An assessment of genetic fingerprinting tools for typing of multidrug-resistant and biofilm forming *Staphylococcus aureus*

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DECLARATION

I, B. MBANGA, declare that the mini-dissertation entitled "**Genetic fingerprinting of multidrug-resistant and biofilm-forming *Staphylococcus aureus* isolated from dairy farm in Mahikeng, North-West Province, South Africa"**, submitted for the degree of Master of Science (Biology) at the North-West University, Mafikeng Campus, and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

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Dr KP Montso (Co-supervisor)

DEDICATION

To the memory of my friend and colleague, Rebecca Qhushikiwe who passed away in December 2021. You will forever live in my heart. This is also your degree.

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ABSTRACT

Staphylococcus aureus is a ubiquitous organism that has been associated with foodborne and nosocomial infections that may be difficult to treat due to the development of multiple antibiotic resistances. Genetic variation may also impact disease severity and transmission in susceptible populations. The occurrence and genetic diversity of multidrug-resistant and biofilm-forming *S. aureus* from drinking water troughs, milk, and milking environments at the North-West University dairy farm, were determined. A cross-sectional study design was employed to collect 39 samples from different sampling sites. Samples were screened by culture and the identity of the isolates was confirmed by PCR amplification of the *Gap* gene. Confirmed isolates were subjected to the Kirby-Bauer antibiotic susceptibility test against 11 antibiotics and antibiotic resistance genes (ARGs) detection by PCR. The biofilm-forming potential of the isolates were determined phenotypically. Genetic fingerprinting assays including REP, ERIC, and RAPD-PCR were used to determine the genetic relatedness of the drug-resistant of *S. aureus*. Out of 144 putative isolates, 124 (86.11%) harboured the *gap* gene. These isolates showed varied resistance abilities to cefoxitin (51.25%) penicillin (32%) and clindamycin (24,22%). Three ARGs, namely, *mecA*, *blaZ*, and *tetO* were amplified in 31.14%, 21.95% and 3.65% of the isolates respectively. The dominant MAR phenotypes were CLI-PEN-TET-FOX and PEN-ERY-RIF-FOX each in 15.38% of the isolates. Proportions of 34.83%, 42.86%, and 61.29% of the *S. aureus* isolates were capable of producing biofilms at 4 ºC, 25 ºC, and 37 ºC respectively. PCR-based DNA fingerprinting grouped the 33 *S. aureus* isolates into 19 distinct profiles. The study findings revealed MDR and biofilm-forming strains of *S. aureus* with high genetic diversity from various sampling sites, which could serve as a reservoir for the dissemination of severe and difficult-to-treat infection in susceptible populations.

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DEFINITION OF CONCEPT

Antibiotic: A compound that kills or inhibits the growth of microorganism**.**

Antibiotic resistance: it is the ability of microorganism to develop resistance to a specific antibiotic.

Antibiotic resistance phenotypes: A given overview of the antibiotic resistance profile of a bacteria strain which can be achieved without any genetic alteration.

Antibiotic resistance genes: Are mobile genetic elements that can be transferred from cell to cell by conjugation, transformation, or transduction via horizontal gene transfer.

Biofilms: Are complex structure of microbial communities that attach to and grow on a surface forming single or multiple type of cells. The outcome of this process is sometime an alteration in the phenotype of the organisms with respect to growth rate and gene transcription.

Cluster analysis: The grouping of a set of bacterial isolates in order to determine their phenotypic or genotypic relatedness.

Genetic fingerprinting: A technique that provides indirect access to DNA sequence polymorphism in order to assess species or clonal identity of bacterial organisms. It is also in order to study bacterial genome composition and genetic relatedness.

Methicillin resistance: A bacteria that does not respond to beta-lactam antibiotics including methicillin.

Pathogen: A disease causing microornagism.

Pathogenesis: The origin and development of a disease.

Pathogenicty: The ability to cause disease.

Whole genome sequencing: A techique that reveals the complete DNA make-up of an organism, enabling an understanding of variations both within and between species.

ABBREVIATIONS

CA-MRSA : community-associated MRSA

CNS **:** Coagulase-negative staphylococci

CPS **:** Coagulase-positive staphylococci

D **:** Discriminatory indices

ERIC **:** Enterobacterial repetitive intergenic consensus

Fab **:** Fragment antigen-binding

FC **:** Fragment crystallizable region

HA-MRSA **:** Hospital-associated MRSA

Ig **:** Immunoglobulin

LA-MRSA **:** livestock-associated MRSA

MLST **:** Multiple locus sequence typing

MRSA **:** Methicillin-resistant *S. aureus*

PCR **:** Polymerase chain reaction

PFGE **:** Pulsed-field gel electrophoresis

RAPD **:** Random amplified polymorphic DNA

Rep-PCR **:** Repetitive element-based PCR

SCC*mec* **:** Staphylococcal cassette chromosome *mec*

SpA **:** Staphylococcal protein A

UPGMA **:** Unweighted pair group method with arithmetic mean

UV **:** Ultraviolet

WHO **:** World Health Organization

CHAPTER ONE

INTRODUCTION AND PROBLEM STATEMENT

1.1 General Introduction

Milk and dairy products are important sources of vital nutrients for humans (Fusco *et al.*, 2014). The unique properties and composition of milk makes it an excellent growth medium for bacterial strains and a potential source for the transmission of food-borne pathogens resulting in various infections in susceptible consumers (Claeys *et al.*, 2013). Studies have shown that milk-borne pathogens pose a serious threat to human health, and account for a large proportion (approximately 90%) of all diseases associated with the contact with diary animals or the consumption of contaminated dairy products including milk (Berhe *et al*., 2020).

Amongst the organisms that are frequently associated with contamination in milk and milking environments, *Staphylococcus aureus, Salmonella* species, *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Campylobacter* are the notable bacterial species (Gonzales-Barron *et al*., 2017). The challenges faced with the management of diseases caused by these pathogens coupled with the high reliance on raw milk especially in developing and rural communities highlights the need to enforce, adhere or consider the health status of the animals in the dairy farm, farm management practices used, environmental and personnel hygiene standards as well as milk storage temperatures (Food Standards Australia New Zealand, 2009) as these contribute to the quality of the product.

Staphylococci are Gram-positive cocci, catalase-positive commensal bacteria belonging to the family *Micrococcaceae* and occur in great numbers as normal flora on the mucous

membranes such as oropharynx, intestines, nasal cavity and skin of humans and other warm-blooded animals (Zhang *et al*., 2022). These organisms are non-spore-forming, non-motile and facultative anaerobes. Staphylococci may be classified into two groups based on their ability to produce the extracellular enzyme coagulase, with coagulase-positive staphylococci (CPS) such as *S. aureus* considered as the principal pathogenic strain (Omar Mat-Kamir, 2018; Heo *et al*., 2020; Sigudu *et al*., 2023). However, coagulase-negative staphylococci (CNS) including *S. epidermidis* and *S. saprophyticus* were previously known to be non-pathogenic due to their extremely low clinical significance (Omar Mat-Kamir, 2018; Heo *et al*., 2020; Sigudu *et al*., 2023).

Recent studies have revealed that clinical significance of these CNS especially *S. epidermidis* and *S. saprophyticus* have increased and are frequently isolated from diseased cases (Manandhar *et al.,* 2021). Coagulase-negative staphylococci have been associated with infections in dairy animals and humans (Shrestha *et al*., 2020; Manandhar *et al.,* 2021) thus requiring significant attention.

Staphylococcus aureus remains a significant pathogen due to its association with a wide variety of complications in humans and animals (van Belkum *et al*., 2009; Otto, 2012). *S. aureus,* a facultative intracellular pathogen, colonizes the anterior nares or nasal canals of their hosts, invade and replicate within the hosts cells resulting to its spread to other tissues and the blood stream (Balasubramanian *et al*., 2017; Becker, 2018). The potential to colonize tissues by this pathogen does not necessarily guarantee the establishment of staphylococcal disease in the host, therefore, colonized individuals may serve as asymptomatic carriers (Chmielowiec-Korzeniowska *et al.,* 2020). Under favourable conditions these organisms may cause a number of local infections making the hosts prone to the development of a variety of

clinical complications (Merino *et al*., 2009; van Belkum *et al*., 2009). This trait significantly contributes to the persistence of *S. aureus* in host tissues, their protection from antibiotics and other antibacterial agents as well as enhancing their potential to evade host immune defence mechanisms (Fraunholz and Sinha, 2012; Becker, 2018). In addition, the potential of this organism to produce a variety of extracellular and cell surface virulence factors enhances its pathogenicity (Sarowska *et al*., 2019), thus making it an organism of severe public health concern.

To date, *S. aureus* is a major human pathogen that is associated with a wide variety of infections and is a frequent cause of nosocomial infections worldwide (Komodromos *et al*., 2022). Diseases associated with *S. aureus* range from superficial skin infections such as pimples, boils, impetigo, cellulitis, folliculitis, carbuncles, and abscesses, to life-threatening conditions, such as bacteremia, meningitis, osteomyelitis, endocarditis, cow mastitis, pneumonia, and toxic shock syndrome (Merino *et al*., 2009; Giersing *et al*., 2016; Balasubramanian *et al*., 2017; Lubna *et al*., 2023). *S. aureus* strains have also been associated with post-surgical infections such as surgical wounds, bloodstream and urinary tract infections especially in catheterized patients, as well as medical devices such as feeding tubes (Ikonomidis *et al*., 2009; Guggenbichler *et al*., 2011). The wide spread of *S. aureus* in environmental sources, animals and humans even in countries with more advanced public health and healthcare facilities is facilitated by its high genetic diversity, its ability to acquire exogenous genetic materials and its capability to resist a variety of conventional antibacterial agents (Rice *et al.*, 2007; Palavecino, 2014). Despite the availability of potent antibiotics, *S. aureus* remains an important opportunistic pathogen responsible for high morbidity and mortality in humans (Breves *et al*., 2015, Prakash *et al*., 2017).

According to the World Health Organization (WHO), *S. aureus* has been categorized as one of the "high priority" pathogens with increasing emergence of antimicrobial resistance, and this places it as a pathogen of significant public health concern globally (WHO, 2023). The misuse of antibiotics in veterinary medicine especially in food-producing and companion animals has also resulted in the selection, and emergence of resistant bacteria and the spread of antibiotic resistance determinants (Caneschi *et al.*, 2023). Exposure to such contaminated livestock products or contaminated food processing equipment may enhance the spread of resistant bacterial strains from animals to humans (Lubna *et al*., 2023). Among the resistant staphylococci, methicillin-Resistant *Staphylococcus aureus* (MRSA) has been shown to be resistant to almost all β-lactam antibiotics including cloxacillin, penicillin and methicillin (Frieri *et al*., 2017; Nandhini *et al*., 2022). The clinical significance of MRSA is enhanced by their ability to also exhibit resistance to other classes of antibiotics (Schito, 2006; Smith, 2015). The organism is, therefore, considered as a "superbug" due to the high health risks associated with persistent infections as well as the high morbidity and mortality on humans (Lowy, 2003; Frieri *et al*., 2017).

The pathogenicity of *S. aureus* is also amplified by their ability to form biofilms (Tamar *et al.*, 2022). Biofilm-formation entails attachment to surfaces and growth to produce a complex structure of single or multiple cell types surrounded by an extracellular matrix (Lim *et al.*, 2013; Cassat *et al*., 2014; Vestby *et al*., 2020). *Staphylococcus* species can form biofilm in bulk-tanks that are used to store milk (Lianou *et al*., 2021). This may affect the quality and quantity of milk thus resulting in huge economical loss and severe health implications on consumers (Lianou *et al*., 2023). During biofilm formation, the extracellular polymers produced by the microorganisms facilitate matrix formation and attachment to solid surfaces (Rhoads *et al*., 2012; Moormeier and Bayles, 2017; Bumunang *et al*., 2020). In addition, to

enhance the growth, biofilms alter the phenotype and gene transmission patterns of the now "complex" microbial communities. Therefore, biofilm-forming ability of bacteria species further complicates the global antibiotic resistance challenge (Lim *et al.*, 2013; Cassat *et al*., 2014; Vestby *et al*., 2020) especially with the acquisition of the potential to escape host immune responses by the strains (Cha *et al*., 2013; Kot *et al*., 2018; Chen *et al*., 2020; Vestby *et al*., 2020).

The 2030 Agenda for Sustainable Development, adopted by all United Nations Member States in 2015 (WHO, 2015), provides a shared blueprint with seventeen Sustainable Development Goals (SDGs), formulated as an urgent call for action by all countries in a global partnership approach. Of great importance, Goal 3 that aims to "Ensure healthy lives and promote well-being for all at all ages" is affected by health care challenges including but not limited to the growing bacterial resistance and their enhanced pathogenicity. In order to contribute to some of the adopted intervention strategies such as the Global action plan on antimicrobial resistance (WHO, 2015), and specifically the objectives; to strengthen the knowledge and evidence base through surveillance and research, to reduce the incidence of infection through effective sanitation, hygiene and infection prevention measures and to optimize the use of antimicrobial medicines in human and animal health, microbial source tracking may be useful in determining points of contamination.

Microbial source tracking (MST) methods are intended to discriminate between human and non-human sources of microbial contamination (Griffith *et al*., 2003). They allow for the identification of sources that is critical for management of microbial contamination. Microbial typing methods that range from phenotypic to genotypic techniques provide valuable information for monitoring the occurrence, spread, and diversity of pathogens and

these generate data that may be relevant in supporting healthcare systems in the management of infections (Montso *et al*., 2022; Woodford *et al*., 2011). Molecular typing methods provide an understanding of the origin of bacteria strains, their clonal relatedness and epidemiological differences, as well as their dissemination between various ecological niches (Singh *et al*., 2006; Rhoads *et al*., 2012; Li *et al*., 2017). Various molecular methods have been used in typing *S. aureus* strains which include but are not limited to Pulsed-Field Gel Electrophoresis (PFGE) (Golding *et al.,* 2015), Multi Locus Sequence Typing (MLST) (Saunders and Holmes, 2007), Spa typing, staphylococcal cassette chromosome *mec* (SCC*mec*) (Goudarzi *et al.,* 2016), Repetitive element-based (REP) PCR (Arabestani *et al.,* 2022), Enterobacterial repetitive intergenic consensus (ERIC) PCR (Candan *et al.,* 2013), and Random amplified polymorphic DNA (RAPD) PCR (Pereira *et al.,* 2002). According to Murchan *et al*. (2002), the reliability of a typing method depends on its reproducibility, typeability, discriminatory power, stability, ease of use as well as its interpretation. Although these typing methods may differ from one another with respect to their reproducibility and discriminatory powers (Strommenger *et al*., 2007; Van Belkum *et al*., 2007), they play an important role in accurately distinguishing strains from different clones based on variations in the fingerprints generated. In addition, these methods also provide reliable information on potential cross-contamination for isolates from different and interconnected sources (Murchan *et al*., 2002; Hallin *et al*., 2007; Stefani *et al*., 2012).

1.2 Problem statement

Staphylococcus aureus is a human pathogen that causes several infections worldwide and is commonly associated with nosocomial infections (Merino *et al*., 2009; Giersing *et al*., 2016; Balasubramanian *et al*., 2017; Lubna *et al*., 2023). In addition, the global increase in the emergence of antibiotic resistance among bacteria species is a severe public health challenge

(Moormeier and Bayles, 2017; Bumunang *et al*., 2020). *Staphylococcus aureus* has developed resistance to several antimicrobial agents over the years ranging from penicillin (1942) to methicillin (MRSA) (1961) as well as vancomycin (1997) (Kumar *et al*., 2020; Nandhini *et al*., 2022). The production of beta-lactamases (penicillinase) changes the bacterial surface receptors thus reducing the binding ability and permeability of the antibiotics (Ateba *et al*., 2010; Foster, 2017). Biofilm formation has been reported in *S. aureus* strains and according to Kwon *et al*. (2008), it may be the leading cause of persistent infections associated with the pathogen. The correlation between biofilm forming potentials and antibiotic resistance in bacteria also play a significant role in enhancing the persistence of the pathogenic strains (Kumar, 2012; Akindolire *et al*., 2015; Vestby *et al*., 2020).

Antibiotic resistance in *S. aureus* first emerged in health care settings, then spread to community as well as animal environments. It is worth noting that antibiotics have been used as animal growth promoters in livestock including dairy farms (Kateete *et al*., 2019; Lianou *et al*., 2023; Lubna *et al*., 2023). The misuse of antibiotics as well as the development of tolerance to the drugs are the main leading factors accounting for the increasing acquisition and spread of antibiotic resistance. Studies have shown that milk-borne pathogens pose a serious threat to human health, and account for a large proportion (approximately 90%) of all diseases associated with diary animals or the consumption of contaminated dairy products (Ryser, 1998). *Staphylococcus aureus* is one of the frequently implicated bacterial species in mastitis, a disease that does not only result in severe economic loss, but also imparts negative consequences on human health if milk products from mastitis cows are contaminated with the bacteria species. The subsequent spread of the contaminating organisms to healthy individuals or uncontaminated sources is concerning (Fortuin-de Smidt *et al*., 2015; Fri *et al*., 2020; Akinboye *et al*., 2023). Although various studies have been carried out on

Staphylococcus aureus in South Africa (Ateba *et al*., 2010; Akindolire *et al*., 2015; Fri *et al*., 2020), very little has been reported on the occurrence of MRSA, the genetic strains circulating especially in the dairy industry. This study aimed to assess the antimicrobial resistance profiles, biofilm-forming potentials, and genetic relatedness of *S. aureus* from a milk and milking environment in a dairy farm in the North-West Province of South Africa.

1.3 Research aims and objectives.

1.3.1 Aim of the study

This study aimed to determine the genetic diversities of multidrug-resistant and biofilm-forming *S. aureus* isolated from drinking water troughs, milk and milking environments of a dairy farm in the North-West Province.

1.3.2 Objectives of the study

The specific objectives of the study were:

i) To isolate and identify *S. aureus* from drinking water troughs, milk and milking environment of the dairy farm in the North-West Province using specific PCR assays and sequencing.

ii) To determine the phenotypic and genotypic antimicrobial resistant profiles of *S. aureus* isolates.

iii) To determine the phenotypic biofilm production potentials of the *S. Aureus.*

iv) To determine the genetic relatedness of *S. aureus* using MLST, ERIC, REP, RAPD, and SCC*mec* typing (for MRSA).

CHAPTER TWO

LITERATURE REVIEW

2.1 General Characteristics of *Staphylococcus aureus*

Staphylococcus aureus is a facultative anaerobic, Gram-positive, non-motile, and non-spore forming cocci measuring about 0.5-1.0 µm in diameter and mostly occurring as irregular grapelike clusters. It was first described in 1880 and later differentiated from other Staphylococci by Rosenbach in 1884 by its yellow colony pigmentation. *S. aureus* is a member of the family Staphylococcaceae and genus *Staphylococcus* consisting of about 42 species. *S. aureus* strains are coagulase positive, catalase positive and oxidase negative (Harris *et al*., 2002; Plata *et al*., 2009; Oliveira *et al*., 2018). The size of its genome is approximately 2.8 Mb and according to the genome databases at least seven strains have been reported including 8325, COL, Methicillin Susceptible *Staphylococcus aureus* (MSSA), Methicillin Resistance *S. aureus* (MRSA), N315, Mu50, and MW2 (Foster, 1996; Harris *et al*., 2002; Le Loir *et al*., 2003; Plata *et al*., 2009; Foster and Geoghegan, 2015). Of these, MRSA is a strain of utmost importance causing severe infections in hospitals and the community at large due to the development of resistance to various antimicrobial agents making treatment difficult. In the last two decades, MRSA has been reported in livestock as well as companion, and wild animals (Moellering, 2012; Otto, 2012; McDanel, 2013).

2. 2 Carriage and Transmission of *S. aureus*

Staphylococcus aureus is a commensal bacterium found on the skin and mucosa membranes of humans and warm-blooded animals. Although the ecological niches of *S. aureus* are mostly the skin, mouth, anterior nares, perineal area, pharynx, gastrointestinal tract, genital,

and anal areas, the moist squamous epithelium of the anterior nares is the most frequently colonized site (Baptista *et al*., 2016; Kim *et al*., 2018). Any superficial disruption or damage of the skin may facilitate the penetration of *S. aureus* into the host cells which can reach different sites such as the heart, blood, lungs, and other deep tissues causing a wide variety of infections (Chambers, 2001; Kuroda *et al*., 2001; Foster, 2005; Frank *et al*., 2010; Sakr *et al*., 2018). Mortality and morbidity rates associated with infections are very high in bacteria such as *Pseudomonas aeruginosa* and *S. aureus* ranging between 40% and 20% to 25% respectively (Wood *et al*., 2023; Zhang *et al*., 2023). In general, humans and animals are persistent carriers of *S. aureus* with approximately 20-30% of the population whereby the bacterium is harmless and causes no disease (Plata *et al*., 2009). These carriers are more prone to staphylococcal infections as well as serving as reservoirs for the spread of *S. aureus* strains in the population. Nonetheless, at least 80% of carriers who get infected with the pathogen are health care workers, hospitalized patients, immunocompromised individuals, and persons with chronic disease (Bien *et al*., 2011; Otto, 2014; Oliveira *et al*., 2018).

Generally, *S. aureus* strains can be transmitted through person-to-person contact with infected animals, and the consumption of contaminated products (Grema *et al*., 2015). The pathogen can also be transmitted from health care environments, workers, patients (for example infected surgical wounds) and instruments (such as contaminated feeding tubes and catheters) to other patients or workers. *S. aureus* can also spread from mother to child following delivery (Schaumburg *et al*., 2014). It has also been reported that children above 5 years play an important role in the transmission of the bacteria due to their age-related activity-associated behavior including frequent sharing of items with peers (Sakr *et al*., 2018). In livestock, *S. aureus* can easily spread from an infected animal to healthy workers or consumers and *vice-versa*. Consumption of contaminated animal products is also a

contributor to the acquisition of the pathogen (Cooper *et al*., 2004; Vanderhaeghen *et al*., 2010). The major public health concern is the emergence of MRSA for which *S. aureus*-colonized persons are at higher risk to develop MRSA infections. It is also worth noting that biofilm-forming strains play an important role in the persistence of the pathogen and thus increase the likelihood of their transmission (Marshall et *al*., 2004; Cepeda *et al*., 2005; Smith *et al*., 2018).

2.3 Epidemiology and diseases caused by *S. aureus*.

S. aureus easily switches from being a normal flora to a pathogen and is therefore opportunistic in nature and has been associated with many outbreaks worldwide (Solberg 2000; van den Honert *et al*., 2021; Strasheim *et al*., 2021). Several studies conducted in South Africa have reported the occurrence of *S. aureus* in hospital environments, faeces, meat, pork, poultry, water, and soil, representing a health risk to those exposed and susceptible population (Cotton *et al*., 2008; Akanbi *et al*., 2017; Amoako *et al*., 2020; Shi *et al*., 2021; Sineke *et al*., 2021). MDR *Staphylococcus aureus* containing enterotoxin genes have also been isolated from retail foods and vegetables (Wang *et al*., 2017; Wu *et al*., 2018), a source of severe health risk to consumers (Mourabit *et al*., 2021).

MRSA infections have increased recently due to the overuse of various antibiotics to treat infections (SA Health, 2016; Smith *et al*., 2018; Borg and Camilleri, 2021). The prevalence of vancomycin resistance *S. aureus* (VRSA) has also increased over the years, mostly in Asia and Africa, from 2% in 2006 up to 7% in 2020 (Wu *et al*., 2021). *Staphylococcus aureus* food-borne disease outbreaks have been reported in several countries including Germany, Belgium, Denmark, United States, United Kingdom, and some Asian countries (Chen and Huang, 2014; Smith *et al*., 2018). Senegal, Ghana, Nigeria and South Africa have also

reported a high prevalence of antimicrobial resistant-associated with *S. aureus* infections (Egyir *et al*., 2014; Akindolire *et al*., 2015; Owusu-Kwarteng *et al*., 2020; Leone *et al*., 2022). This high incidence of infection in developing countries is thought to be due to inappropriate use of antibiotics, mostly resulting from self-medication (Jamhour *et al*., 2017; Gras *et al*., 2020). Migration across countries may also accelerate the spread of *S. aureus*. The first significant outbreak was reported in Michigan, USA in 1884 and it affected a considerable population due to the consumption of Staphylococci-contaminated cheese (Kadariya *et al*., 2014). In 1914, SFP due to the consumption of unrefrigerated milk from mastitis cows was reported and in 1936, food poisoning was reported in students who had eaten contaminated cream puffs (Hennekinne *et al*., 2012). To date, there is still a constant increase of outbreaks that involve Staphylococci species. Most recently *Staphylococcus capitis* has also been reported to be associated with food-borne diseases (Heath *et al*., 2023).

Food-borne diseases associated with *S. aureus* are due to the consumption of food or water contaminated with enterotoxigenic strains of *Staphylococcus* spp. These toxins are resistant to conditions such as high temperatures, pH, gamma irradiation, freezing, dehydration and high sodium chloride concentration (Angelillo *et al*., 2000; Hazariwala *et al*., 2002; Argudín *et al*., 2010; Kadariya *et al*., 2014; Xu *et al*., 2016). It is mostly associated with hand-prepared food for direct consumption (Akindolire *et al*., 2015; Xu *et al*., 2016). Generally, there is a potential for an increased food contamination outbreak in developing countries due to the conditions under which these foods are handled. Improper processing, preparation, production, and distribution of food may enhance the number of pathogens on the product (Hennekinne *et al*., 2012; Kadariya *et al*., 2014). For SFP to occur, there should be (i) an enterotoxin source which can be raw materials, or healthy or infected *S. aureus* carrier, (ii) transmission of the enterotoxin to the food due to unsterilized or unclean food preparation

equipment, (iii) enrichment of food with favorable physicochemical element for *S. aureus* growth, (iv) adequate conditions (temperature, time or pH) to produce toxin and lastly (v) the consumption of food with enough toxins to incite symptoms (Angelillo *et al*., 2000; Hennekinne *et al*., 2012). Manifestation of SFP is rapid, approximately one to five hours after ingestion of food with toxins. The most common symptoms of SFP are abdominal cramps, nausea, vomiting and sometimes followed by diarrhea (Hazariwala *et al*., 2002; Le Loir *et al*., 2003; Kadariya *et al*., 2014).

2.3.1 Mastitis

Dairy farms are agricultural environments where activities such as milk production, soil and pasture management, water irrigation and abstraction, farm management and animal health take place (Groot and van't Hooft, 2016; Cabrera and Fadul-Pacheco, 2021; Singh *et al*., 2021). Products from these farms are widely consumed worldwide such as milk, and beef. One of the frequently reported diseases associated with milking animals is mastitis. Mastitis is an infection that damages milk-producing tissue of mammals, therefore affecting the quality of the milk. *S. aureus* is one of the major causes of mastitis in dairy cattle due to its ability to produce toxins that damage cell membranes (Ateba *et al*., 2010; Petersson-Wolfe *et al*., 2010; Akindolire *et al*., 2015). Milk from mastitis cows, therefore, poses a health risk to the consumers, especially in cases involving antibiotic resistant strains. Mastitis also causes huge economical loss in the milking industry (Moellering, 2012; Otto, 2012; Tanih *et al*., 2015; Perillo *et al*., 2022).

2.4. Antibiotic resistance in *S. aureus*

Antibiotics are widely used in modern medicine for the treatment of animal and human bacterial infections. However, there is an increasing prevalence of antibiotic resistance with a negative consequence on public health. Increasing emergence of antibiotic resistance has

been attributed to their extensive use as growth promoters in animal food production, and their inappropriate use as therapies (Kumar, 2012; Turner *et al*., 2019; Fri *et al*., 2020). Some bacteria have developed resistance not only to one class of antibiotics but many. These multidrug-resistant bacteria are associated with an increased morbidity, hospital stay and mortality (Frieri *et al*., 2017; Silva *et al*., 2020). Although the mechanism of antibiotic resistance in *S. aureus* is not fully understood, studies have revealed that horizontal gene transfer by plasmids and chromosomal mutation of genes are the main mechanisms of acquisition for which the misuse of or prolonged antibiotic therapy are promoting factors (Tenover, 2006; Pantosti *et al*., 2007; Foster, 2017).

Antibiotics act in various ways which include interfering in (1) cell wall synthesis (2) integrity of the plasma membrane; (3) synthesis of nucleic acids; (4) ribosomal activity; and (5) the synthesis of folates. The bacteria cell wall has Penicillin-Binding Proteins (PBPs) that reduce affinity to antibiotics, especially the beta-lactams and glycopeptides (Dörr *et al*., 2019). Antibiotic efflux pumps lead to a low concentration of the antibiotics in the cell, a mechanism that drives macrolides, tetracyclines and fluoroquinolones resistance (Douafer *et al*., 2019). At the nucleic acid site, antibiotic resistance is associated with mutation. Clinical pathogens are able to produce enzymes (topoisomerase and RNA polymerase) that block the effect of antibiotics (fluoroquinolone and rifampicin) (Lade and Kim, 2021). For the ribosomes target site, the function of antibiotic is to inhibit the protein synthesis between the host and bacteria. These antibiotics include macrolides, lincosamides, aminoglycosides, and tetracyclines (Foster, 2017; Lade and Kim, 2021).

2.4.1 Evolution of antibiotic resistance in *S. aureus*

2.4.1.1 Penicillin resistance

The discovery of penicillin by Alexander Fleming in 1928 was a great success in healthcare as there was a great decline in the high mortality caused by *S. aureus* (Ventola, 2015) This antibiotic was commonly used in the 1940s for the treatment of various infections including staph infections. A few years after the introduction of penicillin, *S. aureus* developed resistance primarily due to the production of beta-lactamase that hydrolyses penicillin (Moellering, 2011; Otto, 2012). Plasmids may play an essential role in the production of penicillinase and are transmitted by transduction and conjugation among *S. aureus* strains (Foster, 2017). *BlaZ* gene encodes β-lactam antibiotics in *S. aureus* and is carried by transposon which can be incorporated in the large plasmid or in the bacteria chromosome (Kumar, 2012; Dweba *et al*., 2018). Secondly, resistance can be due to the inhibition of the transpeptidation domain of PBPs (penicillin-binding proteins) which can block the synthesis of bacteria cell wall. The reduced affinity of antibiotic on bacterial surface receptors is associated with the chromosomal expression. In this case the surface receptors have the low affinity to bind to the beta-lactam antibiotics (Pantosti *et al*., 2007). An example is the emergence of MRSA strains which have a low affinity to PBP2a. This strain has emerged mostly in hospital units and spread to animals, especially livestock and community environment (Stapleton and Taylor, 2002; Kumar, 2012). Also, *S. aureus* develops tolerance to various antibiotics by staying for a long time in the dormant stage to avoid being killed (Kumar, 2012).

2.4.1.2 Methicillin resistance

To find a quick solution against penicillin resistant *S. aureus* (PRSA), methicillin was introduced by Beecham as an alternative in 1959 to treat staph infections. Within two years

after its introduction, resistance rapidly emerged giving rise to methicillin resistance *S. aureus* (MRSA) (Moellering, 2012; Otto, 2012). The resistance developed against methicillin was related to the acquisition of the *mecA* gene, which is carried on the mobile genetic element, the staphylococcal chromosome cassette *mec* (SCC*mec*) (Hiramatsu, 2001; Enright *et al*., 2002; Moellering, 2012). The *mecA* gene encodes Penicillin-binding protein (PBP2a) which has a low-affinity for penicillin and other β-lactam antibiotics. MRSA is one of the MDR bacteria that cause life-threatening diseases which are difficult to treat with conventional antibiotics (Appelbaum, 2007; Otto, 2012; Chon *et al*., 2017). MRSA was initially thought to have developed in health care environments, termed Hospital-acquired MRSA (HA-MRSA) (Bai *et al*., 2021; Liu *et al*., 2021), then spread to the community, namely, Community-acquired MRSA (CA-MRSA) (Aires-de-Sousa, 2017; Mitevska *et al*., 2021) as well as in animals, known as Livestock-associated MRSA (LA-MRSA) (Doulgeraki *et al*., 2017; Chon *et al*., 2017).

2.4.1.3 Vancomycin resistance and other antibiotic resistance

Due to high prevalence of methicillin resistance in staphylococcal infections, as rescue, vancomycin which targets bacteria cell wall synthesis was introduced in the 1980s. The emergence of vancomycin resistance took a long time to be identified. The first case was identified in 1996 in Japan (Hiramatsua *et al*., 1997). The development of vancomycin resistance is due to reduced affinity to the target site preventing cell wall destruction by the antibiotic. In addition, mutation has been involved in vancomycin resistance and at least six mutations may occur in different genes involved in cell wall for resistance to occur (Appelbaum, 2007; Sakoulas and Moellering, 2008; Foster, 2017).

Daptomycin, linezolid, teicoplanin and quinupristin/dalfopristin are therefore the antibiotics of choice for the treatment of MRSA infections (Welte and Pletz, 2010; Nigo *et al*., 2017).

2.5 Molecular typing of *Staphylococcus aureus*

Staphylococcus aureus has emerged widely giving rise to many new strains arising from the same ancestor that may differ according to the loci on the DNA chromosome. Many typing methods have been used to distinguish unrelated strains of *S. aureus* including 16S rRNA, MLST, REP, ERIC and RAPD typing.

2.5.1 16S rRNA sequencing

Pathogens can be quickly and precisely identified in cases of suspected infection which can be helpful for the treatment of patients. The 16S ribosomal RNA (rRNA) is among the most conserved bacterial regions that can be used for identification and characterisation (Kai *et al*., 2019; Marizzoni *et al*., 2020). It contains fewer evolutionarily restricted areas and variable regions. The sequences of these regions can facilitate the comparison of phylogeny and taxonomic pathogenic bacterial species from the environment. 16S rRNA can be used to classify organisms into new clades, identify new pathogens, or reclassify strains (Johnson *et al*., 2019; Kai *et al*., 2019)

There are many technologies involved in DNA sequencing of 16S rRNA, which include Illumina's MiniSeq, Sanger, Ion Torrent Proton, Roche 454 sequencing, and MinION. They are more flexible, rapid, and give good result even if they are expensive (Sanschagrin and Yergeau, 2014; Pichler *et al*., 2018; Kai *et al*., 2019; Fujiyoshi *et al*., 2020).

2.5.2 Multilocus Sequence Typing (MLST)

Multilocus Sequence Typing (MLST) has been developed to determine the levels of diversity on a nucleotide sequence present in a loci of species (Trindade *et al*., 2003). MLST has been used to investigate the epidemiology evolution of *S. aureus*. It is highly discriminatory and is

based on the sequence of seven internal fragments of housekeeping genes. The analysis of the housekeeping genes sequence data allows the understanding of the differences among *S. aureus* strains (Cooper and Feil, 2004; Melles *et al*., 2004; Sit *et al*., 2017). MLST can be used to track the source of epidemic associated with animal or environmental reservoirs as well as the emergence and spread of antibiotic resistance (Cooper and Feil 2004).

2.5.3 REP-, RAPD- and ERIC-PCR

Repetitive Extragenic Palindromic sequence-based polymerase chain reaction (Rep-PCR) was first described in 1984 by Stern and co-authors (Krych *et al*., 2018). It is a reliable typing method that greatly explains the genome elements present in bacteria strains. The REP sequence is distributed in multiple copies on the bacteria chromosome (Johnson and O'Bryan, 2000). Rep-PCR is useful for molecular diagnosis as it can establish relationships among bacteria strains, and investigate disease outbreaks (Ishii and Sadowsky, 2009; Naghoni *et al*., 2017; Hossain *et al*., 2022).

To study the microbial genomes of bacteria, the repetitive elements have been divided into three techniques which include repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, and BOX elements (Rampadarath *et al*., 2015). Both REP and ERIC sequences are highly conserved regions with 30-40 bp and 124-127 bp respectively. They are located in the noncoding but transcribed bacteria chromosomes. While Rep is located on the palindromic stem, ERIC is found on the intergenic region of the bacteria genome (Rademaker *et al.,* 2004). Many studies carried out on *Staphylococcus aureus* have used REP- or ERIC-PCR to understand the genetic relatedness between isolates and their implication in the origin or spread of infections (Reinoso *et al*., 2007 and 2008; Zhong *et al*., 2009; Mkhize *et al*., 2021; Arabestani *et al*.,

2022). Due to its wide distribution among species. Enterobacterial Repetitive Intergenic Consensus sequence-based polymerase chain reaction is mostly used to determine the relatedness among strains.

Randomly Amplified Polymorphic DNA (RAPD)-PCR was described by Williams *et al*, in 1990. This technique is a useful in identifying genomic polymorphism. The method is based on the amplification of multiple genomic loci using a single short sequence of oligonucleotide primer usually 10 nucleotides (Neela *et al*., 2005; Kumar and Gurusubramanian, 2011). At low annealing temperature, the primer is likely to detect several sequences within the template DNA (Williams *et al*., 1990). RAPD can be used to identify species and subspecies as well as the genetic diversity in *S. aureus* (Hermans *et al*., 2000; Yoon *et al*., 2022). It has frequently been used to identify the spread of infection associated with *S. aureus*. Isolates are classified according to the band patterns obtained and can differentiate closely related isolates (Otokunefor *et al*., 2020). These aforementioned PCR techniques have advantages such as their ease of use, speed, low cost and high discriminatory power. However, they are less reproducible (Olive and Bean, 1999; Candan *et al*., 2013; Ranjbar *et al*., 2017).

2.6 *S. aureus* and Biofilm formation

Staphylococcus aureus can form biofilms which is one of the main causes of treatment failure (Kwon *et al.*, 2008; Lim *et al*., 2013; Bazzoun *et al.*, 2014). Formation of biofilm involves five phases/steps: adherence, attachment, accumulation, maturation, and dispersal. In the first step, a substrate is needed for adherence to occur which could be biotic or abiotic. Such substrates include cartilage, bone, catheters, teeth, orthopaedic devices, and other equipment. The adherence can be reversible or irreversible and involves the production of specific

adhesive molecules such as the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). *Staphylococcus aureus* can produce approximately 20 matrix proteins that enhance its initial attachment (Cassat *et al*., 2014; Asmaa *et al*., 2016). The second step, attachment, is an irreversible action where bacteria floating cells attach to a surface. These bacteria floating cells constitute extracellular polymeric substance (EPS), lipopolysaccharides (LPS), fimbriae, pili, and flagella (Lim *et al*., 2013; Cassat *et al*., 2014). During the accumulation phase, bacteria attach to each other to form a thin layer. This is facilitated by the polysaccharide intercellular adhesin (PIA) determinant through intercellular adhesion *ica* operon (icaABCD). Other proteins that are involved include, biofilm associated protein (Bap), *S. aureus* surface protein C (SasC), *S. aureus* surface protein G (SasG), and accumulation-associated protein (Aap) (Foster and Geoghegan, 2015; Rabin *et al*., 2015). During maturation, microorganisms produce adhesive matrix that mediate multiplication and enhance bacteria to form multi-layers of micro-colonies. During this stage, there is a high rate of bacteria replication that promotes the transmission of genes from one species to another including the transfer of antibiotic resistance genes and virulence genes to susceptible species (Beenken *et al*., 2004; Vasudevan, 2014; Gebreyohannes *et al*., 2019). Once biofilm has formed, bacteria are able to disperse and colonize new niches where nutrient and oxygen are available and the cycle begins again (Cassat *et al*., 2014).

In industries, including dairy, dentistry and other healthcare, food, water and mining, biofilms can develop on every surface and equipment and cause product spoilage, corrosion, infections, pipe blockages, and equipment failure that causes huge economic losses. Once bacteria in biofilms have attached to a substrate, they are very difficult to remove using conventional procedures such as sanitizing due to their resistance to chemical components. Most *S. aureus* isolated from clinical settings and equipment have been shown to be strong

biofilms producers (Donlan, 2002; O'Toole, 2003; Garrett *et al*., 2008; McCarty *et al*., 2014; Rabin *et al*., 2015).

Staphylococcus aureus produce various adhesin proteins such as elastin, clumping factor, collagen, fibronectin binding proteins which belong to MSCRAMMs element that enhance its ability to form biofilms. Many studies have identified *S. aureus* as a cause of recalcitrant biofilm limiting the efficacy of antimicrobial agents (Brady *et al*., 2011; Hanke *et al*., 2013; Jia *et al*., 2014; Sakr *et al*., 2018).

2.6.1 Biofilms and the dairy industry

Biofilms can affect the quality and safety of food products because biofilms can form on the surfaces of devices used in food production. Biofilms associated with milk are very challenging to eliminate by sterilization (Reffuveille *et al*., 2017; Avila-Novoa *et al*., 2018). Dairy animals are exposed to a wide range of infections associated with biofilms. Mastitis in cows has been reported to be associated with biofilms, where *S. aureus* strains were the major implicating organism. Infection of the mammary gland causes a huge loss in animal reproduction, their products, and the economy. In such a scenario, inadequate treatment of milk may pose a health threat to humans (Raza *et al*., 2013; Zapotoczna *et al*., 2016).

2.6.2 Biofilm and Antibiotics resistance

Biofilms, particularly the polysaccharide in the matrix that enhances physiology, prevents antibiotics from sufficiently penetrating the bacteria cells as well as escaping the host immune responses (Cha *et al*., 2013; Cassat *et al*., 2014). Therefore, biofilm producing organisms are the leading cause in persistent infections. In addition to antibiotics, biofilm-disrupting vaccines have been used for therapy against infections associated with biofilms. A combination of antibiotics and vaccines may show considerable reduction in pathogenic

biofilm bacteria as well as antimicrobial resistant bacteria compared to antibiotic treatment alone (Brady *et al*., 2011; Lim *et al.*, 2013; Buchy *et al*., 2020).

2.7 Virulence factors and pathogenesis of *S. aureus* infections

The ability of *Staphylococcus aureus* to establish infection depends on the expression of cell wall proteins, which consist of adherence factors and exoproteins (Arvidson and Tegmark, 2001; Matsumoto *et al*., 2007). The exoproteins include toxins and enzymes that mediate host cell lysis whereas adherence factors are associated with colonization and evasion of the host immune system (Bien *et al*., 2011; Moran *et al*., 2019).

2.7.1 Adherence factors

Staphylococcus aureus makes use of various adhesins to attach to host surfaces either via blood plasma or extracellular matrix (Harris *et al*., 2002; Frantz *et al*., 2010). Most of these adherence factors are found on the bacteria's cell wall and are referred to as surface proteins or cell wall-anchored (CWA) proteins (Lacey *et al*., 2019). The CWA proteins are covalently attached to cell wall peptidoglycan by the enzyme sortase A. Approximately 25 CWA proteins are secreted by *S. aureus*, and they play an important role in adhesion, as well as in invasion, biofilm formation, or evasion of host immune responses (Foster *et al*., 2014; Jan-Roblero *et al*., 2017). Currently, *S. aureus* CWA proteins are classified into four major families: (i) microbial surface components recognizing adhesive matrix molecule (MSCRAMM); (ii) near iron transporter (NEAT) motif (iii) the three-helical bundle and (iv) G5-E repeat (Paharik and Horswill, 2016; Foster, 2019).

The MSCRAMM family is the largest group of CWA proteins which are extracellular polysaccharides (poly-N-acetylglucosamine (PNAG) or polysaccharide intercellular adhesins (PIA). *S. aureus* adhesins mainly consist of collagen-binding protein, clumping factor (Clf) A

and B, fibronectin-binding proteins A and B (FnbpA and FnbpB) and staphylococcal protein A (SpA) (Frantz *et al*., 2010; Bien *et al*., 2011; Costa *et al*., 2013).

Clumping factors are cell wall-anchored proteins that enhance the virulence mechanism of *S. aureus*. They are the surface receptors of host fibrinogen with a high affinity to attach to host surfaces. Therefore, clumping factor enhances the clumping of blood plasma of the host when exposed to *S. aureus* (Undas and Ariens, 2011; Lacey *et al*., 2019). Diseases associated with clumping factors include arthritis, endocarditis, sepsis, and thrombosis. In addition, clumping factors play an important role in the formation of biofilms, mediating the attachment of the bacteria on a surface and to each other (Wang *et al*., 2017; Wolz *et al*., 2020). Two types of clumping factors, protein A and B, have been described which have the same molecular structure with a high homologue in their genomic sequence (Herman-bausier *et al*., 2018).

Clumping factor A (ClfA) immobilizes fibrinogen or fibrin on coated-surfaces, initiating infection. ClfA has been shown to mediate the clumping of *S. aureus* when exposed to an implanted biomaterial coated with fibrinogen in an *in vitro* experiment. (Josefsson *et al*., 2001; Li *et al*., 2016). ClfA is associated with staphylococcal septic arthritis, sepsis, endocarditis, and skin infection. (Ni Eidhin *et al*., 1998; Luczak-Kadlubowska *et al*., 2006; Lacey *et al*., 2019). Clumping factor A consists of various regions that facilitate the adherence of *S. aureus* to the cell wall of the host. These regions contain an N-terminal secretory signal peptide which contains the fibrinogen binding domain, the 520-residue region A which has the ligand-binding region that bind to the γ-chain of fibrinogen, the R region whose function is to expose the ligand binding domain on the cell surface and the C-terminal domain that allows the anchoring of the ClfA protein to the cell wall of host surface (Ni Eidhin *et al*., 1998; Higgins *et al*., 2006; Herman-bausier *et al*., 2018). Clumping factor B (ClfB) mediates the colonization of *S. aureus* to the nasal cornified envelope of the host. ClfB can bind to proteins such as the plasma fibrinogen, cytokeratin and loricrin contained in the

squamous cells of the host. Skin and soft tissues infection (SSTI) results from the interaction of these ligand-binding proteins (Sakr *et al*., 2018; Lacey *et al*., 2019).

Fibrinogen/fibrin is a major plasma protein that plays an important role in blood coagulation when exposed to a bacterium as well as for host immune defense. However, microorganisms have developed various mechanisms to interrupt blood coagulation by binding to a specific receptor, the fibrinogen-binding protein (McDevitt *et al*., 1994; Kot *et al*., 2018). Fibrinogen is crucial for the adherence of bacteria to the host during the development of biofilms as well as for the clumping of blood during the pathogenesis of *S. aureus*. Exposure of *S. aureus* to fibrinogen results in diseases such as wound infection, bacteraemia, septic arthritis and endocarditis (Tristan *et al*., 2003; Geoghegan *et al*., 2013; Pickering *et al*., 2019).

Fibronectin-binding protein (FnBP) facilitates the binding mechanism to elastin, fibronectin and plasminogen during bacteria invasion of the host. This ligand-binding protein facilitates the adhesion of multiple bacteria to the fibronectin receptor of the host by avoiding the detection of bacteria by the immune system (Joh *et al*., 1998; Burke *et al*., 2010). FnBPs is associated with skin and soft tissue infections, urinary tract infections, pharyngitis, and infective endocarditis (Geoghegan *et al*., 2013; Lacey, *et al*., 2016; Jan-Roblero *et al*., 2017).

Collagen-binding protein (cna) is one of the main constituents important in the adherence and invasion of *S. aureus* through the host immune system. It also plays a role in the accumulation phase of *S. aureus* to form biofilms (Foster and McDevitt, 1994). Collagens are mostly found in fibrous tissues such as the bones, tendons, muscles, ligaments, cartilage, blood vessels, surface of damaged heart valves, skin, and aortic tissues. Diseases associated with cna are therefore endocarditis, keratitis, mastitis, septic arthritis, and osteomyelitis (Hienz *et al*., 1996). ‘Dock, Lock and Lactch’ (DLL) is a ligand-binding mechanism that has a high affinity to collagen. *Staphylococcus aureus* used the DLL to facilitate the adhesion of

collagen-binding protein to the host. However, antibodies against *cna* can inhibit the binding process to collagen as well as block the formation of biofilms (Zong *et al*., 2005; Foster, 2019).

Staphylococcal protein A (SpA) is *S. aureus* cell wall anchored protein. SpA plays an important role in the virulence process of *S. aureus*, mediating the adhesion and invasion of the host immune system, therefore inducing pathogenesis. The SpA structure is composed of five immunoglobulin-binding domains (A, B, C, D and E) which can bind to different host ligand proteins (Falugi *et al*., 2013; Lacey *et al*., 2016). The *Spa* gene has a conserved polymorphic variable repeat region which is widely used to classify *S. aureus* strains. These adhesin factors interact with the host Fc-region of immunoglobulin G (IgG) (Foster, 2005). The ligand-binding interaction between SpA and the Fc binding site protects *S. aureus* from being recognized by neutrophils Fc receptor due to the incorrect orientation of IgG antibody. This interaction induces the suppression of the host B-cells and therefore prevents the destruction of the bacterium by blocking the phagocytosis and opsonization of that bacterium (Claßen *et al*., 2011; Jan-Roblero *et al*., 2017). SpA is involved in the development of SSTIs, staphylococcal pneumonia infections, keratinocytes infections, skin inflammations and tissue (Falugi *et al*., 2013; Foster *et al*., 2014; Foster, 2019).

2.7.2 Toxins

Toxins produced by *S. aureus* result in cell lysis of a target cell or tissues. The toxins are grouped into either pore-forming toxin (PFTs), exfoliative toxins (ETs) or superantigens (SAgs). Among these, α-Toxin, β-Toxin, δ-Toxin, Panton-Valentine Leucocidin, Enterotoxin, Exfoliative Toxin and Toxic Shock Syndrome Toxin are mostly associated with Staphylococcal Food Diseases (SFD) outbreak. They have been reported in various parts of the world which includes Canada, England, France, Japan, and United States, Taiwan as well as in Africa (Akindolire *et al*., 2015; Baptista *et al*, 2016; Kong *et al*., 2016; Zhang *et al*.,

2017; Oliveira *et al*., 2018; Samutela *et al*., 2021). These toxins have been identified from *S. aureus* isolated from mastitis cattle, goats, and sheep. Toxins are mostly active in the gastrointestinal tract where they cause the degranulation of intestinal mast cells (Grumann *et al*., 2014). This result in emesis, endocarditis, sepsis, kidney injury, and gastroenteritis. The ability of *Staphylococcus aureus* to cause staphylococcal food poisoning is a major problem in the food industry because SEs are not easily destroyed during the sterilizing process (Kadariya *et al*., 2014; Otto, 2014).

2.7.3 Enzymes

Staphylococcus aureus can produce a wide range of extracellular enzymes that play important roles in disease pathogenesis. These enzymes are able to breakdown the host cells and tissues and convert them into nutrients for bacteria growth. These enzymes include coagulase, hyaluronidase, lipase, nuclease, protease and staphylokinase involved during the infection process as well as in the survival of *S. aureus* during biofilm formation, virulence mechanism, and abscess formation (Edwards-jones and Foster, 2002; Oogai *et al*., 2011; Costa *et al*., 2013).

2.8 Control and prevention of *S. aureus* infections

Staphylococcus aureus is a strain that is difficult to control due to high morbidity, multidrug resistance and biofilm formation. To monitor the spread of that bacteria, isolation of infected patients may have a great impact on the dispersal of the pathogen, cleaning and disinfecting the environment also plays an important role (Bamberger and Boyd, 2005; Coia *et al*., 2006). Regulation in the regular intake of antibiotics may play an important role in reducing the resistance factor. The use of vaccines may enhance the prevention of invasive biofilm-forming strains (Leonard and Markey, 2008; Dayan *et al*., 2016). Hand hygiene and environmental cleaning are methods used to prevent the spread of MRSA strains. Preventing

patient-to-patient contact also plays a great role on the propagation of MRSA (Hare and Thomas, 1956; Morgan *et al*., 2010; Kadariya *et al*., 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical consideration

Ethical clearance for the study was obtained from the North-West University, Faculty of Natural and Agricultural Science (FNAS) Research Ethics Committee and an ethics number NWU-01723-20-A9 was assigned to the study. The study commensed only after the approval was granted.

3.2 Study design, area and site

This was a cross sectional study conducted from March 2021 to June 2021. Samples were collected from the North-West University (NWU) dairy farm, in Molelwane, Mafikeng, North-West Province, South Africa (-25° 51' 54.79" S, 25° 38' 39.16" E). The North-West province spans an area of approximately 106 000 km^2. Livestock production is a major component of agriculture in the area. Products such as milk and meat that are produced from livestock are a source of food for humans and also serve as a source of income to Southern Africa including Mahikeng residents (Rocha *et al.*, 1991).

3.3 Sample collection

The samples were collected three times within a three-month period to estimate the occurrence, antibiograms, biofilm forming potentials and genetic relatedness of *S. aureus* in milk, milking environments and milking personnel. Unpasteurized raw milk samples were collected directly from dairy cattle and storage tanks in the dairy farm while ensuring that strict aseptic procedures were enforced to prevent cross-contamination. Pasteurized milk,

drinking water from troughs, swab samples from the milking environments and personnel as well as the udder of animals were also obtained.

Before sampling, the udder, teat ends and hands samplers were washed with soap, dried and disinfected with cotton soaked in 70% (v/v) ethanol. The milk samples were taken from lactating dairy cows (Figure 3.1) according to an earlier protocol (Quinn *et al.,* 2002). Approximately 1-2 mL of first milking streams were discarded from individual cattle before aseptically collecting 5-10 mL of the composite milk into properly labelled sterile screw cap bottles. The bulk milk samples were obtained after agitation of the tank. Drinking water used by animals was collected from the top and bottom of the troughs into 250 mL sterile bottles. Swab samples were also collected from the teat of each cow prior to milk collection; and from milking equipment (suction pipes liners, bulk tank, pasteurized tank) using sterile cotton-tipped swabs. The cotton swab samples were placed in sterile phosphate-buffered saline (PBS). The milk-room floor drains were also sampled. All samples were transported on ice in cooler boxes to the Antimicrobial Resistance and Phage Biocontrol Research Laboratory for analysis. Table 3.1 shows the number and type of samples collected during the study.

Table 3.1: Source and number of samples collected during the study.

Sampling type	**Number of samples**
Raw	11
Pasteurized milk	2
Drinking water from troughs	4
Teat swabs	8
Swab of bulk tank	4
Swab of the suction pipes	8

Mixed wastewater from the parlour	7
Milking parlour floor drainage	3
Total	**47**



Figure 3.1: Suction pipes from which milk samples were collected.

3.4. Bacterial Isolation

In order to isolate staphylococci from the samples, 1 mL of each milk sample was added into 9 mL sterile 2% (w/v) peptone water (Biolab, South Africa). An aliquot of 100 µL of the diluted milk samples was spread-plated on mannitol salt agar (MSA) (Merck, Darmstadt, Germany) and the plates were incubated aerobically at 37 ˚C for 24 hours. Similarly, aliquots of 100 mL from each water trough samples were aseptically filtered through 0.45µm sterile membrane filters (Pall Corporation, USA) using a pressure pump (Model Sartorius 16824). Sterile forceps were used to transfer the membrane filters onto MSA (Biolab, South Africa) and the plates were incubated aerobically at 37 ˚C for 24 hours. Swab samples were directly streaked onto MSA plates and incubated aerobically at 37 °C for 24 hrs. After incubation, isolates with typical large pink to yellow-white domed morphotypes were considered as presumptive *S. aureus* and these colonies further purified by sub-culturing onto fresh

prepared MSA plates (Biolab, supplied by Merck). The plates were incubated aerobically at 37 °C for 24 hours. Pure isolates were Gram stained based on a standard procedure (Cruikshank *et al*., 1975) and later subjected to specific biochemical tests (catalase test, coagulase test and mannitol fermentation). Isolates that were Gram-positive cocci that appear in clusters, catalase positive, and coagulase positive were further preserved in 25% (v/v) glycerol/water (stabilizes the frozen bacteria, preventing damage to the cell membranes and keeping the cells viable) and stored at -80 °C for further analyses.

3.5. Bacterial control strains

Staphylococcus aureus (ATCC 25923) and *S. aureus* (ATCC 43300) were used as positive control for MRSA strains while *Escherichia coli* (ATCC 43888) was used as a negative control for all analytical procedures.

3.6 Confirmation of identities of isolates

3.6.1 Genomic DNA extraction

Genomic DNA was extracted from all the presumptive *S. aureus* isolates and control strains using a modified CTAB method as previously described (Minas *et al*., 2011). In brief, pure cultures of the presumptive isolates were inoculated in 5 mL of Tryptic Soy Broth (TSB) and incubated at 37 °C for 24 hours. The optical density of the cultures was monitored to ensure that exponential phase cells were used in the DNA extraction procedure. Approximately 1.5 mL of exponential phase cells were transferred into 2 mL eppendorf tubes and centrifuged at 13500 × g for 15 minutes. The supernatant was discarded and the procedure was repeated three times to increase the quantity of the pellet. A volume of 200 µL of Cetyltrimethylammonium bromide (CTAB) lysis buffer was added to each pellet, vortexed

briefly (Scientific Industries SI-D258 Disruptor Genie Analog Mixer, Europe) and incubated in a Digital Dry Bath (BIO-RAD Techne, Taiwan), at 65 ^{o}C for two hours. After incubation, 200 µL of chloroform: isoamyl alcohol (24:1) was added to the tube, mixed thoroughly and centrifuged at 8000 *xg* for 15 minutes to create a phase separation. The upper aqueous phase was carefully transferred to a new tube. The process was repeated twice, and the final aqueous phase was used for further downstream procedures. An aliquot of 180 µL of the top layer was transferred to clean microcentrifuge tubes. After this, 360 µL of chilled isopropanol was added to the top layer to allow the precipitation of DNA and incubated overnight at -20 ^{o}C. The following day, tubes were centrifuged at 8000 x g for 15 minutes and the supernatant discarded. The pellet was washed twice with 360 µL of chilled 70% (v/v) ethanol, centrifuged at 8000 × g for 15 minutes and the supernatant was discarded on each occasion. The pellet was air-dried at room temperature (25 ^{o}C) and 60 µL of chilled TE buffer was added to the tubes and vortexed in order to reconstitute the DNA. The DNA were stored at -20^{o}C for separation by agarose gel electrophoresis and further use in PCR assays.

3.6.2 Amplification of 16S rRNA gene

DNA from presumptive isolates were subjected to bacterial 16S rRNA PCR analysis using universal primer sequences and according to the protocol of Zwart *et al*. (2002). The primer sequences used in the amplification process are shown in Table 3.2. The PCR was prepared as standard 25 µL volumes containing 12.5 µL master mix, 0.5 µL of both forward and reverse 16S rRNA primers, 7 µL of nuclease-free water and 5 µL of template DNA. The PCR amplification was conducted in a T3000 thermocycler (Bio-Rad, USA). PCR products were resolved by agarose gel electrophoresis.

3.6.3 Species-specific PCR

The *gap* gene fragments encoding glyceraldehyde-3-phosphate dehydrogenases were targeted for confirming the identities of potential staphylococci. The oligonucleotide primer sequences and cycling conditions that were used in these amplification procedures are shown in Table 3.3. PCR reactions were prepared as 25 µL volumes consisting of 7 µL of nuclease-free water, 12.5 µL of 2X master mix, 0.25 µL of each primer, and 5 µL of template DNA.

3.7 Antibiotic resistant profiles of isolates

3.7.1 Antibiotic susceptibility of *S. aureus*

Confirmed *S. aureus* isolates were subjected to antibiotic susceptibility testing using the Kirby-Bauer disk diffusion technique on Mueller-Hinton agar as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines. The antibiotic resistance profiles of the isolates was determined against a panel of eleven antimicrobial agents (Table 2). These antibiotics were chosen based on their frequent use in the treatment of *S. aureus* and other bacterial infections as well as their inclusion in the susceptibility testing for Staphylococcaceae according to CLSI (CLSI, 2018). For each confirmed *S. aureus* isolate, a fresh culture was grown from the glycerol stock by sub-culturing on Tryptic Soy Agar (TSA) and the plates were incubated aerobically at 37 °C for 24 hours. Bacterial suspensions were prepared by inoculating colonies of each isolate in 0.9% (v/v) saline solution. The turbidity of the broth culture was adjusted to 0.5 McFarland standard (equivalent to 1.0×10^8 cfu/mL). Aliquots of 100 µL of each bacterial suspension was spread-plated onto Muller Hinton Agar (MHA) plates. The discs impregnated with various but recommended concentrations of antibiotics were aseptically placed on the inoculated plates. The plates were incubated aerobically at 37 °C for 24 h. After incubation, the diameter of the zones of inhibition were measured and recorded. These diameters were classified to classify isolates as resistant,

intermediate resistant, or susceptible to a particular antimicrobial agent based on standard reference values (CLSI, 2018).

3.7.2 Detection of Methicillin Resistant *S. aureus*

Drug resistant *S. aureus* were screened to detect the presence of methicillin resistant traits using the Kirby-Bauer disc diffusion method (Kirby *et al.,* 1966). The antibiotic disc Cefoxitin (30 µg) obtained from bio-Mérieux, USA) was used to assess the resistance profiles of the isolates and hence their MRSA status according to the CLSI guidelines (CLSI, 2018). Cefoxitin and moxalactam have been reported as surrogate markers for the detection of methicillin resistance especially in *S. aureus* (Felten *et al.*, 2002; Skov *et al.,* 2003).

Table 3.2: List of antibiotic disks used in the study.

Group	**Antibiotics**	**Abbreviation**	**Concentration of antibiotic in the disc**
Quinolone	Ciprofloxacin	CIP	5 µg
Lincosamide	Clindamycin	CLI	2 µg
β-lactam	Penicillin G	PEN	10 unit
Oxazolidinones	Linezolid	LZD	10 µg
Macrolide	Erythromycin	ERY	15 µg
Aminoglycoside	Gentamycin	GEN	10 µg
Sulfonamide	Trimethoprim-sulfamethaxaxole	SXT	1.25/23.75 µg
Chloramphenicol	Chloramphenicol	CHL	30 µg
Tetracyclines	Tetracycline	TET	30 µg
Rifamycin	Rifampicin	RIF	5 µg
2nd generation Cephalosporins	Cefoxitin	FOX	30 µg

3.7.3. Multi-drug resistance and Multiple Antibiotic Resistance Index (MARI)

Multidrug resistance in *S. aureus* defined as isolates showing resistance to 3 or more antibiotic classes was determined (Rota *et al*., 1996). MARI was used to determine the health

risks associated with antibiotic resistance (Kruperman *et al*., 1983). The formula below was used to determine the MAR index for each isolate.

MARI = a/b; where 'a' represents the number of antibiotics to which an isolate was resistant to and 'b' the total number of antibiotics to which the isolate was exposed to or tested against.

An MARI > 0.2 implies that the antibiotic usage is high and vice versa in the environment where the bacteria strain originates from.

3.7.4 Molecular detection of antimicrobial resistance determinants

S. aureus isolates that were classified as multidrug resistant were subjected to PCR for the detection of the antibiotic resistance genes *mecA*, *blaz*, and *tetO* using specific oligonucleotide primer sequences that appear in Table 3.3 (Kunishima *et al.,* 2010; Al-Abbas, 2012). Each PCR reaction was prepared as 25 µL volumes consisting of 7 µL of nuclease-free water, 12.5 µL of 2X master mix, 0.25 µL of each primer, and 5 µL of template DNA.

Table 3.3: Oligonucleotide primers and cycling conditions used for amplification of bacterial 16S rRNA gene fragments and molecular confirmation of *S. aureus* isolates as well as antibiotic-resistance genes in the isolates.

Gene	**Primer Sequence 5'-3'**	**Amplicon size (bp)**	**Cycling Conditions**
16S rRNA	27F-AGAGTTTGATCCTGGCTCAG 1492R-GGTTACCTTGTTACGACTT	1492	Initial denaturation at 95 ºC for 1 min, followed by 35 cycles of 95 ºC for 40 s, 58 ºC for 1min, 72 ºC for 2 min. Final elongation for 10 min at 72 ºC
gap	F-ATGGTTTTGGTAGAATTGGTCGTTTA R-GACATTTCGTTATCATACCAAGCTG	933	Initial denaturation at 95 ºC for 3 minutes, 35 cycles of 94 ºC for 30 s, 50 ºC for 45 s, 72 ºC for 1 min. Final elongation at 72 ºC for 5 min
mecA	F-AACAGGTGAATTATTAGCACTTGTAAG R-ATTGCTGTTAATATTTTTTGAGTTGAA	174	Initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 64.1 °C for 45 s and 72 °C for 1 min. Final extension at 72 °C for 10 min

blaZ	F-CAAAGATGATATAGTTGCTTATTCTCC R-TGCTTGACCACTTTTATCAGC Ferreira *et al*., 2017	421	Initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 56.8 °C for 45 s and 72 °C for 1 min. Final extension for 10 min at 72 °C.
tetO	F-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA (Abd El-Razik *et al*., 2017)	515	Initial denaturation hot start of 5 min at 94°C was followed by 35 cycles 94°C for 30 s, 51°C for 45 s, 72°C for 60 s. Final extension for 10 min at 72°C.

3.8. Determination of the Biofilm forming potential of *S. aureus* isolates

The biofilm formation potentials of the isolates were determined using the microtiter plate method (Cha *et al*., 2013; Chen *et al*., 2020; Montso *et al*., 2021). An overnight culture of *S. aureus* was prepared at 37 °C in tryptic soy broth (TSB) supplemented with 0.5% glucose. The turbidity of the broth culture was adjusted to an absorbance of 0.08-0.1 at a wavelength of 630 nm using a spectrophotometer (Merck Chemicals, Pty, Ltd, USA). The cell suspension (200 µL) was inoculated in triplicates into wells of a 96 well polystyrene flat-bottom microtiter plate (Lasec, South Africa). The plates were incubated such that each isolate was subjected at 4 °C, 25 °C and 37 °C incubation for 24h, 48h and 72h. After incubation, nonadherent cells (planktonic bacteria) were are rinsed away by gently inverting the microtiter plate on a paper towel. The wells were rinsed gently three times with 200 µL of sterile phosphate buffer-saline [PBS 1X (pH 7.4)] and dried for 30 minutes at room temperature. Bacteria culture attached on the surface of the well were stained with 200 µL of 1 % crystal violet for 15 minutes, then washed five times with PBS, and dried at room temperature. Plates were destained with 200 µL of 95 % ethanol for 10 minutes at room temperature. Then, the cell suspension was measured spectrophotometrically at an optical density (OD) of 600 nm in a Micro-ELISA auto plate reader (MB-580, Zhengzhou, China). The isolates were classified as follows: non adherent: OD ≤ ODc, weak formers: ODc <

OD600 ≤ 2X ODc; moderate formers: 2xX ODc < OD600 ≤ 4X ODc; and strong formers: 4xODc ≤ OD600. The cut-off-value (ODc) was defined as three times the standard deviation (SD) above the mean OD of the negative control. *Pseudomonas aeruginosa* (ATCC 10145) was used as positive control in this experiment (Ghosh *et al*., 2022).

3.9. Molecular typing of *S. aureus*

3.9.1 Multilocus Sequence Typing (MLST)

MLST types can be classified based on the difference in the sequence types (STs) found between each combination of the seven housekeeping loci. The sequences of the seven internal fragments of housekeeping genes, carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*) phosphate acetyltransferase (*pta),* triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*) were amplified by PCR using specific primers listed in Table 3.4 (Enright *et al*., 2000; Saunders and Holmes, 2014). The amplified products were analysed on a 1.5 % (w/v) agarose gel to confirm the presence of the desired products.

Table 3.4: Oligonucleotide primer sequences used for MLST typing.

Gene	**Primer Sequence 5′–3′**	**Amplicon size (bp)**	**References**
arc	F: TTGATTCACCAGCGCGTATTGTC R: AGGTATCTGCTTCAATCAGCG	570	Enright *et al*., 2000 Leeuwen *et al*., 2003 Saunders and Holmes, 2014
aroE	F: ATCGGAAATCCTATTTCACATTC R: GGTGTTGTATTAATAACGATATC	536	
glpF	F: CTAGGAACTGCAATCTTAATC R: TGGTAAAATCGCATGTCCAATTC	465	
Gmk	F: ATCGTTTTATCGGGACCATC R: TCATTAACTACAACGTAATCGTA	429	
Pta	F: GTTAAAATCGTATTACCTGAAGG R: GACCCTTTTGTTGAAAAGCTTAA	575	
Tpi	F: TCGTTCATTCTGAACGTCGTGAA R: TTTGCACCTTCTAACAATTGTAC	402	
yqiL	F: CAGCATACAGGACACCTATTGGC	598	

	R: CGTTGAGGAATCGATACTGGAAC		

3.10. Genetic fingerprinting of *Staphylococcus aureus*

The genetic relatedness of *S. aureus* isolates was determined using the typing methods REP, ERIC, and RAPD PCRs (Candan *et al*., 2013; Montso *et al*., 2022). PCRs were prepared as 25 µL reaction mixtures containing 12.5 µL mastermix, 0.5 µL oligonucleotide primer sequences (Table 3.5), 4 µl of template DNA and nuclease-free water to adjust the final volume. Amplification was conducted in a T3000 thermocycler (Bio-Rad, USA) using cycling conditions indicated in Table 3.5. Aliquots of 5 µL of the amplicons were analysed by electrophoresis using 2% w/v agarose gels stained with ethidium bromide run at 100 V, for 2 hours and visualized using UV light. Based on PyElph software 1.3 (Python Software Foundation, Wilmington, DE, USA) updated by Pavel and Vasile (2012), a phylogenetic tree was constructed from the gel image. The fingerprints were compared and analysed for the presence, absence, and intensity of band data obtained. The phylogenetic trees were constructed based on uncorrected data using UPGMA (Unweighted Pair Group Method with Arithmetic Mean). The arithmetic averages of the measure of dissimilarity, whole-gene alignment, and concise alignment were taken into consideration by the software. The band matching and percentage similarity were estimated at 70%. The discriminatory indices of isolates were determined using Hunter and Gaston's (1988) formula.

Table 3.5: Oligonucleotide primers and cycling conditions used for REP, ERIC and RAPD typing.

Primer	**Sequence 5'-3'**	**Amplicon size (bp)**	**Cycling Conditions**

RW3A	TCGCTCAAAACAACGACACC	Variable	Initial denaturation hot start of 5 min at 94°C was followed by 35 cycles 94°C for 30 s, 51°C for 45 s, 72°C for 60 s. Final extension for 10 min at 72°C.
ERIC2	AAGTAAGTGACTGGGGTGAGCG	Variable	Initial denaturation at 95°C for 2 minutes, 30 cycles of 94°C for 3 seconds, 50°C for 1 minute, 65°C for 8 minutes. Final extension for 8 minutes at 65°C.
RAPD 1283	CCGCAGCCAA	Variable	Initial denaturation at 95°C for 5 minutes, 45 cycles of 94°C for 3 seconds, 36°C for 1 minute, 72°C for 1 minute. Final extension for 10 minutes at 72°C.

3.11. Agarose gel electrophoresis

All other PCR products were separated by electrophoresis on a 1.5% (w/v) agarose (Seakme®, Rockland, USA) gel using Tris-acetate-EDTA (1 X TAE) (40 mM Tris, 1 mM EDTA and 20 mM glacial acetic acid; pH 8.0) in a horizontal agarose gel equipment (BiocomDirect, Bridge of Weir, UK). A volume of 1 µg/ml of ethidium bromide (Bio-Rad Laboratories, Canada) was added to each cooled gel (about 50 ^{o}C) prior gel casting. Five microliters (5 µL) of each PCR product were loaded in each well and electrophoresis was performed at 80 V for 60 minutes. A 100 bp or 1 kb DNA ladder (Fermentas, Glen Burnie, USA) was included in each gel run as a molecular weight standard. A ChemiDoc ™ MP Imaging system (Bio-Rad, Hercules, USA) was used to visualize and capture the gel images.

CHAPTER FOUR

RESULTS

4.1. Occurrence of *Staphylococcus aureus* in dairy farms

4.1.1 Phenotypic and Biochemical screening outcomes

Selective and differential isolation of staphylococci and *S. aureus* using MSA was achieved with 144 isolates identified based on colonial morphologies. Detailed results of the colonial morphologies of the isolates appear in Appendix 1A. The cellular morphologies based on Gram staining revealed that a large propotion (128) of the isolates were Gram-positive cocci occuring in clusters, thus presumptively *S. aureus* (Appendix 1B). Specifically, 128 (88.9%) of the isolated strains were able to ferment mannitol, by forming yellow colonies on MSA, whilst 16 (11.11%) strains were mannitol-negative thus producing light pink colonies. Catalase test, which is essential to differentiate *Staphylococcus* spp. (catalase positive) from *Enterococcus* spp. (catalase negative), gave positive results for 116 (90.62%) isolates. All the isolates were oxidase negative and displayed positive results for the presence of staphylocoagulase (Table 4.1)

Table 4.1: Proportion of *S. aureus* isolated from various sources in the dairy farm.

Sampling source (number of samples)		Presumptive isolates	Gram Positive	Catalase Positive	16S rRNA	*gap*	*mecA*	*blaZ*	*tetO*	*aroE*	*pta*
Raw Milk (*n* =11)		33	29	27	21	20	9	6	2	5	9
Pasteurizise milk (*n* =2)		6	4	3	2	0	0	0	0	0	0
Swabs	Cow teat (*n* =8)	24	23	23	22	20	2	0	0	1	2
	Equipment (*n* =12)	39	33	29	24	16	0	0	0	0	0
Parlour mixed wastewater (*n* = 7)		21	20	16	14	14	12	4	1	8	14
milking parlour drain (*n* = 3)		9	8	8	6	5	3	2	0	2	3
Drinking water (*n* = 4)		12	11	10	8	5	0	0	0	0	0
Total (N = 47)		144	128	116	97	80	26	12	3	16	26

4.1.2. Molecular confirmation of *Staphylococcus* species

The 116 presumptive *S. aureus* were preliminary confirmed as bacteria species by screening for the presence of bacterial 16S rRNA gene fragments. Figure 4.1 is a representative gel indicating 16S rRNA PCR products.

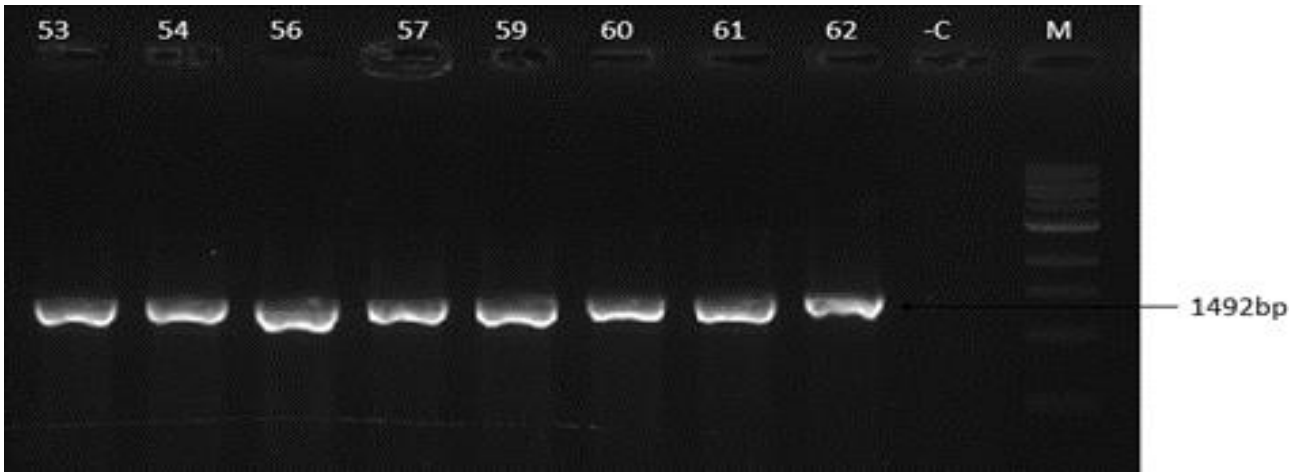


Figure 4.1: A 1.5 % (w/v) agarose gel image of bacterial 16S rRNA gene fragments (1492 bp) amplified from the isolates. Lane M: 1 kb DNA molecular weight marker; Lane -C: negative control; Lane 53: 16S rRNA gene fragment amplified from *S. aureus* ATCC 25923; Lanes 54–62: bacterial 16S rRNA gene fragments amplified from representative presumptive *S. aureus* isolates.

The confirmation of isolate identities through amplification of the *gap* gene revealed that a large proportion (80; 82.47%) of the isolates were *Staphylococcus* species (Table 4.1). No *Staphylococcus* spp. was detected in pasteurized milk from bulk tank samples. The distribution of isolates is summarized in Table 4.1 and Figure 4.2 is a representative gel showing the *gap* gene PCR products.

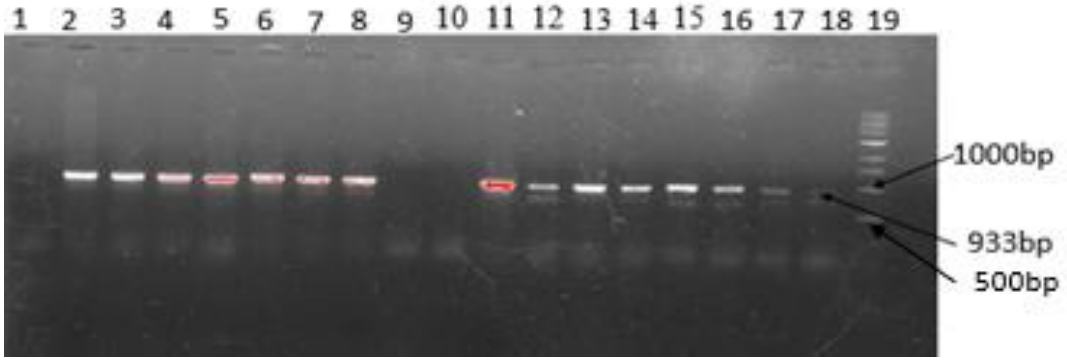


Figure 4.2: A 1.5 % (w/v) agarose gel image of staphylococci *gap* gene fragments (933 bp) amplified from the isolates. Lane 1: negative control PCR reaction from DNA of *E. coli* (ATCC 43888) control strain; Lane 2: *gap* gene fragment amplified from the *S. aureus* positive control strain (ATCC 25923); Lanes 3–8 and 11-18: *gap* gene fragments amplified from staphylococci isolates; Lane 19: 1 kb molecular weight marker.

4.2 Antibiotic resistant profiles of *Staphylococcus* spp.

4.2.1 Phenotypic antibiotic resistance profiles

All 80 *Staphylococcus* isolates were subjected to antibiotic susceptibility tests and the zone of inhibition was taken (Appendix 2). Generally, large proportions of the isolates were often resistant to β-lactam antibiotics including cefoxitin (41; 51.25%) and penicillin (32; 40%). In addition, these isolates were also resistant to clindamycin (30; 37.5%). On the contrary, small proportions were resistant to erythromycin (11; 13.75%), rifampicin (7; 8.75%), tetracycline (5; 6.25%), ciprofloxacin (3; 3.75%), chloramphenicol (2; 2.5%) and trimetroprim-sulfamethozaxole (1; 1.25%) (Figure 4.3). However, all the isolates were susceptible to linezolid and gentamicin.

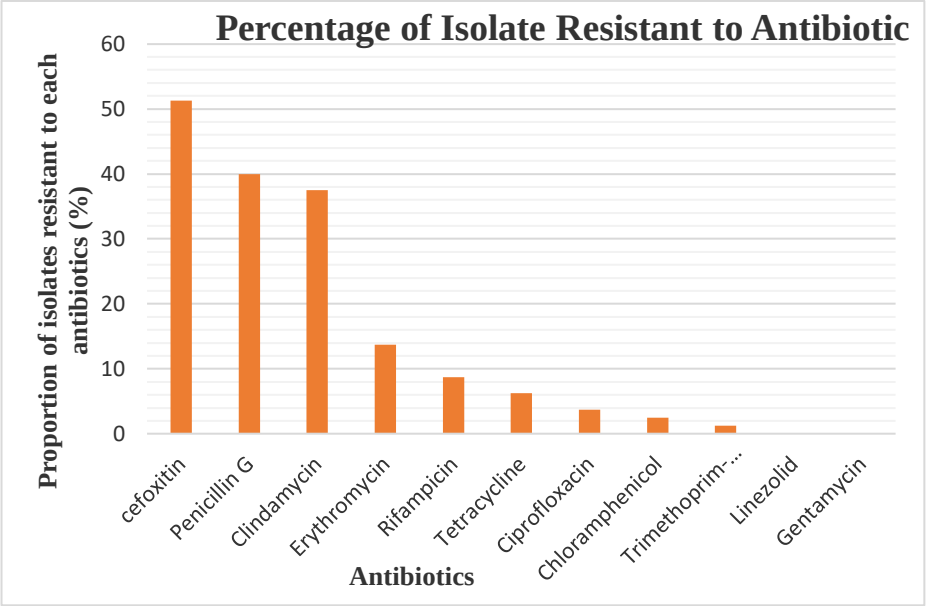


Figure 4.3: Percentage antibiotic-resistance profiles of *Staphylococcus aureus* isolated in the study (n = 80). Percentages were calculated by dividing the number confirmed as *S. aureus* resistant to a particular antibiotic by the total number of isolates tested.

Detailed antibiotic resistance results are shown in Figure 4.4. Based on sample type and as shown in Figure 4.4, the isolates from milk displayed the highest resistance to cefoxitin (12/20), penicillin (10/20), clindamycin (7/20) and erythromycin (5/20). A lower number of isolates from none to two were resistant to the other antibiotics. Raw milk was the only sample with resistant isolates to trimethoprim-sulfamethaxaxole. Ten out of the 36 (27.8%) swab sample isolates were resistant to clindamycin, 8 (27.8%) to penicillin, 6 (16.7%) to cefoxitin, and 5 (13.9%) to erythromycin. Isolates from mixed wastewater were also most often resistant to cefoxitin (10/14), penicillin G (8/14) and clindamycin (7/14). Isolates from drinking water and the milking parlour drain were most often susceptible to the antibiotics especially linezolid and gentamycin with less than five isolates showing resistance to particular antibiotics. Chloramphenicol resistance was only detected among drinking water isolates (Figure 4.4).

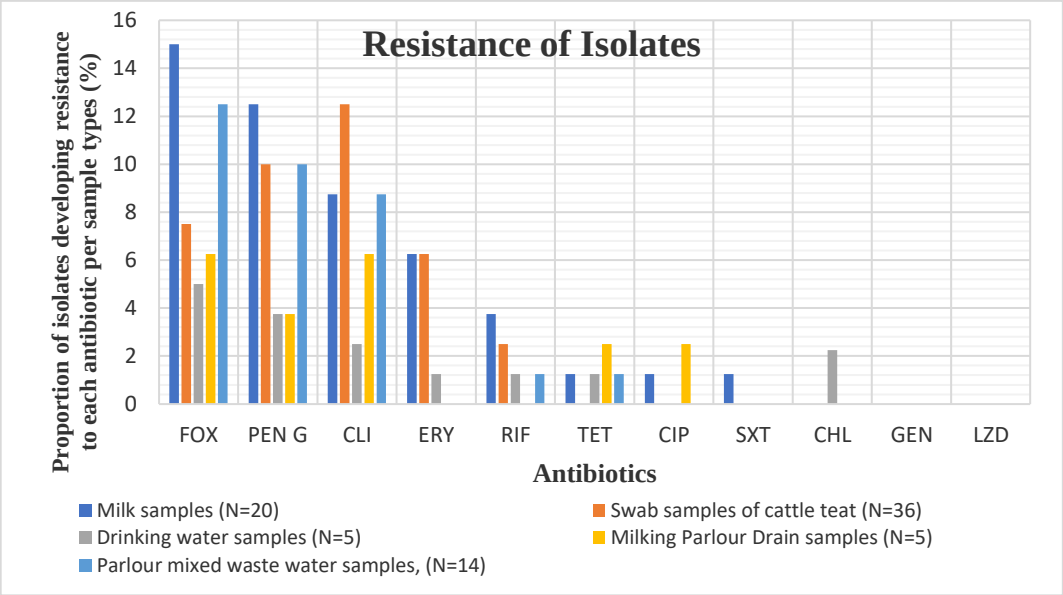


Figure 4.4: Percentages of antibiotic resistance to *Staphylococci* species recovered from different samples types. Ciprofloxacin (CIP), Clindamycin (CLI), Penicillin G (PEN), Linezolid (LZD), Erythromycin (ERY), Gentamicin (GEN), Trimethoprim-sulfamethaxaxole (SXT), Chloramphenicol (CHL), Tetracycline (TET), Rifampicin (RIF), Cefoxitin (FOX).

4. 2.2 Multiple antibiotic resistance and MAR Index-related health risk

Generally, a total of 13 isolates (16.3%) were multi-drug resistant (displayed resistance to 3 or more classes of antibiotics). Resistance to three, four, five and seven antibiotic classes were recorded in 3, 6, 3 and 1 isolate(s) respectively. From milk, 4 isolates were resistant to 3 or more antibiotic classes, specifically to 3, 4, 5, and 7 antibiotics respectively. Similarly 4 swab isolates were multiple antibiotic resistant, two each displaying resistance to 3 and 4 antibiotic classes. Two isolates each from drinking water and milking parlour drain were resistant to 4 and 5 antibiotics classes respectively (Table 4.2). In addition, one isolate from mixed wastewater from the palour was resistant to five different antibiotic classes. Two MAR phenotypes of PEN-ERY-RIF-FOX and CLI-PEN-TET-FOX stood as the most frequent phenotype detected in two isolates each. In general, 11(13.75%) and 27 (33.75%) out of 80 of

the isolates in this study had a MARI greater 0.2. One isolate was resistant to up to 7 classes of antibiotics displaying a MAR index of 0.6 (Table 4.2).

Table 4.2: Multiple antibiotic resistance (MAR) phenotypes and MAR index of *Staphylococcus* isolates.

Source of the sample	Phenotypes	Number of isolates	MAR Index (> 0.2)
Milk	PEN-ERY-RIF-FOX	1	0.4
	CLI-PEN-ERY-RIF-FOX	1	0.45
	CLI-PEN-ERY-SXT-TET-RIF-FOX	1	0.6
	CIP-CLI-FOX	1	0.3
Swab	CLI-PEN-ERY	1	0.3
	CLI-RIF-FOX	1	0.3
	PEN-ERY-RIF-FOX	1	0.4
	PEN-ERY-CHL-FOX	1	0.4
Drinking water	PEN-ERY-CHL-RIF-FOX	1	0.45
	CLI-PEN-TET-FOX	1	0.4
milking parlour drain	CLI-PEN-TET-FOX	1	0.4
	CIP-CLI-PEN-FOX	1	0.4
Parlour mixed waste water	CLI-PEN-TET-RIF-FOX	1	0.45

4.3.3 Distribution of antibiotic resistance genes

All 80 isolates that were resistant to antibiotics phenotypically were subjected to PCR analysis to detect the presence of *mecA*, *blaZ* and *tetO* antibiotic resistance genes (Section 3.8.1 - Materials and Methods). A large proportion (26/80; 32.5%) of isolates harboured the *mecA* gene. The *mecA* positive isolates comprised 14 from wastewater collected in the milking parlour, 9 isolates from milk, 3 from drain system of the milking parlour, and 2 isolates from swab samples. The *blaZ* gene was haboured by a samller proportion (12/80;

15%) of the isolates comprising 6 from milk, 4 from parlour mixed water, and 2 from the drain system of the milking environment. Screening for the chromosomally or transposonally encoded tetracycline resistance gene *tetO* whose mode of resistance involves ribosomal protection revealed a very small proportion (3/80; 3.75%) of the isolates harbouring this gene. Two of these isolates were from milk and while one isolate was from parlour mixed waste water. Table 4.1 indicates the distribution of antibiotic resistance genes detected in the study while figues 4.5, 4.6 and 4.7 are reprsentative agarose gel indicating *mecA*, *blaZ* and *tetO* PCR gene fragments amplified from the *Staphylococcus* respectively .

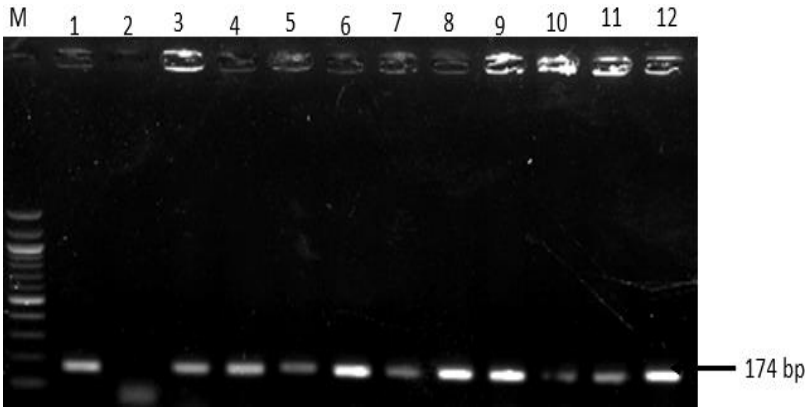


Figure 4.5: Image of the *mecA* gene fragments (174 bp) that were amplified from staphylococci in the study. Lane M: 100 bp molecular weight marker, Lane 1: *mecA* gene fragment amplified from *S. aureus* ATCC 43300 as positive control strain, Lane 2: *mecA* negative PCR reaction using DNA from *E. coli* (ATCC 43888) control strain, Lanes 3-12: *mecA* gene fragments amplified from staphylococci in the study.

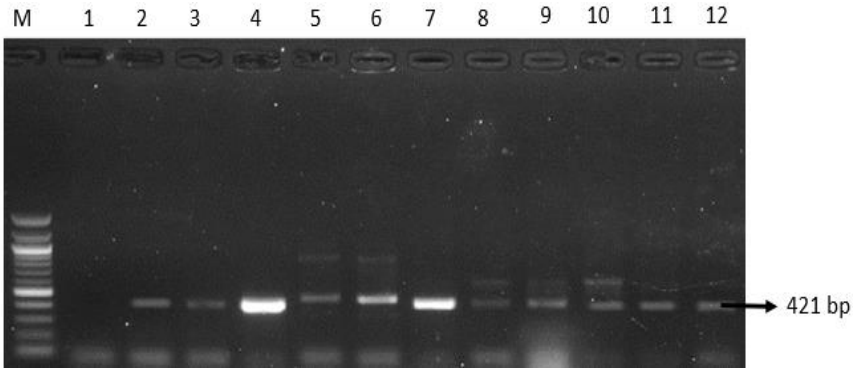


Figure 4.6: Image of the *blaZ* gene fragments (421 bp) that were amplified from *staphylococci* in the study. Lane M: 100 bp molecular weight marker, Lane 1: *blaZ* negative PCR reaction using DNA from *E. coli* (ATCC 43888) control strain, Lane 2: *blaZ* gene fragment amplified from *S. aureus* ATCC 43300 positive control stain, Lanes 3-12: *blaZ* gene fragments amplified from *staphylococci* in the study.

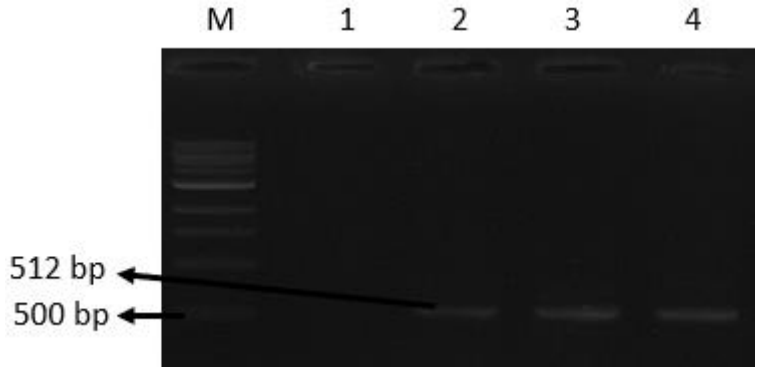


Figure 4.7: Image of the *tetO* gene fragments (512 bp) that were amplified from *staphylococci* in the study. Lane M: 100 bp molecular weight marker, Lane 1: *tetO* negative PCR reaction using DNA from *E. coli* (ATCC 43888) control strain, Lanes 3-4: *tetO* gene fragments amplified from *staphylococci* in the study.

A comparison of the antibiotic resistance gene profiles of the isolates are shown in Figure 4.10.

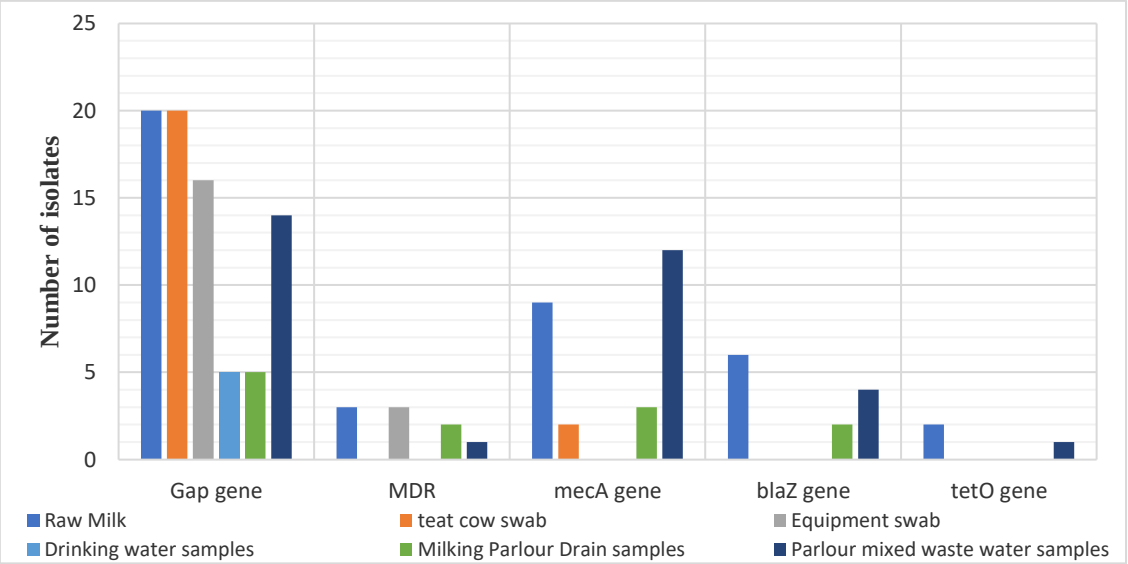


Figure 4.10: Relationship between *S. aureus* and the resistance genes detected in the study.

4.3 Biofilm formation potentials of *Staphylococcus* species

A total of 75 *Staphylococcus* species were subjected to phenotypic detection of biofilms at 4, 25, and 37 °C for over 24, 48, and 72 hours of incubation. The isolates were classified as non-biofilm forming, weak, moderate, or strong biofilm-forming strains. Based on this classification, almost half (36/75; 48%) of the isolates were able to form biofilms at 4 °C, 25 °C and 37 °C after 24, 48 and 72 hours of bacteria growth. Despite the fact that a large proportion (39/75; 52%) of these isolates were non-biofilm formers at all the temperatures assessed in this study, a significant proportion (20/75; 26.67%) were weak biofilm formers. Although a small proportion (10/75; 13.33%) of the isolates in this study were moderate biofilm formers only 6 (8%) isolates were able to form strong biofilms (Table 4.3). Table 4.4 gives an overview of the proportion of isolates with different biofilm forming potentials at different incubation periods used in this study.

Table 4.3: Distribution of isolate associated with temperature.

Biofilm forming potential / incubation temperatures	4°C	25°C	37°C	Total (n=75)

non-forming	46	42	31	39
weak	21	20	19	20
moderate	5	10	14	10
strong	3	3	11	6

Table 4.4 Proportion of biofilm formation by *S. aureus* isolates at 4, 25, and 37 °C during 24, 48, and 72 h periods.

Incubation Temperature	Incubation Time (h)	Non-Adherent (%)	Weak (%)	Moderate (%)	Strong (%)
4 °C	24	42 (56)	21 (28)	9 (12.)	3 (4)
	48	55 (73.33)	16 (21.33)	2 (2.67)	2 (2.67)
	72	42 (56)	26 (34.67)	4 (5.33)	3 (4)
25 °C	24	58 (77.33)	11 (14.67)	4 (5.33)	2 (2.67)
	48	15 (20)	34 (45.33)	21 (28)	5 (6.67)
	72	53 (70.67)	15 (20)	4 (5.33)	3 (4)
72 °C	24	33 (44)	22 (29.33)	13 (17.33)	7 (9.33)
	48	17 (22.67)	20 (26.67)	19 (25.33)	19 (25.33)
	72	42 (56)	17 (22.67)	10 (13.33)	6 (8)

Figure 4.11 shows that (46/75; 61.78%) of the isolates were non biofilm formers at 4°C with 21 (28%), 5 (6.67) and 3 (4%) of these isolates classified as weak, moderate and strong biofilm formers (Figure 4.11). *S. aureus* isolates produced moderate biofilms (10/75; 13.33%) at 25°C after 24 hours, with the biofilm-forming potentials decreasing following 48 and 72 hrs incubation periods (Figure 4.12). Strong biofilm formation was demonstrated by 11 (14.67 %) isolates at 37 °C after a 48 h incubation period. At 37 °C, the production of

biofilm was strong after a 48 hours incubation period, and the biofilm production of some isolates decreased after 72 h ($p < 0.05$), as shown in (Figure 4.13).

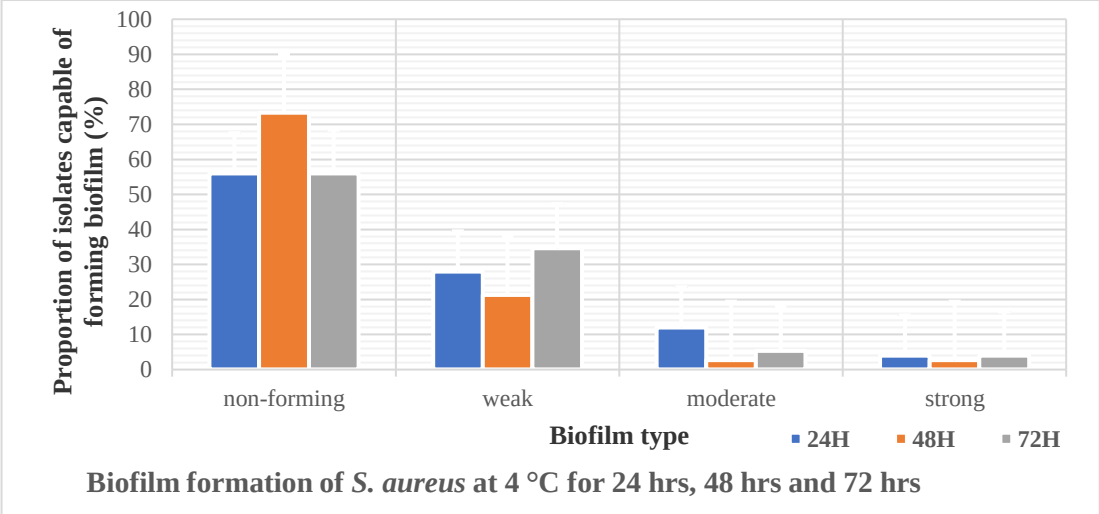


Figure 4.11: Phenotypic distribution of biofilm forming on 96-well microtiter plates. Biofilm formation by *S. aureus* isolates when incubated at 4 °C for 24, 48 and 72h. Cut-off values 0.40 at OD_{600nm} was used for the classification of isolates as followed: non-biofilm former: $OD \leq ODc$; weak biofilm former: $ODc < OD \leq 2$ x ODc; moderate biofilm former: 2 x $ODc < OD \leq 4$ x ODc; strong biofilm former: 4 x $ODc < OD$.

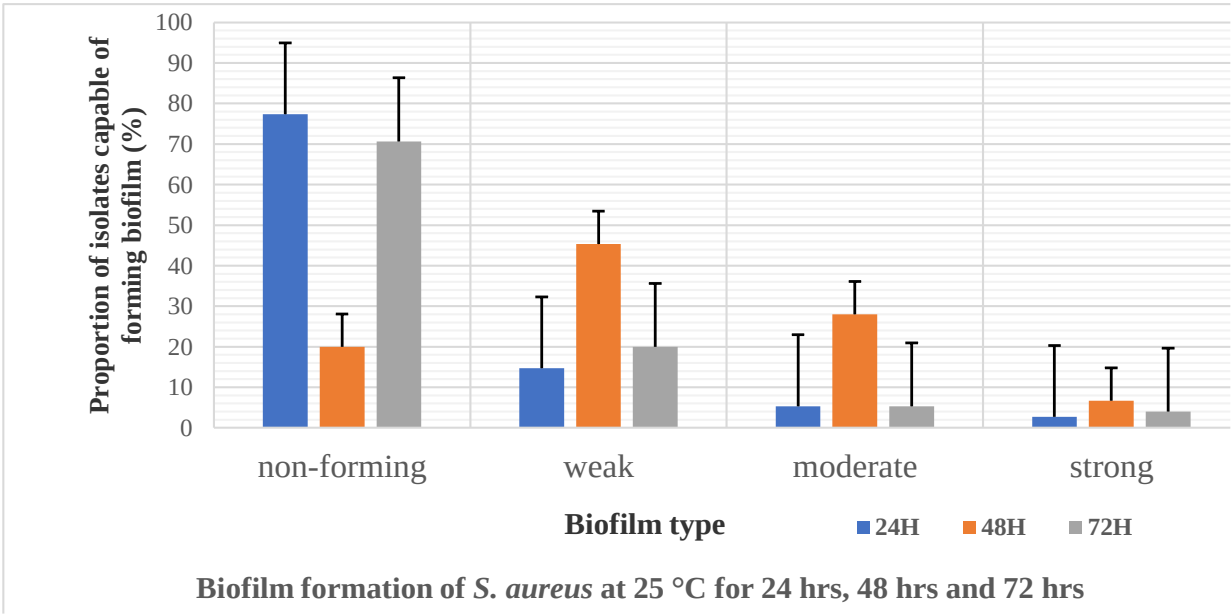


Figure 4.12: Phenotypic distribution of biofilm forming on 96-well microtiter plates. Biofilm formation by *S. aureus* isolates when incubated at 25 °C for 24, 48 and 72 h. Cut-off values 0.40 at OD_{600nm} was used for the classification of isolates as followed: non-biofilm former: $OD \leq ODc$; weak biofilm former: $ODc < OD \leq 2$ x ODc; moderate biofilm former: 2 x $ODc < OD \leq 4$ x ODc; strong biofilm former: 4 x $ODc < OD$.

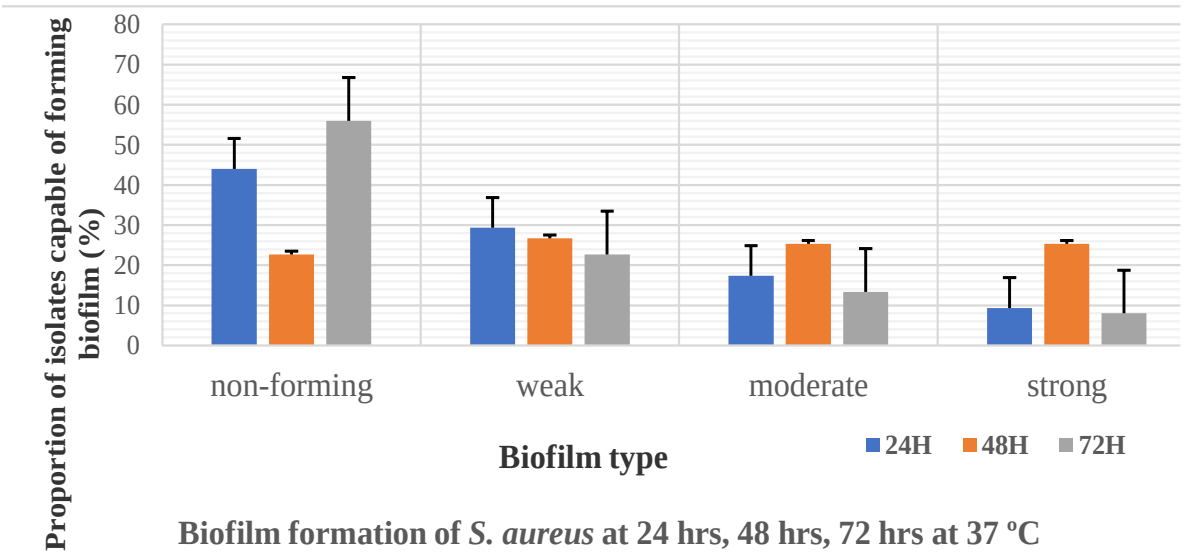


Figure 4.13: Phenotypic distribution of biofilm forming on 96-well microtiter plates. Biofilm formation by *S. aureus* isolates when incubated at 37 °C for 24, 48 and 72 h. Cut-off values 0.40 at OD_{600nm} was used for the classification of isolates as followed: non-biofilm former: $OD \leq ODc$; weak biofilm former: $ODc < OD \leq 2$ x ODc; moderate biofilm former: 2 x $ODc < OD \leq 4$ x ODc; strong biofilm former: 4 x ODc < OD.

The mean standard deviation of the optical density (OD) for isolates showing significant differences ($P < 0.05$) with respect to their biofilm-forming potentials are summarized in Tables 4.4, 4.5, and 4.6. The OD_{600} values for the *Staphylococcus* isolates ranged from 0.15 ± 0.01 to 4.01 ± 0.01. Table 4.4 outlines the the interaction between the biofilm forming potential of isolates and incubation temperatures. For isolate 67, the OD decreased with increase in the incubation temperature, for isolate 134, an increase in the OD was observed with increase in the incubation temperature. However, for the reamaining isolates, maximum OD was attained at 25 °C and these reduced with increase in temperature respectively.

Table 4.4: Effect of incubation temperature on optical density for *Staphylococci* spp during biofilm formation from milk and milking environment.

	Incubation temperature (°C)			
Isolate	4	25	37	**SEM[1]**
67	0.37	0.31	0.18	0.037
81	0.35	0.45	0.27	0.046
94	0.39	2.24	1.21	0.117
134	0.32	0.38	1.54	0.129

[1]SEM: Standard error of the means.

The time of incubation was seen to have a great impact on the OD of isolate during biofilm forming (Table 4.5) as the p-value was greater than 0.05. From Table 4.5, isolates 74 and 132

reached their maximum OD at 48h and after that, these decreased. On the contrary, isolate 94 has a high OD after 24h, and this dropped at 48h and then increased at 72h of incubation. The OD for isolate 122 was highest after 24h of incubation and decreased throughout the experiment.

Table 4.5: Effect of incubation time on optical density for *Staphylococci* spp during biofilm formation from milk and milking environment.

Isolate	**Incubation time Means**			**SEM[1]**
	24 h	48h	72h	
74	0.28	0.49	0.25	0.039
94	1.18	1.08	1.56	0.117
122	0.49	0.42	0.24	0.046
132	0.51	0.58	0.26	0.076

[1]SEM: Standard error of the mean

Based on the interaction between incubation time and temperature, the ability of isolates to form biofilms revealed a significant correlation with p-values greater than 0.05. Table 4.6, revealed that a large proportion (35/75; 46.7%) of the isolates comprising isolates 57, 61, 62, 63, 64, 68, 69, 72, 75, 78, 79, 85, 86, 87, 88, 89, 90, 92, 95, 96, 98, 101, 104, 105, 113, 116, 121, 124, 125, 128, 130, 133, 136 and 141 had a low OD_{600} after 24h of incubation, with ODs increasing after 48h and then later decreasing at 72h for temperatures 25 °C and 37 °C, respectively. On the contrary, the remaining isolates either had high OD_{600} at 24h of incubation which decreased 48hrs or had low OD_{600} at the initial incubation period and these increased and attained maximum at 72h. At 4 °C, 28 isolates demonstrated high OD_{600} at 24h which decreased at 48h and 72h (22 isolates). There is a small proportion (11) of isolates that displayed high OD_{600} at 48h while a few isolates (22) that began with low OD_{600} at 24h and 48h had OD_{600} that reach optimum at 72h (Table 4.6).

Table 4.6: Interactive effects of incubation temperature and time on optical density for *Staphylococci* spp during biofilm formation from milk and milking environment

	4°C			25°C			37°C			
Isolate	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	SEM[1]
57	0.32	0.34	0.46	0.22	0.61	0.22	0.24	1.81	0.22	0.109
59	0.56	0.35	0.51	0.31	0.62	0.35	0.34	0.28	0.33	0.041
60	2.21	0.33	0.32	0.33	1.43	0.33	0.25	0.41	0.27	0.068
61	0.52	0.35	0.31	0.31	1.11	0.28	0.31	0.56	0.37	0.069
62	0.41	0.34	0.31	0.31	1.34	0.29	0.31	0.64	0.41	0.069
63	0.29	0.32	0.29	0.23	0.71	0.21	0.21	0.96	0.31	0.072
64	0.41	0.33	0.27	0.35	1.01	0.21	0.19	0.35	0.25	0.063
65	0.77	0.49	0.57	0.75	-*	0.41	0.53	0.38	0.43	0.088
66	0.34	0.32	0.26	0.23	-*	0.24	0.23	1.01	0.42	0.107
68	1.24	0.33	0.38	0.22	0.33	0.21	0.17	0.26	0.18	0.071
69	0.42	0.31	0.33	0.29	0.76	0.21	0.24	0.29	0.19	0.047
72	0.36	0.31	0.43	0.28	0.84	0.18	0.17	0.87	0.21	0.111
73	0.31	0.26	0.29	0.29	0.68	0.22	0.21	0.22	0.27	0.062
75	0.32	0.43	0.43	0.99	1.06	0.43	0.55	1.23	0.42	0.132
78	0.48	0.88	0.54	0.33	0.57	0.21	0.23	0.24	0.18	0.057
79	0.41	0.25	0.27	0.22	0.91	0.27	0.19	0.22	0.19	0.102
80	0.48	0.25	0.26	3.04	2.17	2.06	0.41	2.19	1.37	0.234
83	0.36	0.31	0.27	0.23	0.41	0.41	0.37	0.74	2.64	0.129
85	0.85	0.46	0.76	0.49	1.23	0.52	0.47	2.13	0.59	0.253
86	0.48	0.51	0.34	0.62	1.12	0.68	0.53	2.38	0.83	0.106
87	0.45	0.26	0.46	0.98	1.29	0.71	0.94	2.81	1.01	0.153
88	0.36	0.23	0.29	0.43	1.46	0.51	0.76	2.93	0.43	0.183
89	0.23	0.24	0.33	0.19	0.58	0.26	0.26	2.32	0.18	0.241
90	0.28	0.31	0.53	0.21	0.44	0.21	0.24	1.51	0.23	0.173
91	0.21	0.18	0.41	0.21	0.79	0.31	0.46	1.25	1.57	0.126
92	0.25	0.22	0.71	0.31	0.71	0.39	0.44	0.74	0.41	0.109
95	0.46	0.43	0.34	0.59	1.31	1.17	0.43	2.91	0.24	0.096
96	2.71	2.14	3.39	0.21	0.28	0.27	0.25	0.33	0.24	0.111
98	0.38	0.32	1.01	0.21	0.31	0.22	0.37	2.11	1.48	0.204
101	1.54	0.53	1.35	0.83	1.11	0.79	0.58	1.33	1.04	0.112
104	0.25	0.38	0.59	0.44	0.44	0.22	0.34	1.25	0.24	0.163
105	0.23	0.21	0.73	0.21	0.23	0.17	0.23	1.71	0.21	0.133
111	0.36	0.49	0.53	0.19	0.21	0.22	1.42	3.08	2.69	0.154
113	0.23	0.31	0.42	0.17	0.31	0.31	0.28	1.36	0.42	0.179
114	0.24	0.28	0.48	0.15	0.25	0.29	0.21	2.86	0.35	0.112
115	1.05	1.94	4.01	0.26	0.36	0.61	0.23	1.06	0.27	0.165

116	1.69	1.42	1.39	0.43	1.21	1.02	1.14	2.79	2.24	0.236
117	0.17	0.18	0.51	1.36	0.31	0.21	0.31	0.71	0.24	0.075
118	0.31	0.34	0.36	0.21	2.73	0.25	1.17	1.05	0.63	0.131
119	0.41	0.34	0.35	0.61	0.71	3.04	3.38	0.15	3.22	0.088
121	0.45	0.32	0.61	0.17	3.07	0.82	0.65	2.95	2.89	0.169
123	0.81	0.25	0.18	0.23	1.07	0.59	2.05	0.51	0.25	0.137
124	0.61	0.23	0.22	0.23	0.61	0.27	0.56	1.25	0.42	0.079
125	0.31	0.31	0.24	0.24	0.52	0.28	0.45	1.06	0.36	0.061
126	0.22	0.29	0.23	0.18	0.53	1.41	1.52	2.39	0.74	0.241
127	0.31	0.35	0.31	0.18	0.91	0.25	1.31	0.15	0.31	0.194
128	0.35	0.41	0.43	0.18	0.73	0.23	0.27	0.33	0.19	0.071
129	0.41	0.35	0.29	0.25	0.58	0.28	0.84	0.61	0.45	0.058
130	0.92	0.31	0.21	0.19	0.51	0.28	0.44	0.69	0.35	0.143
131	0.29	0.26	0.17	0.31	0.46	0.71	3.05	1.11	1.27	0.099
133	0.32	0.47	0.23	0.52	1.21	0.22	0.73	2.13	0.69	0.079
135	1.56	0.31	0.45	0.19	1.66	0.52	2.01	0.41	1.19	0.314
136	0.35	0.35	0.32	0.16	2.24	0.21	0.27	0.37	0.21	0.055
137	0.26	0.46	0.25	0.66	0.65	0.19	1.71	2.36	0.22	0.231
138	0.22	0.44	0.22	0.21	0.44	0.18	0.61	0.51	0.21	0.051
139	0.59	0.71	0.34	0.22	0.32	0.44	0.98	0.41	0.22	0.115
140	0.31	0.27	0.18	0.21	0.37	0.33	0.92	0.26	0.22	0.112
141	0.24	0.28	0.22	0.25	1.14	0.24	0.19	0.22	0.13	0.063
142	0.22	0.26	0.26	0.18	0.91	0.23	0.81	0.64	0.37	0.141
143	0.25	0.41	0.32	-*	0.26	0.31	1.39	0.41	0.47	0.112
144	0.24	0.44	0.45	-*	0.51	0.42	2.93	2.77	0.38	0.082

[1]SEM: Standard error of the means, -*: Insufficient suspension culture of the isolates.

From the molecular analysis, antibiotic resistance and biofilm forming; there were some corelation between isolates. It was based on these results that the REP, ERIC and RADP typing were performed.

4.4 Molecular typing of *Staphylococcus* spp.

4. 4.1 MLST genotyping and genetic diversity of staphylococci.

The MLST scheme uses internal fragments to amplify seven house-keeping genes comprising *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase), and *yqi* (acetyle coenzyme A acetyltransferase). In this study, only two genes *pta* and *aroE* encoding

for phosphate acetyltransferase and shikimate dehydrogenase were successfully amplified with PCR. Images of agarose gel pictures of representative *pta* and *aroE* PCR products are shown in Figures 4.8 and 4.9). Further analysis was not undertaken to determine the sequence types.

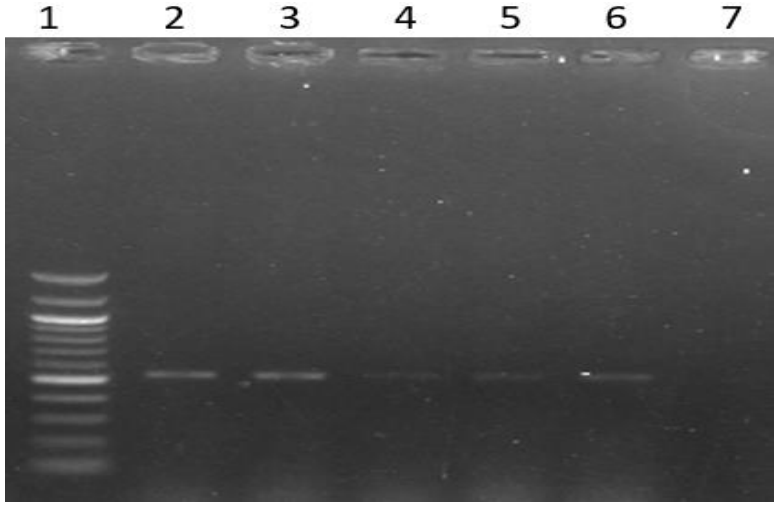


Figure 4.8: Image of the *aroE* gene fragments (536 bp) that were amplified from staphylococci in the study. Lane 1: 100 bp molecular weight marker, Lanes 2-6: *aroE* gene fragments amplified from staphylococci, Lane 7: *aroE* negative PCR reaction using DNA from *E. coli* (ATCC 43888) control strain in the study.

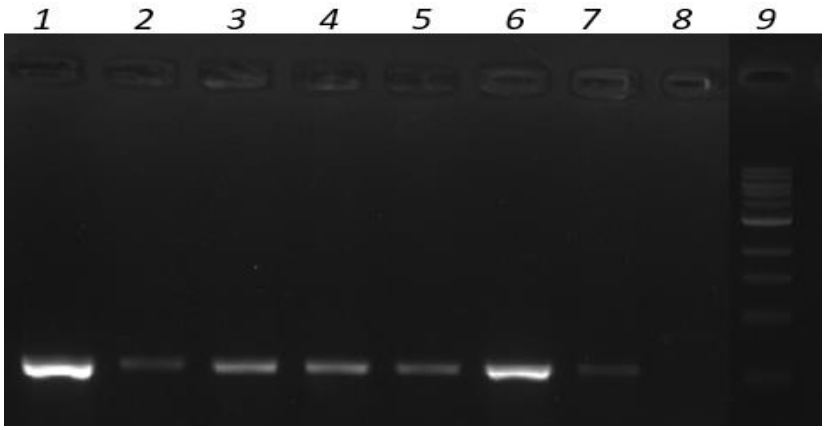


Figure 4.9: Image of the *pta* gene fragments (575 bp) that were amplified from staphylococci in the study. Lane 9: 1 kb molecular weight marker, Lanes 1-7: *pta* gene fragments amplified

from staphylococci, Lane 8: *pta* negative PCR reaction using DNA from *E. coli* (ATCC 43888) control strain in the study.

4.4.2 REP, ERIC and RAPD typing analysis of *Staphylococcus* isolates.

The genetic relatedness of *S. aureus* was determined using fingerprinting patterns generated through REP, ERIC, and RAPD-PCR typing methods. ERIC typing produced band patterns ranging from 1 to 11 bands per isolate with sizes of 150 – 2500 bp. Out of 33 isolates that were subjected to ERIC PCR, isolates were grouped into 6 major clusters designated I-VI at a genetic distance of 08.8%. Cluster I was the largest (48.48%), when compared to cluster III (18.18%), cluster IV (12.12%), clusters II and IV (6.06% each) and cluster VI (3.03%). An assessment of the association of isolates within the different clusters revealed that cluster I had all the isolates from sewage sample as well as a few from the other sources. All the other clusters were highly diverse (Figure 4.14). The discriminatory power of the ERIC typing method was at 0.6667 (Table 4.7) indicating its ability to differentiate among isolates from different sources when compared to the other methods used in this study.

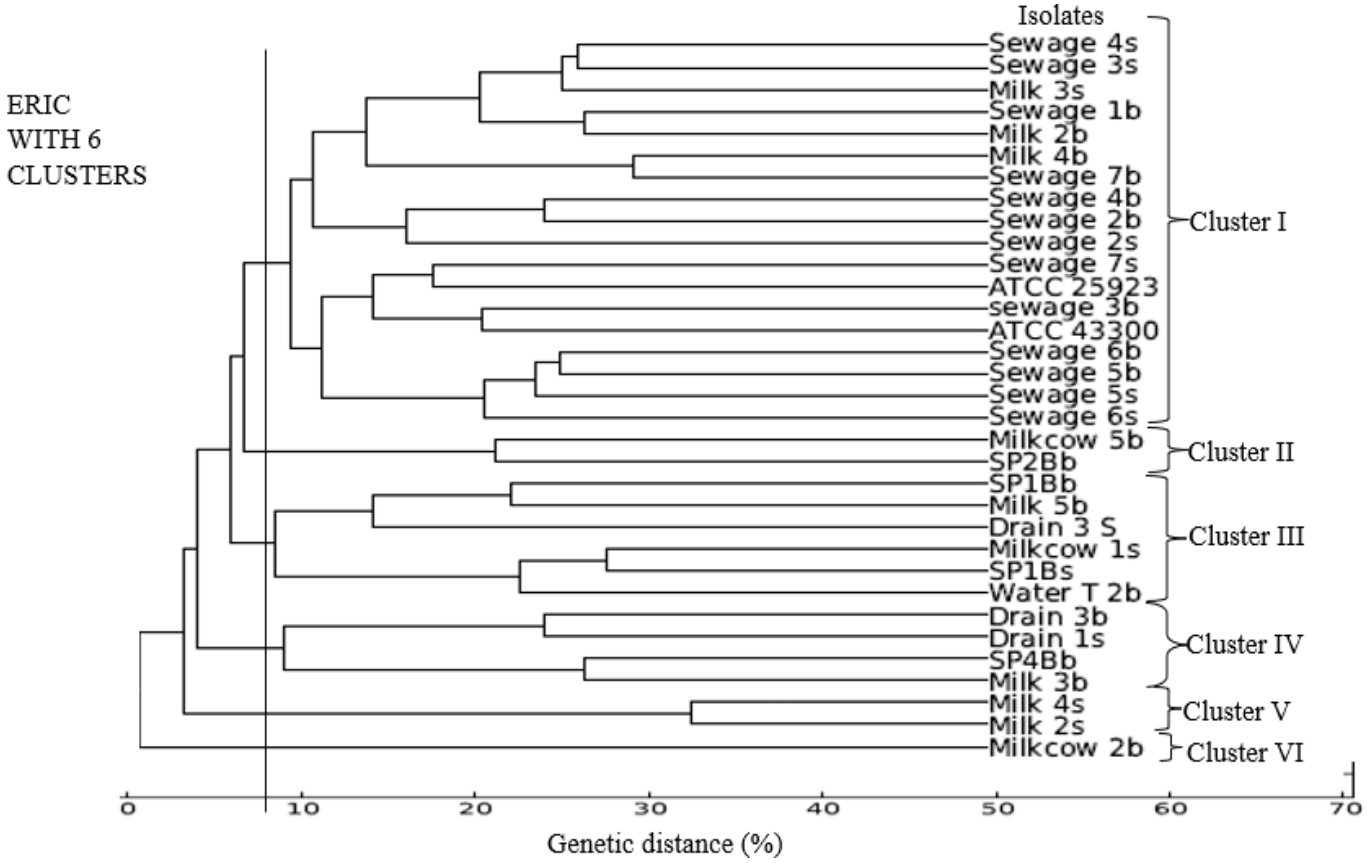


Figure 4.14: Dendrogram generated from ERIC-PCR fingerprinting of 33 *Staphylococcus spp*. Five clusters and 1 single isolate were categorized at a genetic distance of 8.8% (straight line).

RAPD PCR typing generated 0 to 9 band patterns ranging from 200 to 3000 bp per isolate. Based on a genetic distance of 03.5%, six clusters designated I – VI, were generated in this study. The number of isolates per cluster revealed that cluster II with 24 isolates was the largest, followed by cluster I with 4 isolates, cluster III that possessed 2 isolates, and clusters IV, V and VI that contained one isolate each (Figure 4.15). The discriminatory power of RAPD typing was at 0.464 (Table 4.7).

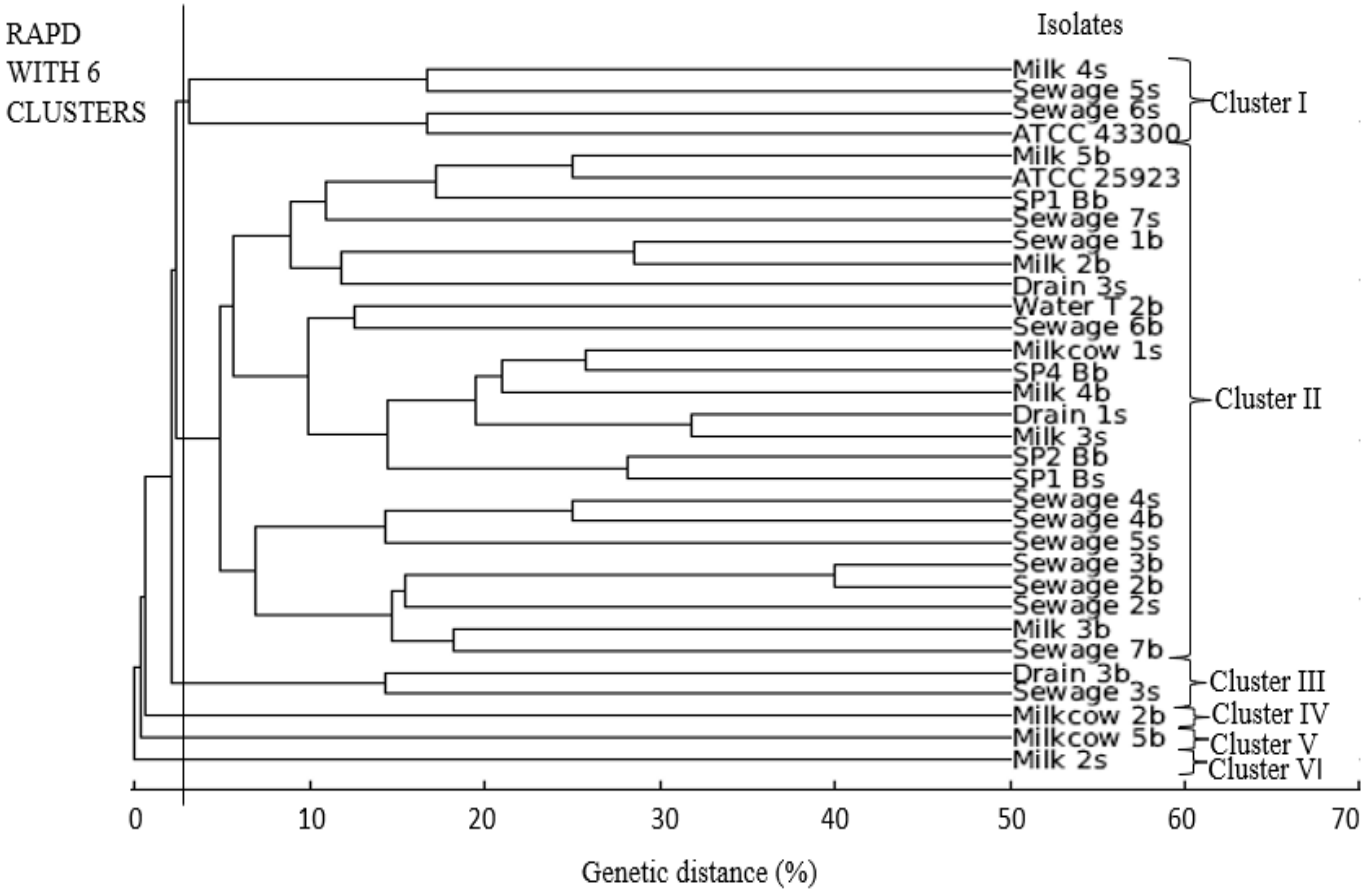


Figure 4.15: Dendrogram generated from RAPD-PCR fingerprinting of 33 *Staphylococcus spp*. Three clusters and 3 single isolates were categorized at a genetic distance of 3.5% (straight line).

Genetic typing with REP revealed that the isolates had 1 to 7 bands with amplicon sizes ranging from 200 – 1800 bp per isolate generated at 0.5% of the genetic distance. Two main clusters (clusters I and II) were obtained, and these had 10 and 23 isolates respectively (Figure 4.16). The discriminatory power of the REP PCR typing method was at 0.4356 (Table 4.7).

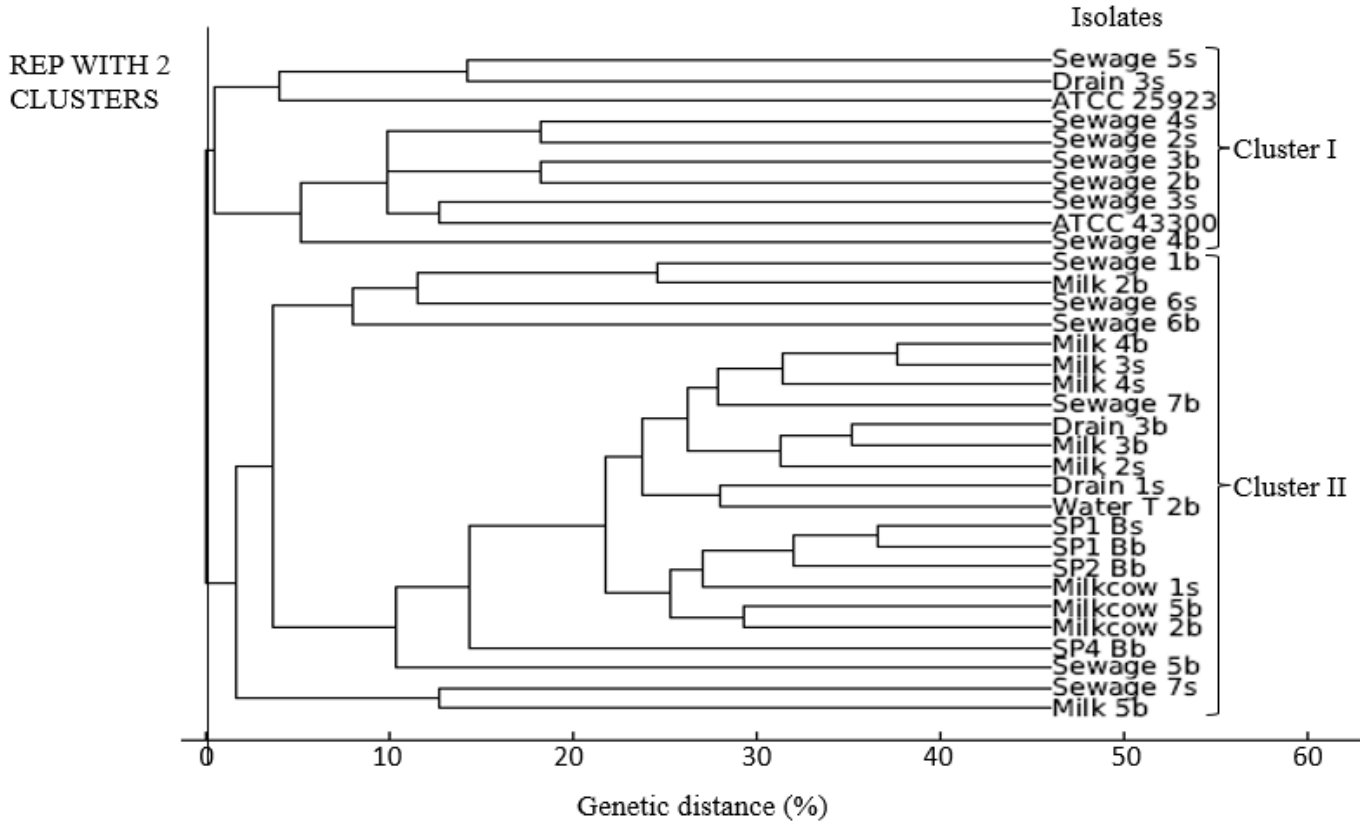


Figure 4.16: Dendrogram generated from REP-PCR fingerprinting of 33 *Staphylococcus spp*. Two clusters were categorized at a genetic distance of 0.5% (straight line).

Table 4.7: Discriminatory indices of ERIC-, RAPD- and REP-PCR in genotyping of *Staphylococcus* isolates (*n* = 33).

Genotyping method	Genetic distance (%)	Number of clusters	Cluster sizes	Number of single isolates	Discriminatory index
ERIC-PCR	8.8	6	18,2,6,4,2,1	1	0.6667
RAPD-PCR	3.5	6	4,24,2,1,1,1	3	0.464
REP-PCR	0.5	2	23,10	0	0.4356

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 General discussion

Milk from dairy animals is one of the most widely consumed foods thus productive systems are constantly being adapted to meet the high demand of milk and dairy products (Akindolire *et al*., 2015). The demand for milk has been associated with its high nutritious nature, which unfortunately makes it an excellent growth medium for microbes, especially bacteria, thus negatively affecting the health of consumers (Muehlhoff *et al.,* 2013). Raw milk may be contaminated by spoilage organisms due to contact with animal faeces, soil, air, feed, water, bedding material, animal hide, infected udder, milking containers and the milking personnel (Moatsou and Moschopoulou, 2015). However, the usually low numbers of spoilage organisms in raw milk, can be easily dealt with by the immune system of consumers implying it should be safe for human consumption (Owusu-kwarteng *et al.,* 2020).

Access to safe and good quality milk is important as this helps in protecting human health and well-being (WHO, 1997). The aim of this study was to determine the genetic types of multidrug-resistant and biofilm-forming *Staphylococcus aureus* that was isolated from drinking water troughs, milk and milking environments of dairy farms in the North-West Province using several molecular tools. In this study, there was a relatively high occurrence (80; 82.47%) of *Staphylococcus* species in milk samples from all the sites. Similar observations were reported by Abraha *et al*., (2018) and Sadat *et al*., (2022) for *S. aureus* detected in raw milk in Ethiopia (26.74%) and Egypt (41.1%), respectively. The environment may play an important role in the spread of *S. aureus* from animals to human, especially to those exposed to unpasteurised contaminated milk products. Therefore, the presence of these pathogens may have resulted due to various factors including contact with contaminated

milking utensils, poor hygiene in the environment as well as anthropogenic activities during the milking process (Jørgensen *et al*., 2005; Abraha *et al*., 2018; Mohamed *et al*., 2022). *S. aureus* was not identified in pasteurized milk and pasteurized bulk tank milk. Similar observations were reported by Bedasa *et al* (2018) in Ethiopia. This may have resulted from the fact that the pasteurization process plays an important role in the reduction of microorganisms in milk (Akindolire *et al*., 2015; Bissong and Ateba, 2020).

Antimicrobial agents are widely used for the treatment of bacterial infections (Fri *et al*., 2020). However, the misuse of antibiotics in both human and veterinary medicine has contributed to the generation of selective pressures in the environment, thus the emergence of multidrug resistant microorganisms (Turner *et al*., 2019). The pace at which antimicrobial resistance is growing coupled with the difficulty of managing multiple drug resistant (MDR) bacterial infections has resulted in its categorisation as a global public health challenge (Bissong and Ateba, 2020; Fri *et al*., 2020). Amongst the pathogens that the WHO has categorized to advocate for the need to urgently develop new antibiotics, *Staphylococcus aureus* and methicillin-resistant *S. aureus* belongs to the "high" priority group thus highlighting the need for continuous surveillance (WHO, 2017). Efforts to contribute to the Sustainable Development Goals (SDG) Agenda 2030, especially Goal 3 aimed to "establish good health and well-being for all", may be enhanced by aligning with the Global Antimicrobial Resistance and Use Surveillance System (GLASS), through surveillance studies (WHO, 2016). The antimicrobial resistance profiles of the isolates revealed that large proportions of the isolates were resistant to β-lactam antibiotics for cefoxitin (51.25%) and penicillin G (40%). A related study reported similar findings for milk obtained from dairy farms (Akindolire *et al*., 2015). β-lactam resistant isolates produce beta-lactamase enzymes that reduce the affinity of antibiotics to bind to target bacteria cells (Dweba *et al*., 2018).

However, the persistence of penicillin resistant in those farms may be due to the fact that this antimicrobial agent is still widely used for the treatment of infections, especially in cattle (Akindolire *et al*., 2015; Duse *et al*., 2021; Şanlıbaba, 2022). On the contrary, a study that was recently conducted in southern Xinjiang, China revealed that none of the isolates displayed resistance to β-lactam antibiotics (Liao *et al*., 2022).

Clindamycin is a last resort antibiotic that is recommended for the treatment *S. aureus* infections especially those resistant to methicillin (Lall and Sahni, 2014). *S. aureus* isolates in this study displayed a significantly high resistance (37.5%) to this drug. Similar observations were reported by Fri *et al*. (2020). Moreover, isolates from this study demonstrated limited (13.75%) resistance to erythromycin. Erythromycin is widely used during therapeutic processes in cattle, especially for cases of mastitis, which may explain its presence amongst staphylococci in this study. Liao *et al*. (2022) revealed similar resistance rates (30.9%) for erythromycin.

The isolates in this study were less often resistant (6.25%) to tetracycline and this was in agreement with a study done by Chai *et al*. (2022). Tetracycline is also widely used in the treatment of mastitis and for the management of other complications in dairy animals (Zhai *et al*., 2023). The persistence of antibiotic resistance pathogens in dairy farms, especially in developing countries in Africa as well as underprivileged communities, demonstrate significantly severe public health impacts on consumers (Phokela *et al*., 2011). Efforts to prevent zoonotic transfer of these pathogens from animals to humans are imperative. Linezolid, an oxazolidinone and suitable alternative to vancomycin, was approved in 2006 for treatment of infections caused by glycopeptide-resistant strains of *Enterococcus faecium*, *S. aureus*, and *S. pneumoniae* (Sáez-Llorens, and McCracken Jr, 2006). Interestingly, all

(100%) the isolates from this study, were susceptible to Linezolid and Gentamicin. These antibiotics may therefore be still effective for the eradication of *S. aureus* infections. Similar observations were reported by Derakhshan *et al*. (2021) and Chai *et al*. (2022). However, these results are in contrast to those by Monecke *et al*. (2023), where the isolates showed a high resistance (90%) to gentamicin and this difference may be due to variation in the geographical locations, antibiotic use, and the source of isolates.

A small proportion (15%) of the isolates in this study, displayed multiple antibiotic resistance, defined as resistance to three or more antibiotics belonging to different classes. The detection of isolates with MARI above 0.2 provide evidence of possible prior exposure to antibiotics in the area. This implies that a significant proportion of these isolates may pose high risks to consumers and the sources from which they were isolated may lead to development of antibiotic resistant bacteria (Fri *et al*., 2018). The effect of drug resistant pathogens is felt by the economy and humans of affected communities. Moreover, in low resource settings, the association between antibiotic resistance and MAR index are used as an important epidemiological tool to monitor drug resistance and usage (Shandhu *et al*., 2016).

The distribution of mobile genetic elements among *S. aureus* strains increases its ability to develop resistance to many classes of antimicrobial agents. In this study, the *mec*A gene which is a biomarker for methicillin resistance in *Staphylococcus aureus* was most often identified (32.5%) in the isolates. Similar results were observed in a study done in Brazil by Dos Santos *et al*. (2023) where the presence of *mec*A gene was detected in 45% of the isolates from piglets. In contrast, a previous study done by Akindolire and colleagues (2015) detected few (2/48) *mec*A determinants amongst *S. aureus*. This increased frequency of the

*mec*A gene determinant may have resulted through horizontal gene transfer (HGT) (Robinson and Enright, 2004; Foster, 2017).

The detection of the *bla*Z genes encoding penicillin resistance in a small proportion of the *S. aureus* isolates is similar to a previous result that was obtained in Nigeria by Okiki *et al*., (2020), except that the gene was harboured by a large proportion (65%) of the staphylococci. On the contrary, Pimenta and co-authors (2023) in Brazil did not detect this gene in all *Staphylococcus* species in their study. Beta-lactam antibiotics are the most often interrupt bacterial cell-wall formation as a result of covalent binding to essential penicillin-binding proteins (PBPs), enzymes that are involved in the terminal steps of peptidoglycan cross-linking in both Gram-negative and Gram-positive bacteria including *Staphylococcus aureus* (Silva *et al*., 2020; Lade and Kim, 2021). Although bacterial resistance to beta-lactams is most often expressed through the production of beta-lactamases, these isolates may have other alternative mechanisms including decreased penetration of antibiotic to the target site, alteration of target site PBPs, and expression of efflux pumps that flush out the antibiotic from the periplasmic space (Ibrahim *et al.*, 2019).

The *tetO* gene, a ribosomal protection protein that is associated with conjugative plasmids and responsible for tetracycline resistance, was detected in three strains that were susceptible to tetracycline by the phenotypic assay. Although the detection of this resistance gene may be affected by its association with conjugative plasmids, the phenotypic findings may have resulted due to drug efflux mechanisms (Palmer *et al*., 2018) with the bacteria inhibiting the potential of the antibiotic to reach its target site.

A further objective of this study was to assess the biofilm forming potentials of *S. aureus* strains and use this to assess public health impacts on humans. Biofilms, an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix (Paharik and Horswill, 2016), thus acts as a semipermeable protective layer that prevents the action of antimicrobial agents (Sharan *et al*., 2023).

The reduced biofilm forming potentials at 4 °C may be due to limited microbial growth at such a low temperature. Although there are limited studies on the biofilm forming potentials at 25 °C, the increase (44%) in the ability to form biofilms at this temperature compared to 4 °C may be due to an increase in the incubation temperature. A similar reason was suggested by Kim *et al*. (2006) in Georgia for isolates from feeding tubes that were compared at 12 °C and 25 °C, respectively.

The frequent detection of strong biofilm formation at 37 °C, a temperature range that is associated with the human body, indicates the public health significance of these isolates. These isolates have the potential to evade the action of antimicrobial agents thus resulting in treatment failures (Tabassum *et al*., 2023). A study conducted in Latvia by Viksne *et al*. (2023) revealed that pathogenic bacteria were moderate and strong biofilm formers at 37 °C. However, the findings of that study were contrary to those obtained in China by Yin *et al*. (2018) in which 25 °C rather than 37 °C was the optimum temperature for biofilm formations. In addition to differences in the geographical locations, the secretion of several virulence factors and the potential to form biofilms in staphylococci are controlled by a sophisticated network of virulence regulators such as the *agr*, *sarA*, *saeR/S* and *arlRS* (Arya and Princy, 2016) that were not investigated in this study.

PCR-based DNA fingerprinting assays that amplify multiple DNA fragments ranging from as small as ≤100 bp to as large as ≥3kb, has been useful in determining the genetic relatedness of *S. aureus* strains and assisting in detailed investigations of the epidemiology of *S. aureus,* especially during mastitis in dairy animals (Matthews *et al.,* 1994). In this study, phylogenetic analysis by the ERIC and RAPD PCR typing methods revealed a greater genetic diversity among isolates when compared to REP PCR typing. A study by Bilung *et al,* (2018) showed that pathogenic *Leptospira* species were genetically diverse as they were distributed in most of the clusters with a diversity that correlated with the genetic distance. Similar to the findings of this study, ERIC PCR was more reliable in assessing the genetic relatedness of pathogenic *E. coli* O157:H7 from different sources (Ateba and Mbewe, 2014; Bilung *et al*., 2018). In addition to ERIC the study by Bilung *et al*. (2018) also highlighted the importance of BOX-PRC in determining genetic diversities among isolates. The finding that ERIC and RAPD typing were more reliable for grouping the isolates in the same phylogenetic tree based on similarities of the genetic fingerprints (Lee *et al*., 2012), may add value in detection of food, human and animal borne contamination with *S. aureus* pathogens, especially in epidemiological studies, medical diagnosis and the identification of new strains and their origins.

5.2 Conclusions

Staphylococci species are a potential cause of mastitis in cows, thus affecting the quality and quantity of milk and therefore resulting in huge economical loss. These organisms are released into the environment and may contaminate either the udder or the milk. The use of antibiotics in the treatment of bacterial infections in humans and animals as well as its use as a supplement in animal feed contributes to the emergence and spread of antibiotic resistant bacteria. Antibiotic resistance is currently a global challenge and resistance by bacteria

pathogens is enhanced by their potential to form biofilms. The challenges caused by antibiotic resistant strains have resulted in the WHO categorising pathogens for which constant surveillance and the need to develop new antibiotics must be prioritised. Amongst the organisms in the different categories, *S. aureus* especially MRSA, is placed in the "high" priority area, thus the motivation for this study. The detection of multidrug resistant and virulent biofilm forming isolates that displayed varying fingerprinting profiles with consistent and more reliable ERIC typing profiles highlights both the need for strict control measures and further studies using more advanced typing tools such as Pulse Field Gel Electrophoresis (PFGE) and Whole genome sequencing. Moreover, strategies to limit cross contamination with this pathogen are critical.

Some limitations for the study include the following;

- Samples were collected from a single farm thus the need for a large-scale study involving many farms.
- The need to employ other recent techniques such as Whole genome sequencing to obtain detailed genetic (resistome and virulome) profiles of the *S. aureus* isolates.

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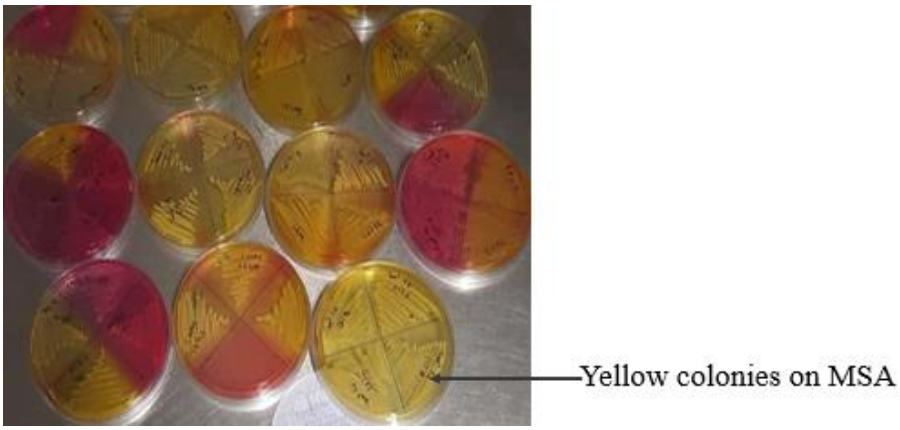
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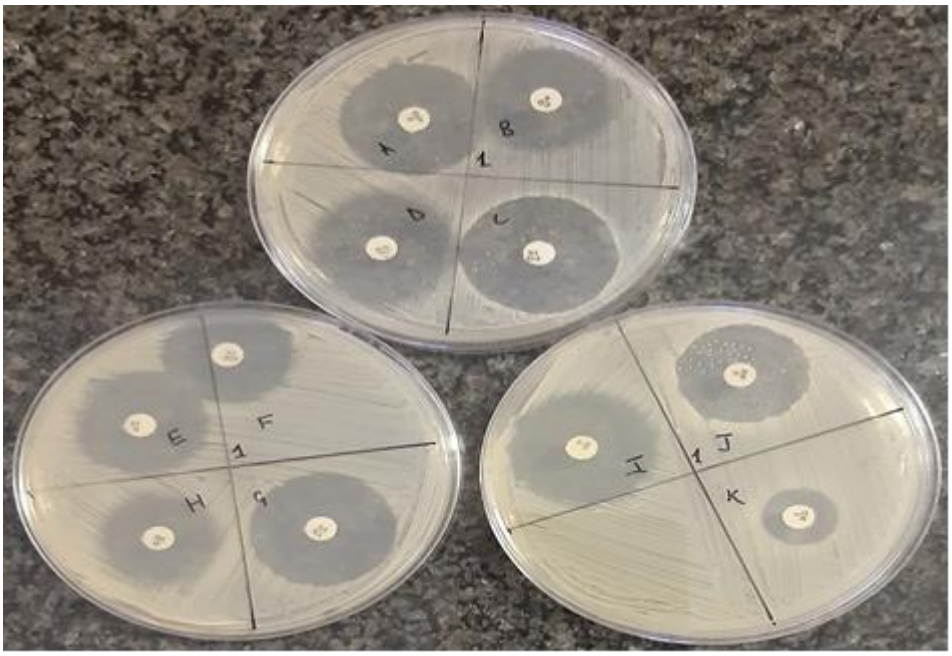
Appendix



Appendix 1A: Golden-yellow of *Staphylococcus aureus* with yellow zones on Mannitol Salt Agar



Appendix 1B: *Staphylococcus* spp. viewed under a microscope with oil immersion after Gram-staining.



Appendix 2: Antimicrobial susceptibility test of *S. aureus* sample; A: Ciprofloxacin, B: Clindamycin, C: Penicillin G, D: Linezolid, E: Erythromycin, F: Gentamicin, G: Trimetroprim-sulfamethozaxole, H: Chloramphenicol, I: Tetracycline, J: Rifampicin, K: Cefoxitin.

Appendix 3: Relationship each isolate associated with antibiotic resistance genes and the ability to form biofilm potentialy.

Sample ID	*mecA*	*blaZ*	*tetO*	MDR	Biofilm
Drain 3S	+	+	-	-	-
Milk 5B	+	+	-	-	-
Milk 2B	+	+	-	+	++
Sewage 1B	+	+	-	-	+++
Sewage 2B	+	-	-	-	++
Sewage 2S	+	-	-	-	+++

Sewage 3B	+	-	-	-	+++
Sewage 3S	+	+	-	-	+++
Sewage 4B	+	-	+	-	++
Sewage 4S	+	+	-	-	++
Sewage 5S	+	+	-	-	+++
Sewage 5B	+	-	-	-	+
Sewage 6B	+	-	-	-	+
Sewage 6S	+	-	-	-	+
Sewage 7S	+	-	-	+	++
Sewage 7B	-	-	-	-	+
Milk 2S	+	+	-	-	+
Milk 3B	+	-	-	-	++
Milk 3S	-	-	-	+	+++
Milk 4B	+	-	+	+	-
Milk 4S	+	+	+	-	-
Drain 1S	+	-	-	+	+++
Drain 3B	+	+	-	+	++
Suction pipe 1bB	-	-	-	-	+++

Suction pipe 1bS	-	-	-	-	+
Suction pipe 2bB	+	-	-	-	+++
Suction pipe 4bB	+	-	-	-	++
Milk cow 1S	+	+	-	-	-
Milk cow 2B	+	-	-	-	+
Milk cow 5B	+	+	-	-	+++
Suction pipe 1aB	-	-	-	+	-
Suction pipe 2aB	-	-	-	+	-
Suction pipe 4aB	-	-	-	+	+++
Milk cow 1B	-	-	-	+	-

$^{-}$isolate not appearing, $^{+}$isolate appearing once, $^{++}$isolate appearing twice, $^{+++}$isolate appearing three times especially for biofilm forming.