental isolates (T27, T28 and T28b) isolates. Pan-genome analysis also gave insights into the variation of environmental samples from distinct geographical locations (Nigeria and South Africa). Pan-genome analysis of *S. lentus* (T20) using four reference *S. lentus* genomes revealed T20 had 2464 genes in homologs and 162 singletons (Figure 8). The singletons consisted of genes conferring virulence/pathogenicity (integrase, super-antigen encoding pathogenicity islands SaPI), antimicrobial resistance (Undecaprenyl-diphosphatase (EC 3.6.1.27), TetM, PBP2a, *MecA*, MecR1, Blal, aadA), adaptations to stress (cadmium resistance protein, EfeB, abortive phage resistant protein, putative cytoplasmic protein), as well as genes associated with mobile genetic elements (anti-restriction protein, proteins associated with Tn7 transposon, a number of mobile element proteins, phage associated proteins, HNH endonuclease). CRISPR-associated protein Cas1/2, Csm1/2/5/6, CRISPR-associated RAMP Csm3/4, CRISPR repeat RNA endoribonuclease Cas6 were also detected among the singletons. 37.7% of the genes were of unknown function while 17.3% were hypothetical proteins. *S. haemolyticus* (T6) had 2158 genes in homologs and 116 singletons (Figure 8). The categories of the singletons were almost similar to those of T20, except that there were no CRISPR-associated proteins. Additional virulence factors like virulence-associated cell-wall-anchored proteins SasH, oxygen

insensitive NAD(P)H nitroreductase, multi antimicrobial extrusion protein, Antiadhesin Pls, binding to squamous nasal epithelial cells were also observed. Genes with unknown functions as well as hypothetical proteins contributed 25% each to the singletons. T27 had a total of 2364 genes in homologs and 200 genes in singletons (Figure 7). The genes of interest include: Tn552 transposase, TetM, ABC transporters, 3-oxoacyl-[acyl-carrier protein] reductase, Secretory antigen SsaA-like protein transposon-related, Chromosome (plasmid) partitioning protein ParA, oly-gamma-glutamate synthase. Genes of unknown functions and hypothetical proteins were dominant. Figure 8 shows a phylogenetic pan-genome accumulation of the *Staphylococcus* genome, showing gain or loss of genes per node. Increase in the number of species resulted in an increase in the number of singletons suggesting that the *Staphylococcus* species used in this study possess an open pan-genome.

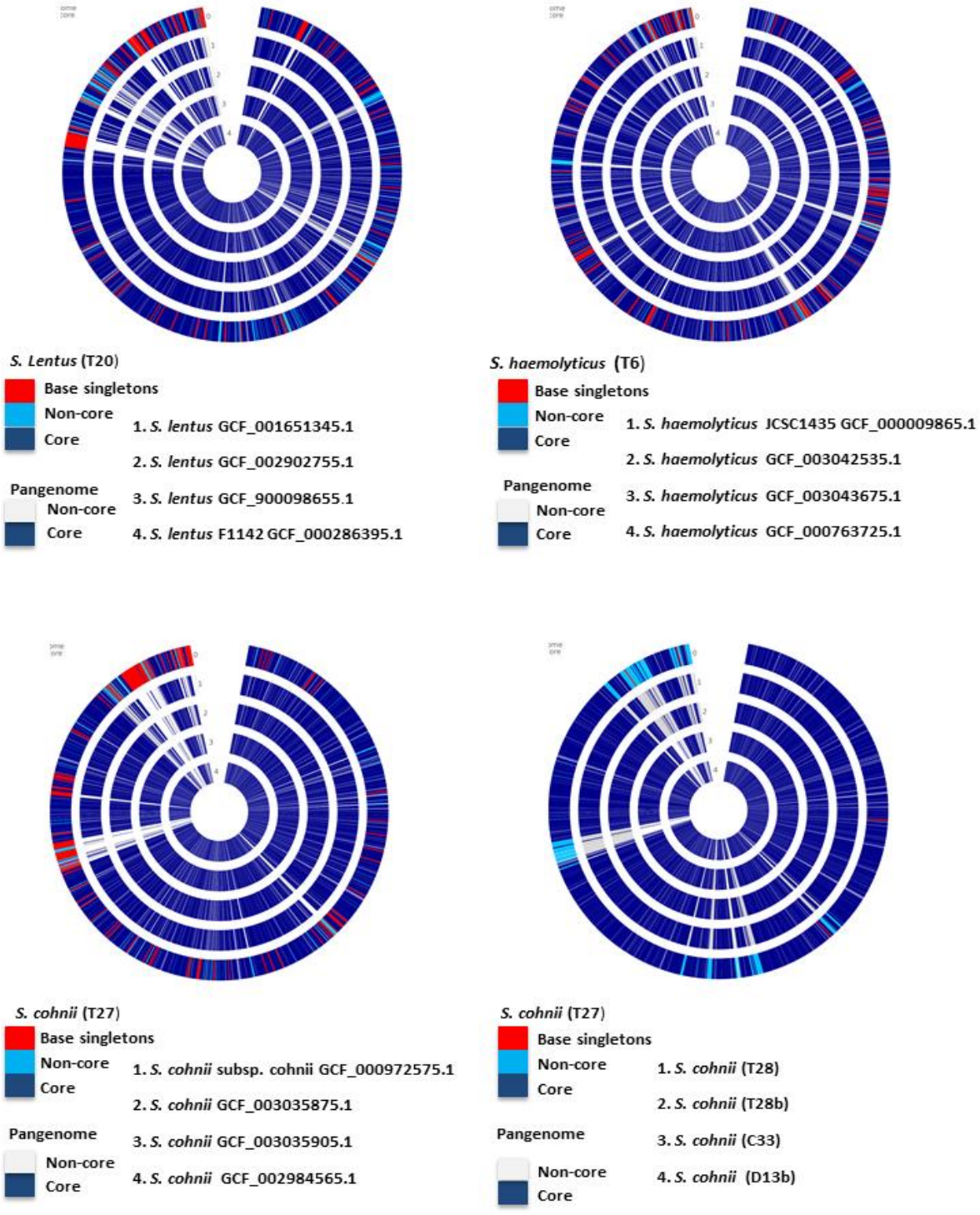


Figure 7: Pan-genome circle plots of the isolates used in this study

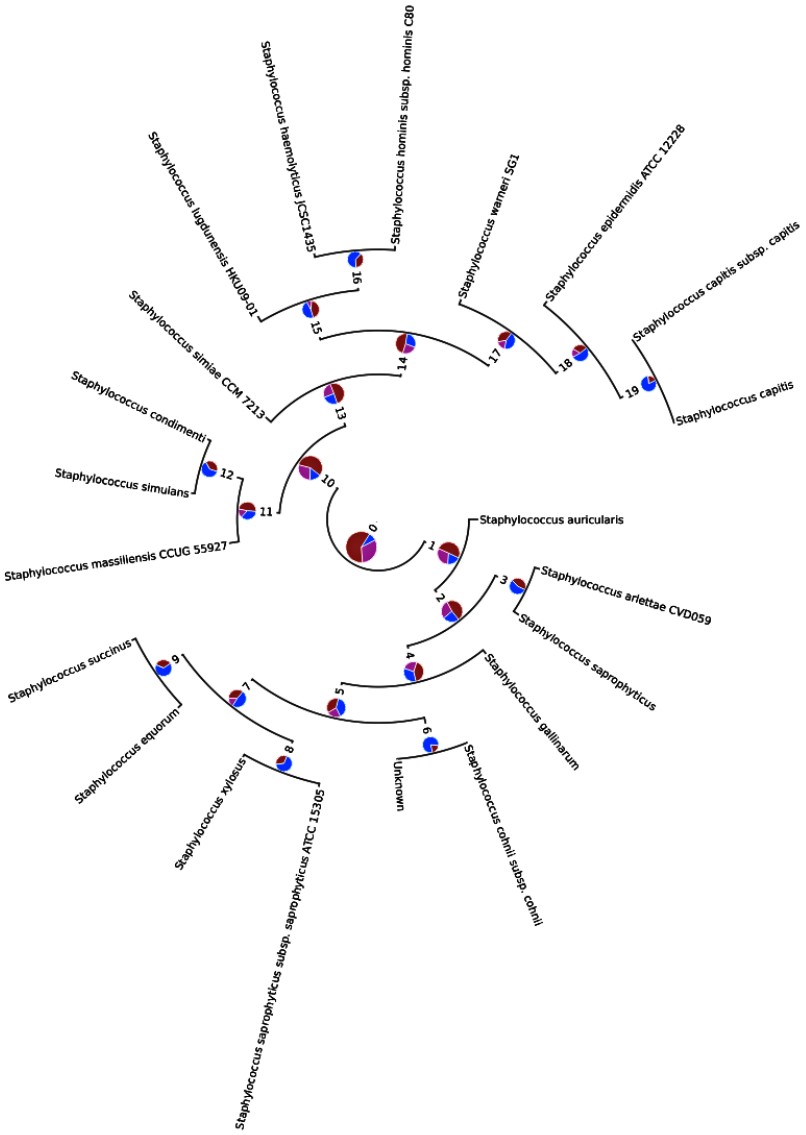


Figure 8: Phylogenetic pan-genome accumulation of the *Staphylococcus* genome showing gain/loss of genes per branch /node

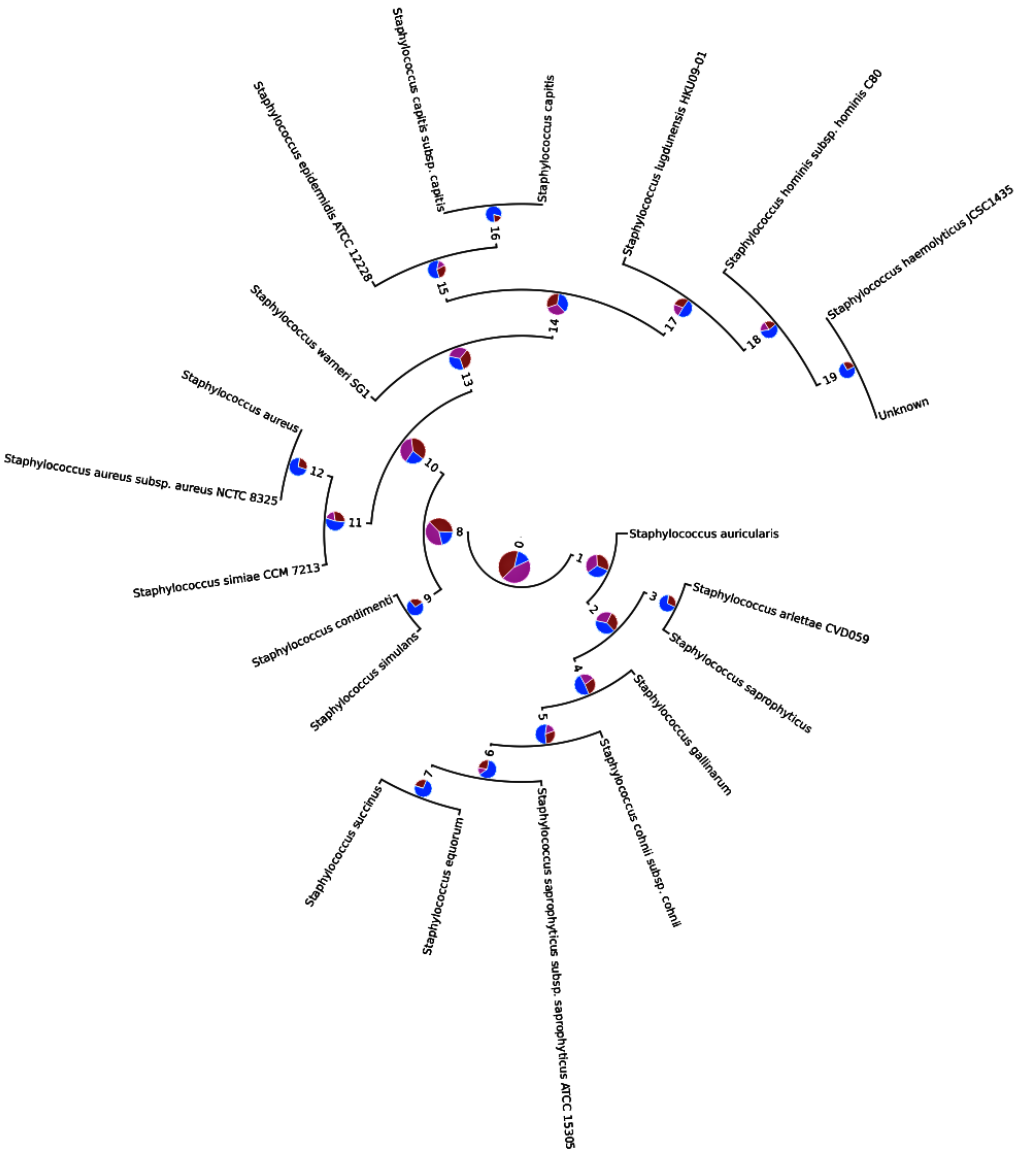


Figure 9: Phylogenetic pangenome accumulation of the genus *Staphylococcus* showing gain and or loss of some genes per branch/node

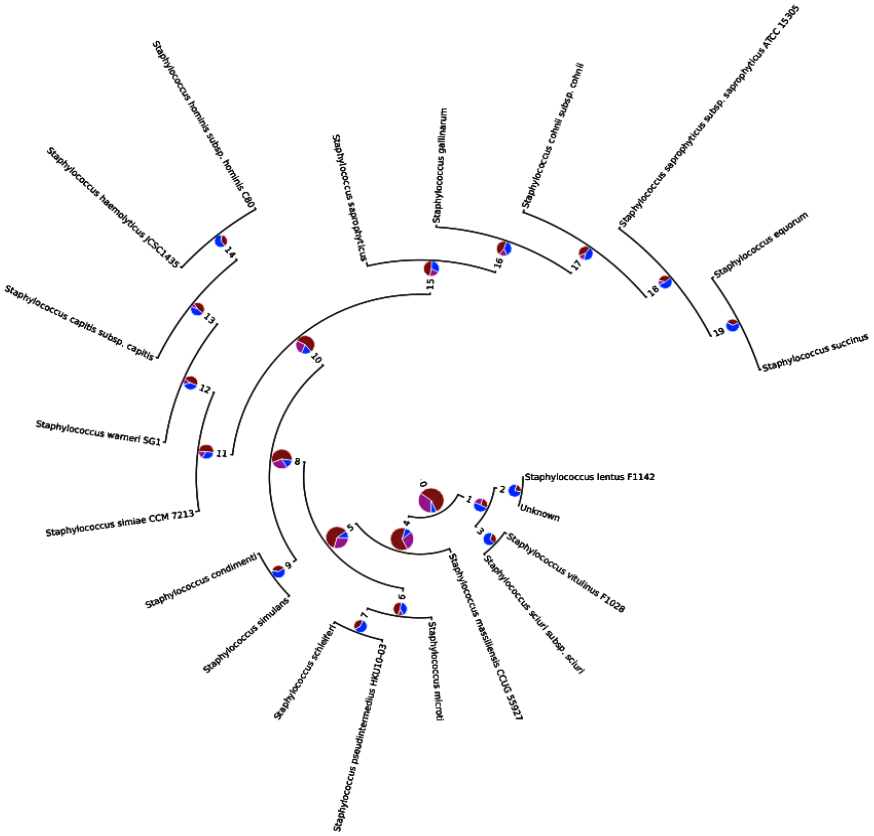


Figure 10: Phylogenetic pan-genome accumulation of the genus *Staphylococcus* showing gain and or loss of some genes per branch/node

5.3.4 Mobile genetic elements

Two intact prophage regions were identified by PHASTER analysis for two (L4 and C33) of the eight isolates. L4 and C33 prophage regions had a total length of 40.1kb and 58.5kb, encoding 59 and 75 proteins, respectively. For L4 the most common phage name was PHAGE_Staphy_StB12_NC_020490, whereas for C33 it was PHAGE_Staphy_phiRS7_NC_022914. Of the proteins encoded by L4 prophage region, peptidoglycan hydrolase, Erf-like recombinase, putative cro/cl-like repressor, LexA repressor, pyrophosphatase, pathogenicity island protein and mobile-element-associated regulatory protein were of interest. In contrast C33 encoded holin, dihydrofolate reductase type 1, protein phosphatase and serine/threonine kinase.

PlasmidFinder detected a total of nine plasmids distributed among five of the eight isolates (Table 12). D13b plasmid was identical to *S. epidermidis* plasmid SAP016A (GQ900381), while that of T6 was identical to *S. epidermidis* plasmid SAP105A (GQ900452). Isolate T27 and T28 had two similar plasmids which were identical to *S. saprophyticus*. subsp. saprophyticus ATCC 15305 plasmid pSSP1 (AP008935) and *Staphylococcus* spp. 693-2 plasmid pLEW6932 (NC_009130.1). The isolate T28b had three plasmids identical to *Staphylococcus aureus* subsp. *aureus* ST398 pS0385-3 plasmid, isolate SO385 (AM990995.1), *Staphylococcus saprophyticus* pSES22 plasmid, isolate 44 (AM159501.1) and *Staphylococcus saprophyticus* subsp. saprophyticus ATCC 15305 plasmid pSSP1(AP008935.1).

5.3.5 Resistome and virulome

A number of antibiotic resistance-associated genes were identified by using the comprehensive antibiotic resistance database (CARD). Table 14 shows the predicted antibiotic resistance genes, the mechanism of resistance as well as the drug class for each isolate. All the isolates except for C33 had at least one hit of the antibiotic resistance genes.

The virulence factors of pathogenic bacteria database (VFDB) was used to determine the potential virulome of the isolates. All the isolates had at least one virulence factor (Tables 15 & 16). Interestingly, the analysis showed that some of the virulence factors were acquired from non-*Staphylococcus* spp (Table 16) suggesting the possibility of these factors being acquired through horizontal gene transfer. The factors covered a broad range of categories which includes adherences, enzymes, immune invasion, toxin, surface protein anchoring, antiphagocytosis and phagosome arrest among other categories. The possibility of strong horizontal gene transfer is supported by few barriers of horizontal gene transfer. Only one out of the eight isolates consisted of the CRISPR-associated proteins which are known to constitute a strong barrier to foreign DNA uptake, particularly plasmid DNA (Louwen et al. 2014; Cao et al. 2016).

Table 14: Antibiotic resistance genes identified from the isolates

Isolate	RGI Criteria	ARO term	AMR Gene Family	Drug Class	Resistance Mechanism	Matching Region	Ref Seq Length
T20	perfect	*mec*I	methicillin-resistant PBP2	Penam	antibiotic target replacement	100	100
	Strict	*tetM*	tetracylin-resistant ribosomal protection protein	tetracycline antibiotic	antibiotic target protection	97.65	100
	Strict	*mecA*	methicillin-resistant PBP2	Penam	antibiotic target replacement	99.85	100
	Strict	*mphC*	macrolide phosphotransferase (MPH)	macrolide antibiotic	antibiotic inactivation	90.64	100
	Strict	*Erm(43)*	Erm 23S ribosomal RNA methyltransferase	macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic	antibiotic target alteration	98.77	100
	Strict	*ErmA*	Erm 23S ribosomal RNA methyltransferase	macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic	antibiotic target alteration	99.59	100
	Strict	*ErmB*	Erm 23S ribosomal RNA methyltransferase	macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic	antibiotic target alteration	98.78	98.79
L4	Perfect	*dfrG*	trimethoprim resistant dihydrofolate reductase dfr	diaminopyrimidine antibiotic	antibiotic target replacement	100	100
	Strict	PC1 beta-lactamase (*blaZ*)	blaZ beta lactamase	Penam	antibiotic inactivation	95.37	100

Table 15: Distribution of virulence factors acquired from *Staphylococcus* genomes

		L4	D13b	T28	T28b	C33	T20	T6	T27	*S. aureus* subsp *aureus*
Adherence										
Autolysin	(*atl*)	+	+	+	+	+	-	+	+	SACOL1062
Clumping factor B	(*clfB*)	+	-	-	-	-	+	-	-	SACOL2652
Elastin binding protein	(*ebp*)	+	+	+	+	-	-	+	+	SACOL1522
Enzymes										
Lipase	(*lip*)	+	+	+	+	-	-	-	+	SACOL2694
Serine V8 protease	(*sspA*)	-	+	+	+	+	+	+	+	SACOL1057
Thermonuclease	(*nuc*)	+	+	+	+	+	-	+	+	
Immune invasion										SACOL0860
Capsule		+ (2)	+ (5)	+ (6)	+ (6)	-	+ (3)	+ (2)	+ (6)	SACOLO136 - 141

Table 16: Distribution of virulence factors likely to have been acquired from other genomes

		L4	D13b	T28	T28b	C33	T20/non T6	T27	Organism
Immune invasion									
Capsule		-	-	+	+	-	-	+	*Acinetobacter*
Polysaccharide capsule		-	-	-	-	-	+	-	*Bacillus*
Toxin									
Cytolisin	(cylR2)	-	-	+	+	+	-	+ (4)	*Enterococcus*
Nutritional factor									
Allantoin utilisation		-	+ (4)	+ (4)	+ (4)	+ (4)	-	+	*Klebsiella*
Serum resistance and immune invasion									
LPS	(wbtE)	-	+	-	-	-	-	-	(*Francisella*)
LPS	(wbtP)	-	-	-	-	-	+	-	(*Francisella*)
Antiphagocytocysis									
Capsule	(uge)	-	-	+	+	-	-	+	*Klebsiella*
Iron uptake									
PBP, ABC transport systems	(vctC)	-	-	-	-	-	+	-	*Vibrio*
Phagosome arresting									
Nucleoside diphosphate kinase	(ndk)	-	-	-	-	-	+	-	*Mycobacterium*
Regulation									
LisR/LisK	(lisR)	-	-	-	-	-	+	-	*Listeria*
Surface protein anchoring									
Lipoprotein diacylglycerol transferase	(lgt)	-	-	-	-	-	+	-	*Listeria*

5.4 Discussions

In the current study, whole genome sequencing of eight CoNS isolates from clinical and environmental settings was performed with the aim of characterising the virulence factors associated with the isolates and the potential threats they may pose. The environmental isolates were from two distinct geographical locations (Supplementary Table 1) which allowed for better comparative genomics. Environmental isolates from South Africa (T20, T27, and T28) carried the tetracycline-resistant ribosomal protection protein *(tetM)* which was not detected from any of the samples from Nigeria (Table 14) regardless of whether they are clinical or environmental samples. Phylo-geographic structure, regional and continental endemism have been demonstrated in a number of bacterial species (Cho & Tiedje, 2000; Zwirglmaier et al. 2008). This is mainly due to the accessory genomes which are essentially for survival under various selective pressures. In the current study, the *tetM* gene was part of the accessory genome, large fraction of the accessory genome are mobile genetic elements (Frost et al. 2005) and they place the host cell in an advantageous position to live under specific conditions (Ussery & Hallin, 2004; Mira et al. 2010). Thus, we propose the over-use of antibiotics particularly tetracycline in the North West province of South Africa led to the acquisition of the necessary accessory genes (*tetM* included). Kanama et al. (2018) detected relatively higher amounts of tetracycline and other antibiotics from wastewater treatment plants in the North West Province, South Africa, suggesting the abuse of these antimicrobials.

Isolates used in this study possess an open pan-genome, increasing the number of species in the analysis resulted in an increase in the number of distinct genes (Figure 7 & Figure 8. This was also augmented by the presence of a number of mobile genetic elements, which include plasmids, phages and pathogenic islands (SaPIs). These MGE

bear *S. aureus* pore-forming toxins and super-antigen enterotoxin coding sequences (Sato'o et al. 2013), which modulate the virulence of CoNS (McCarthy et al. 2014). One (T20) out of the eight isolates used in this study possessed the CRISPR/Cas system, which is a strong barrier to foreign DNA uptake, in particular plasmid DNA (Louwen et al. 2014; Cao et al. 2016). Consistent with this, T20 had no detected phages or plasmids (Table 12), however the singletons from T20 consisted of SaPIs, leading to the hypothesis that this pathogenic island might have been acquired before acquisition of the CRISPR/Cas region. CoNS have been shown to harbour fewer CRISPR/Cas system compared to other bacteria (Rossi et al. 2017). This has been linked to a potential role as reservoir for antibiotic resistance genes for *S. aureus* (Koonin et al. 2017) since they can easily acquire these genes from the environment and pass them to *S. aureus* through horizontal gene transfer. CoNS enhance the evolution and emergence of successfully clones of methicillin-resistant *S. aureus* (MRSA) (Otto, 2013). MGE normally confers a number of adaptive advantages under particular environment and support the virulence of organisms (Bosi et al. 2016). This was also true in this study where dispensable singletons which were on MGE mainly consisted of virulence factors such as integrase, super-antigen encoding pathogenicity islands SaPI), antimicrobial resistance (Undecaprenyl-diphosphatase (EC 3.6.1.27), TetM, PBP2a, *MecA*, MecR1, Blal, aadA), adaptations to stress (cadmium resistance protein, EfeB, abortive phage resistant protein, putative cytoplasmic protein). Intact prophages were only detected in clinical isolates (L4 and C33), no environmental isolate possessed prophages. Prophages are known to increase the virulence potential of pathogenic strains (Varani et al. 2013). There is a positive correlation between the phage related DNA content of a given Enterobacteria and its pathogenicity (Bobay et al. 2013).

A diversity of strategies which is inclusive of adherence, aggression, invasion, persistence, invasion of both innate and adaptive immunity is a vital arsenal essential for pathogenicity of bacteria, however, less is known about these virulence determinants in CoNS (Becker et al. 2014). In *S. aureus*, autolysin (Atl) is known to mediate adherence of bacterial cells to polymer surfaces (Hirschhausen et al. 2012). Homologous proteins to Atl have been described in some CoNS (Hell et al. 1998; Allignet et al. 2001; Yokoi et al. 2008). In the present study, this virulent determinant was present in all isolates except for isolate T20 (Table 15) suggesting the potential virulent nature of the isolates used in this study. Clumping factor B (ClfB) also known as fibrinogen-binding protein (Fbe), a known virulent determinant involved in surface adhesion, was present in T20. This was also detected in L4 (Table 15). In *S. aureus* ClfB was demonstrated to be involved in bacterial adherence to ex vivo human haemodialysis tubing, suggesting its probable involvement in pathogenicity of biomaterial related infections (Pérez-Montarelo et al. 2017). An Fbe deletion mutant resulted in reduced intravascular catheter-associated infection in rats, providing evidence of Fbe being a virulent factor (Guo et al. 2007). Elastin binding protein Epb promotes bacterial colonisation and pathogenesis in *S. aureus* (Park et al. 1999). The Epb gene was also detected in six of the eight isolates, only C33 and L20 lacked this protein (Table 12). Thus, all the isolates used in this study regardless of where they were isolated from had adherence which are crucial virulent determinants involved in the intimal colonisation step.

Moreover, a number of enzymes have been reported in aiding *S. aureus* to invade host tissues, and metastasize to other parts, thIs includes lipase (Li), thermonuclease (*nuc*)

and serine v8 protease (*Sspa*). Hu et al. (2012) demonstrated that deletion of the lipase coding gene in *S. aureus* results in reduced biofilm formation relative to the wild type providing evidence that *S. aureus* lipase plays a pivotal role in its pathogenesis. Based on the findings from this study, *Staphylococcus aureus* and CoNS strains harbouring antibiotic resistance (*mecA*) and other virulence genes such as luk-PVL have been identified in clinical and environmental settings.

Thermonuclease is an enzymatic virulence factor widely used as a marker for the detection of *S. aureus* from clinical samples (Alarcon et al. 2006). These factors are involved in the hydrolysis of DNA and RNA in host cells causing tissue destruction and continuous spread of *S. aureus* (Sandel & McKillip, 2004; Foster, 2005). Furthermore, in the previous Chapters 3 and 4 in this study, the *nuc* gene was amplified and sequenced in all *S. aureus* isolates by PCR. In addition, 7 distinct *spa* types were identified in *S. aureus* strains from this study. This further confirms that there could be a link between the various habitats in clinical and environmental settings.

Serine v8 protease is part of *S. aureus* extracellular proteases which aid in protection against innate system, at both cell dependant and independent levels (Kolar et al. 2013). Table 15 shows the distribution of the described virulence determining enzymes with the isolates identified as *S. cohnii* carrying all the three enzymes regardless of the geographic point of collection. In *S. epidermidis*, serine proteases have been shown to aid the ability to evade the complement defence system and to dysregulate the coagulation cascade (Moon et al. 2001).

When the bacteria enter into the host system, phagocytes engulf these foreign materials. However, virulent pathogens have evolved capsules which effectively mask the bacterial cell surface and surface associated proteins, such as opsonins, from recognition by phagocytic cells (Daffe & Etienne, 1999; Kuipers et al. 2016). All the isolates except for isolate C33 had at least two capsules (Table 15). Of interest were virulence determinants likely to have been acquired from non-*Staphylococcus* species (Table 16) probably through horizontal gene transfer. These virulent determinants covered a broad range of categories which included immune invasion, toxins, nutritional factors, anti-phagocytosis, serum resistance and immune invasion, iron uptake, phagosome arresting and surface protein anchoring.

CoNS are an important reservoir for antibiotic resistance genes, *mecA* gene inclusive. These genes are mainly carried on mobile genetic elements and can be easily transferred to more virulent species like *S. aureus* through horizontal gene transfer (Becker et al. 2004). The continuous spread of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MR-CoNS) strains in clinical and non-clinical environments remain a serious public health concern. This is mainly due to the difficulties associated with the treatment of infections from such organisms as they become resistant to methicillin which is probably considered the prototype of anti-staphylococci penicillin (Lobanovska & Pilla 2017). Methicillin resistance is characterised by an altered penicillin-binding protein (PBP2a) which has a reduced affinity for methicillin rendering the drug ineffective (Eady & Cove, 2003; Turner et al. 2019). PBP2a is encoded by *mecA* which is normally acquired through horizontal gene transfer of a mobile genetic element staphylococci cassette chromosome mec

(SCCmec) (Katayama et al. 2000). In the present study, *mecA* was detected only in isolate T20 designated as *S. lentus* (Table 14). The isolate also had antibiotic resistance genes which included tetracycline-resistant ribosomal protection protein (*tetM*), macrolide phosphotransferase (mphC) Erm 23S ribosomal RNA methyltransferase(s) (Erm43/A/B). This augments the ever-increasing evidence of wastewater treatment plants being a reservoir of multiple antibiotic resistance genes.

A diverse group of antibiotics target the ribosome as the major site of action in the bacterial cell (Poehlsgaard & Douthwaite, 2005), thus altering and protecting these target sites is vital for bacterial survival in the face of antibiotics. Methylation of rRNA has been shown to provide acquired antibiotic resistance (Douthwaite et al. 2004), thus the Erm group provides a crucial role in acquired resistance. Tetracycline, a broad-spectrum of antibiotic agents binds to elongating ribosome and inhibits delivery of the ternary complex to the A-site (Wilson, 2009) thus inhibiting bacterial translation and slowly killing it. Continuous use and abuse of tetracycline resulted in bacteria acquiring resistance determinants to this group of antibiotics (Roberts, 2005). The resistance determinants mainly include ribosome protection proteins with the most prevalent being tetK, tetM and tetO (Chopra & Roberts, 2001; Connell et al. 2003). The tetM was also detected in *S. cohnii* environmental isolates (T27 and T28) Table 14. Remarkably, all the isolates with tetM gene were environmental samples from South Africa. In contrast, no tetM was detected in environmental samples from Nigeria, indicating probably use and abuse of tetracycline in South Africa particularly the North West Province where the isolates were collected. Also, of interest was the detection of genes associated with ß-lactamase activities. The isolates identified as *S. haemolyticus* carried the PC1 beta-

lactamase (blaZ) (Table 14), a gene that encodes ß-lactamase which hydrolyses the ß-lactam ring rendering the ß-lactam antibiotics ineffective. Table 14 gives a clear description of the detected antibiotics, drug class and resistance mechanism.

5.5 Conclusions

In conclusion, this study revealed that most of the isolates from this study possess an open pan-genome with few barriers of horizontal gene transfers which would easily allow for the acquisition of foreign DNA. More interesting was the presence of virulence determinants and multiple antibiotic resistance genes from environmental isolates, demonstrating the importance of wastewater treatments as reservoir for antibiotic resistance genes. Moreover, geographical location and perhaps anthropogenic activities seem to impact the genomic composition of the microbes. This study adds to the baseline for antibiotic surveillance and comparative genomics.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The incidence of antimicrobial resistant (AMR) microorganisms is increasing worldwide and triggers prolonged illness, increased case-fatality, and escalation of treatment costs (Gajdács, 2019). This is particularly worrisome in poorly resourced countries (like Africa) where effective treatment of many infectious diseases is being threatened (WHO, 2017b). It is a serious and growing global health security risk which needs to be prioritized at local and international levels (CDC, 2013). Of particular concern are pathogens such as MRSA and more recently also the CoNS.

Realizing the critical need for action, the World Health Assembly (WHA) embraced the Global Action Plan on Antimicrobial Resistance in May 2015 with 5 key objectives. Of which (4th and 5th) were to optimize the use of antimicrobial agents and to ensure sustainable investment in countering antimicrobial resistance. Subsequently, the WHO global antimicrobial resistance surveillance system (GLASS) and One Health' approach was launched in 2017. This was to strengthen the capacity of regulatory agencies across human, animals, food products and environment among the member states (WHO, 2017).

As a follow-up to this, the National AMR Strategy Framework of the Republic of South Africa was established, with key objectives to slow the development and spread of AMR through better use of antimicrobials (DAFF, 2018). This National AMR Framework with the Departments of Health and Agriculture, Forestry and Fisheries (DAFF) of South

Africa, being a key partner outlined the need to combat and lower the increasing levels of resistance in bacteria. Similarly, the Nigeria National AMR Coordinating Body at the Nigeria Centre for Disease Control (NCDC) was established to step up action with stakeholders in human, animal and environment health. However, these efforts were confronted with the weaknesses of existing control systems and challenges in scaling up interventions (NCDC, 2017).

In addition, several factors have contributed to the incidence, persistence, and spread of AMR in the environment. These include genetic changes in microorganisms, inadequate implementation of infection prevention and control measures, coupled with increased linkage to environmental waters (Tenover et al. 2019). Furthermore, staphylococci are adaptable human pathogens based on their ability to acquire antibiotic-resistance mechanisms and pathogenic determinants, leading to its emergence in hospital, community and environmental settings.

Therefore, a better understanding of the prevalence and distribution of potentially pathogenic *Staphylococcus* spp. from clinical and environmental sources could be an important tool in the development of public health risk mitigation strategies. This thesis has therefore contributed to the body of existing knowledge that opportunistic, pathogenic and ARB such as staphylococci are present in clinical and wastewater sources in Nigeria and in a selected wastewater source in the North West Province, South Africa.

The aim of the study was thus to study the prevalence of *Staphylococcus* species from clinical and environmental sources (water bodies), and, utilizing molecular methods to characterize and confirm the MRSA.

In order to achieve this aim, culture-based and molecular methods were utilized to characterize and confirm the MRSA from this study. Furthermore, a total of 4 specific objectives were formulated to achieve the aim of this study. The findings of each objective are briefly discussed below under specific headings.

6.1.1 A systematic overview of the literature and gaps in research on staphylococci in environmental aquatic sources

The main objective of this review was to assess the prevalence of *mecA* gene in *Staphylococcus* spp. from environmental water and clinical sources. Retrieved articles for the search period (2000 – 2019) indicate that more studies focused on the detection of *mecA* in staphylococcal species and these were conducted in developing countries (84%) compared to developed nations (16%). *Staphylococcus aureus* has been well studied and documented especially in the clinical settings with very little focus on CoNS. There is a scarcity of information on staphylococcal species isolated from wastewaters especially with CoNS where very few studies were found that studied *mecA* genes from environmental sources. In addition, the potential of *S. aureus* and CoNS to exist and invariably serve as reservoirs of ARGs in environmental water (e.g. water receiving wastewaters) are yet to be fully explored. Furthermore, the human and public health implication and dissemination of the *mecA* gene particularly, in wastewater environments are currently insufficiently studied. With the detection of the *mecA* gene in *Staphylococci* from the environmental and clinical settings, the impending global threat and impact of antibiotic resistance on human and public health may have a grave consequence if not studied and monitored more closely.

6.1.2 Isolation and characterization of MRSA from clinical and environmental sources (water bodies receiving hospital wastewater) in Nigeria

The aim of this study was to investigate the prevalence of MRSA in clinical and environmental (hospital effluents and surrounding receiving wastewaters) sources in Ile-Ife, South-western Nigeria. The successful identification of *S. aureus* and other *Staphylococcus* spp. was was done through plate cultural methods and a series of biochemical tests. These include their description as Gram positive, catalase and coagulase-positive bacteria that are capable of growth in the presence or absence of oxygen (facultative anaerobic). Further confirmation of the identities of the *Staphylococcus* spp. obtained from the clinical and environmental sources was done using 16S rRNA gene sequencing analysis. Based on the analyses 76 *Staphylococcus* spp. were identified: 27 were from clinical sources, 14 from hospital effluents and 35 isolates originated from environmental (wastewater) sources. Over 57% were coagulase-negative (CoNS) and 43% coagulase-positive (*S. aureus*). Genes of interest including *mecA, nuc* and *luk-PVL* genes were detected in *S. aureus*, while *mecA* was detected in *S. arlettae, S. sciuri, S. cohnii* and *S. epidermidis*. Finding these *Staphylococcus* species potentially from clinical origin in environmental water is a cause for concern. These are bacterial species not normally tested for in microbiological water quality analyses. This study raised the issue of regulations and testing regimes of environmental water that receives hospital wastewater. It calls for stringent regulations and proper treatment of hospital effluents before disposal to receiving wastewaters. This would reduce the risks posed to human and animal health and safety and may as well also play an important role in mitigation of global antibiotic resistance, particularly of *mecA* associated resistance.

6.1.3 Isolation and characterization of MRSA from a WWTP and the receiving water

The aim of this study was to investigate the prevalence of antibiotic resistant staphylococci and detection of resistance, virulence and *spa* genes in effluent of a South African wastewater treatment plant. Species identified using standard protocols were *Staphylococcus aureus*, *S. lentus*, *S. arlettae, S. cohnii, S. haemolyticus, S. nepalensis, S. sciuri* and *S. xylosus*. Isolates were resistant to methicillin (91%) and other classes of antibiotics. Multiple antibiotic resistance (MAR) indices for the isolates exceeded 0.2 (0.519 – 0.705). Among the isolates, 77% were *mecA*-positive. The *S. aureus* isolates were positive for *nuc* gene and posseses 7 distinct s*pa* types. This includes t061, t7835, t447, t657, t6578 and t5126 with s*pa* type t091 being the most prevalent. Finding such a variety of *spa* types is a potential indication of diverse sources of the isolates and that these could be from different geographical locations. Notably among the *spa* types t657, sequence type (ST) 772 reported in this study was a PVL positive -epidemic clone of MRSA that had been linked to community outbreak of CA-MRSA infections in India (D'Souza et al. 2010). This study emphasized possibility of treated wastewaters being potential reservoir for antibiotic resistant *Staphylococcus* species. This is a cause for concern as wastewater effluents are decanted into environmental waters and these are, in many cases, used for various purposes including recreation (full contact), religious (full body submersion), as well as drinking water for some rural communities and water for livestock. Therefore, a more effective treatment plan or treatment modification procedures of wastewaters may be crucial, especially if the water is to be reused.

6.1.4 Investigation through whole genome sequencing the genetic characteristics of selected staphylococci from Nigeria and South Africa

The main aim of the study was to further explore the virulent and antibiotic resistance determinants associated with the staphylococci isolates form this study. Specifically, the *mecA* positive CoNS. These potentially pathogenic CoNS strains are often overlooked as opportunistic or non–pathogenic in the clinical setting. However, based on the whole genome sequences of the CoNS environmental isolates from this study, we found that these CoNS possessed an open pan-genome which would easily allow for the acquisition of foreign DNA, a typical characteristic of human pathogenic bacterium. Remarkably, the presence of virulence determinants and multiple antibiotic resistance genes from the environmental isolates, demonstrating the importance of wastewater treatment plant as a hotspot for antibiotic resistant bacteria genes. This study added to the baseline for antibiotic surveillance and comparative genomics of potentially pathogenic CoNS. In the current study, the *tetM* gene was part of the accessory genome. A large fraction of the accessory genome is mobile genetic elements (Frost et al. 2005). Thus, we propose that the over-use of antibiotics particularly tetracycline in the North West Province of South Africa led to the acquisition of the accessory genes (such as *tetM*). In addition, geographical location and possibly anthropogenic activities seemed to impact the accessory genome, contributing to variation of isolates within the same species.

6.2. Recommendations:

With reference to this study, the following recommendations are suggested:

1. Wastewater and effluents from hospitals should be adequately treated before discharged into the environmental waters or community based WWTPs. A more effective treatment plan or treatment modification procedures of wastewaters is therefore recommended especially if the water is to be reused. These findings are aspects that the managers of wastewater treatment plants, policy formulators, down-stream users should consider.

2. Surveillance of *Staphylococcus* spp. should be heightened in the hospital settings. Clinical and laboratory diagnosis of staphylococci should focus not only on *Staphylococcus aureus* (HA-MRSA and CA-MRSA) but adequate attention should also be focused on CoNS of medical and environmental importance. Thus, we are proposing the term ‘environmental acquired staphylococci’. This could provide a focus point and more information into the potential of these isolates as reservoirs of antibiotic resistance genes

3. Biochemical techniques used in this study for the identification of staphylococci proved to be restrictive. Sequencing data was more confirmatory and should always be used for the identification of DNA. Molecular techniques such as PCR and sequencing, *spa* typing and whole genome sequencing (WGS), etc. could be used to investigate the resistance and virulence factors associated with these isolates and the potential dangers they might pose.

4. Antibiotic stewardship, a programme that encourages judicious use of antibiotics should be implemented in hospitals on the African continent, particularly in Nigeria and South Africa. Methicillin-resistant *Staphylococcus aureus* (MRSA), and other multi-drug resistant staphylococci isolates in this study were just a few of the ever-growing list of drug resistant bacteria from the hospital, community and environmentally acquired potential pathogens.

5. *Staphylococcus aureus* remains a priority organism (WHO, 2014, 2017b). The detection of a huge proportion of MDR-staphylococci in clinical isolates and environmental waters in Ile-Ife, Nigeria and in wastewater effluents in one South Africa WWTP is a major cause for concern and should be addressed. *S. aureus* is one of the six most common and serious multi-drug resistant (MDR) organisms identified as “ESKAPE pathogens”. Further research and global surveillance of *S. aureus* and MRSA should continue to be in the forefront. This must include adequate monitoring of culture and antibiotic susceptibility data that will help to determine baseline rates for MDR Organisms and monitor the effectiveness of outbreak control measures.

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SUPPLEMENTARY

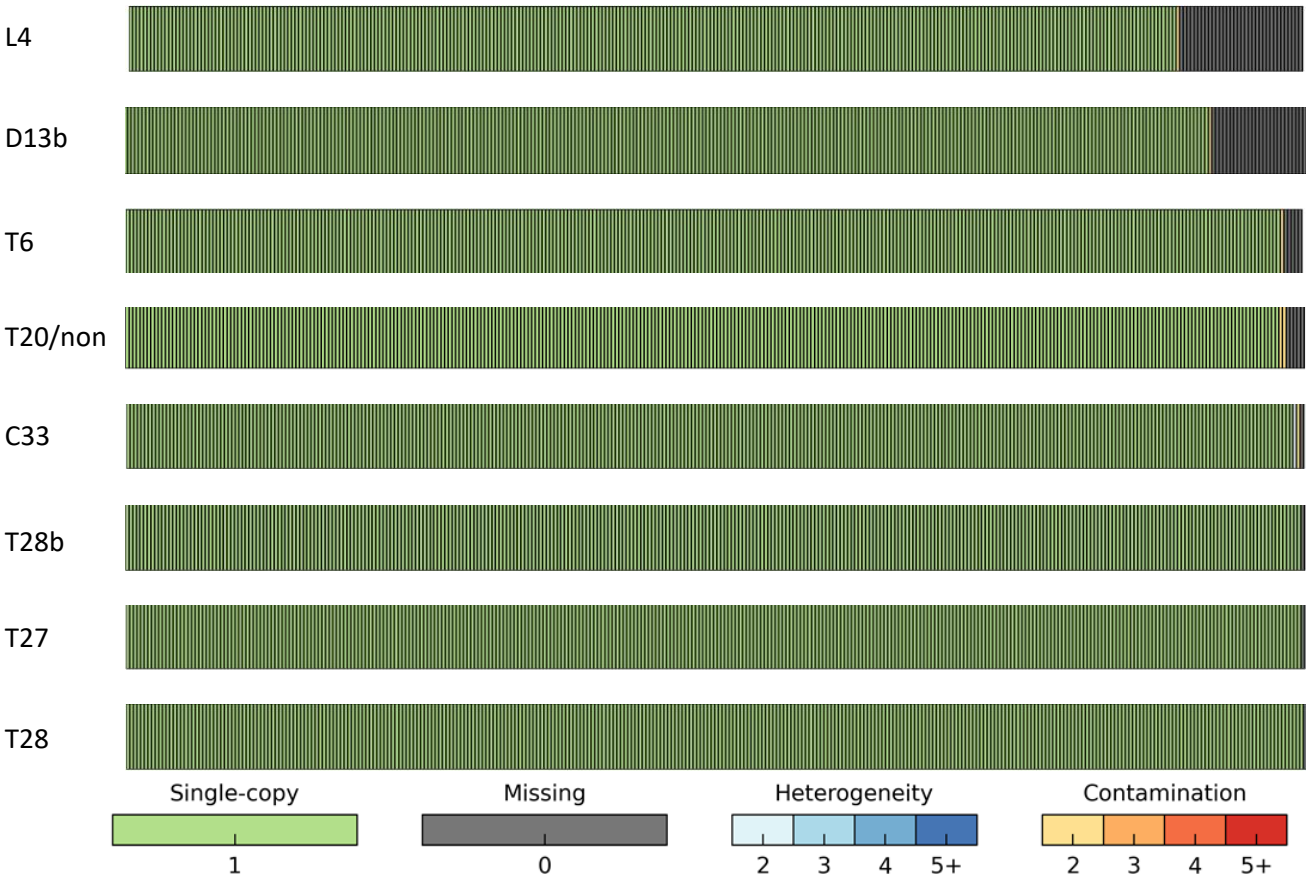
Supplementary Table 1: Description of the isolates' origins

Isolate/Sample ID	Source	Identity	Country of Origin	Settings
T20	WWTP	*S. lentus*	South Africa	Environmental
T27	WWTP	*S. cohnii*	South Africa	Environmental
T28	WWTP	*S. cohnii*	South Africa	Environmental
T6	WWTP	*S. haemolyticus*	South Africa	Environmental
T28b	WWTP	*S. cohnii*	Nigeria	Environmental
C33	Wound Swab	*S. cohnii*	Nigeria	Clinical
L4	Wound Swab	*S. haemolyticus*	Nigeria	Clinical
D13b	Urine	*S. cohnii*	Nigeria	Clinical

WWTP: Wastewater treatment plant

Supplementary Table 2. CheckM quality assessment of the assembled genomes

Isolate	Completeness (%)	Contamination (%)
T28	99.45	0
T28b	99.45	0
T27	99.45	0
T6	98.34	0.55
D13b	96.45	0.55
L4	91.91	0.55
C33	99.45	1.1
T20	98.34	1.1



Supplementary Figure 1: CheckM quality assessment of the assembled genomes of all the isolates. Refer to the text for detailed values