Antimicrobial resistance patterns and biofilm formation of coagulase negative *Staphylococcus* species isolated from cow milk samples.

By

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Dedication

This dissertation is dedicated to my dear parents, Linah and Joseph Phophi and to all my siblings, Tshamaano, Lutendo, Mulalo and Rofhiwa Phophi.

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Abstract

Increased prevalence of antimicrobial resistance, treatment failure, and financial losses have been reported in dairy cattle with coagulase-negative *Staphylococcus* (CoNS) clinical mastitis. However, studies on CoNS are limited in South Africa. Therefore, the objectives of this study were to investigate the antimicrobial resistance patterns and biofilm formation in CoNS isolated from cow milk samples submitted to the Onderstepoort Milk Laboratory. A total of 142 confirmed CoNS isolates were used for this study. Isolates were subjected to the tissue culture plate method for biofilm formation testing and antimicrobial susceptibility testing against a panel of 11 antimicrobials using the disk diffusion method. Biofilm formation was identified in 18% of CoNS tested. *Staphylococcus chromogenes* (11%) had the highest proportion of biofilm formation followed by *S. haemolyticus* 4.0% and *S. epidermidis*, *S. hominis, S. xylosus, and S. simulans* with 1% respectively. Ninety percent (90%) of CoNS isolates were resistant to at least one antimicrobial (AMR) and 51% were multidrug-resistant (MDR). Resistance among CoNS was the highest to ampicillin (90%) and penicillin (89%), with few isolates resistant to cefoxitin and vancomycin, 9% respectively. The most common resistance patterns among the CoNS was penicillin-ampicillin (16%) and penicillin-ampicillin-erythromycin (10%). Forty-two percent (42%) of biofilm positive CoNS were MDR. At the species level, MDR was common among *S. epidermis* (65%), *S. chromogenes* (52%) and *S. haemolyticus* (44%). In conclusion, biofilm formation was uncommon among the MDR-CoNS isolates in this study suggesting that biofilm formation is not a major contributing factor to antimicrobial resistance in this study. In addition, most CoNS isolates in this study were β-lactams resistant. This is concerning as penicillins are used commonly by dairy farmers in treatment of mastitis in South Africa. Nonetheless, the role of antimicrobial use practice in the development of resistance in subclinical mastitis in the dairy industry should be investigated.

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List of abbreviations

AMP: Ampicillin

AMR: Resistance to one antimicrobial

BTA: Blood tryptose agar

C: Chloramphenicol

CIP: Ciprofloxacin

CoNS: Coagulase-negative *Staphylococcus*

DA: Clindamycin

E: Erythromycin

EPS: Extracellular polysaccharide

FOX: Cefoxitin

IMI: Intramammary infection

MDR: Multidrug resistance

MHA: Muellar Hinton agar

MRSA: Methicillin resistant staphylococcus aureus

OB: Cloxacillin

OD: Optical density

ODc: Optical cut-off density

OT: Oxytetracycline

PEN: Penicillins

S: Streptomycin

TSB: Trypticase soy broth

QS: Quorum sensing

95% CI: 95 percent confidence interval

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Chapter 1

1.1 Introduction

1.1.1 Background

Coagulase-negative *Staphylococcus* species (CoNS) are among the most frequently isolated bacteria from clinical mastitis cases in dairy herds, they are commensal opportunistic pathogens normally found on the skin (Birgersson, Jonsson and Holmberg, 1992; Sawant, Gillespie and Oliver, 2009). It is said that nine out of 16 species or subspecies of CoNS investigated cause clinical mastitis in dairy cattle (Bush and Jacoby, 2010). Of these, five have been extensively reported, namely *Staphylococcus. chromogenes, Staphylococcus epidermidis, Staphylococcus simulans, Staphylococcus haemolyticus* and *Staphylococcus xylosus* (De Visscher *et al.*, 2014; Vanderhaeghen *et al.*, 2015).

The majority of animals infected with CoNS are subclinical and may remain a source of infection for susceptible animals (Piessens *et al.*, 2011; Tayyar *et al.*, 2017). Clinical signs of mastitis in dairy cattle include swollen and painful udders, decreased milk production, change in the consistency of the milk including flakes or clots (Tayyar *et al.*, 2017) and increased levels of somatic cell count (Heever and Giescke, 1967; Taponen *et al.*, 2006; Steeneveld *et al.*, 2008; Petzer *et al.*, 2009; Sudhan and Sharma, 2010; Alekish, 2015). Treatment of mastitis cases associated with CoNS is mainly through the use of antimicrobials (Raspanti *et al.*, 2016). However, there are reports of an increased prevalence of antimicrobial resistance among the CoNS (Lowy, 2003; Steeneveld *et al.*, 2008). For example, Raspanti (2016) has reported an increased prevalence of resistance to ampicillin, penicillin, ceftriaxone, cefazolin, amoxicillin-clavulanic acid and erythromycin among CoNS. In addition, methicillin and multidrug resistance CoNS among clinical mastitis cases in dairy cattle have also been reported (Lowy, 2003). The increased prevalence of resistance is of great clinical concern as these may lead to high levels of treatment failure (Sharma, Jindal and Devi, 2010).

The increased prevalence of resistance has been attributed to factors such as the presence of resistance genes, inherent resistance or biofilm formation (R.R. Marples, 1986; Taponen *et al.*, 2006; Mao *et al.*, 2012; Tayyar *et al.*, 2017). The presence of biofilm in CoNS is described as one of the main contributing virulence factors to treatment failure in clinical mastitis cases (Sawadogo-Lingani *et al.*, 2007; Pyörälä and Taponen, 2009; Katarzyna and Lis, 2014; Seng *et al.*, 2017). Biofilms are surface-associated bacterial communities that are embedded in a self-synthesized extracellular polymeric substance matrix (EPS).

Bacteria that form biofilm are able to protect themselves from antimicrobials and the immune system (Felipe *et al.*, 2017; Zapotoczna *et al.*, 2018), leading to recurrent or persistent CoNS infections (Seng *et al.*, 2017). Among CoNS species *S. chromogenes*, *S. hominis*, *S. kloosi,* and *S. xylosus* species have been reported to have the strongest biofilm formation, while *S. epidermis* and *S. simulans* have the weakest biofilm formation (Tremblay *et al.*, 2012).

1.2 Justification

Although studies show that CoNS infection among dairy cattle is on the increase (Taponen, Björkroth and Pyörälä, 2008; Petzer *et al.*, 2009; Thorberg *et al.*, 2009; Kudinha and Simango, 2012; Tayyar *et al.*, 2017), limited information is available in South Africa on the antimicrobial resistance patterns and biofilm-forming CoNS from subclinical and clinical udder infections in dairy cows.

1.3 Aim

The aim of this study is to investigate antimicrobial resistance patterns and biofilm formation among CoNS isolated from cow milk samples of subclinical dairy cattle submitted to the Onderstepoort milk laboratory.

1.4 Objectives

1. To investigate the prevalence and antimicrobial resistance patterns of CoNS isolated from subclinical mastitis cases in dairy cattle
2. To investigate the prevalence of biofilm formation in CoNS isolated from subclinical mastitis cases in dairy cattle

1.5 The benefit of the study

This research will give insight into antimicrobial resistance patterns and biofilm formation of CoNS that were isolated from milk samples submitted to the Onderstepoort milk laboratory, South Africa. The results of the resistant patterns and biofilm will be used to guide CoNS udder health management and mastitis treatment.

1.6 Structure of the dissertation

This dissertation comprises of four chapters. The first chapter provides the general background, aim, objectives, and structure of the dissertation. The second chapter is a literature review which outlines published

studies on CoNS, biofilm formation, and antimicrobial resistance. The third chapter comprises of the methodology of the study, results, and discussion. The last chapter will then outline the findings of the study and will make recommendations based on the results obtained in the study.

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Chapter 2

2.1 Literature review

2.2 Mastitis

Mastitis is the inflammation of the intramammary tissue, and it is defined based on the level of Somatic Cell Count (SCC) (Sykes *et al.*, 2007; Schukken *et al.*, 2009; Parada *et al.*, 2011; Sharma, Singh and Bhadwal, 2011; Alekish, 2015). There are contradicting statements on the SCC threshold level used to describe normal milk compared to subclinical or clinical mastitis (Heeschen, 2010). Nonetheless, an udder with SCC of ≤100 000 cells/ml from which no microorganisms are isolated and without a history of recent infection is considered to be normal (Matthews, Harmon and Langlois, 1992). Whereas an udder with an SCC of >100 000 cells/ml but <200 000 cells/ml is indicative of an inflammatory response and is likely to be infected (De Vliegher *et al.*, 2001; Fry *et al.*, 2014). Cows with SCC <200 000 cells/ml are often asymptomatic and are said to be subclinical (Petzer *et al.*, 2017). While cows with SCC ≥200 000 cells/ml are said to be clinical (De Vliegher *et al.*, 2001). Clinical mastitis cows display signs such as watery milk containing flakes, clots, or pus, and udder clinical signs such as swelling, heat, hardness, redness, or pain (Pyörälä and Taponen, 2009; Mørk *et al.*, 2010; Sinha, Thombare and Mondal, 2014; Taponen *et al.*, 2017). In some cases, these animals may show systemic signs such as fever, depression and inappetence (Myllys and Rautala, 1995; Steeneveld *et al.*, 2008; Barlow, Zadoks and Schukken, 2013; Hosseinzadeh and Dastmalchi Saei, 2014; Santman-Berends *et al.*, 2016; Sharma *et al.*, 2018).

Infectious subclinical and clinical mastitis have a huge financial burden in the dairy industry worldwide (Wellnitz *et al.*, 2016; Schewe and Brock, 2018) and continue to be a challenge in South African dairy herds (Petzer *et al.*, 2009). Globally, $35 billion annual loss is attributed to mastitis in the dairy industry (Ynte Schukken, David Wilson, Francis Welcome, Linda Garrison-Tikofsky, 2003) and losses differ by country or farming systems (El-Jakee *et al.*, 2013; Vanderhaeghen *et al.*, 2015; Dolder *et al.*, 2017). More than 250 different microorganisms that have been isolated in mastitis cases (Abebe *et al.*, 2016) with some organism being opportunistic and others pathogenic (Wellnitz *et al.*, 2016; Schewe and Brock, 2018). Species identified include *Staphylococcus* spp., *Streptococcus* spp.*, Escherichia* spp.*, Mannheimia* spp.*, Arcanobacterium* spp.*, Pasteurella* spp.*,* and *Corynebacterium* spp. (El-Jakee *et al.*, 2013). Among *Staphylococcus* species, coagulase negative *Staphylococcus* species (CoNS) are said to be emerging, environmental, opportunistic, and a contagious cause of subclinical mastitis (Pulverer, 1990; Barlow, Zadoks and Schukken, 2013; Becker, Heilmann

and Peters, 2014; Bexiga *et al.*, 2014; Kayitsinga *et al.*, 2017) characterized by a lower level of SCC compared to other *Staphylococcus* species (Barrett *et al.*, 2005; Pyörälä and Taponen, 2009). In South Africa, CoNS are among the most isolated mastitis-causing pathogen in dairy cattle (Petzer *et al.*, 2009).

2.3 Coagulase Negative *Staphylococcus* species

Staphylococci are gram-positive cocci about 0.5-1.0 µm in diameter growing in clusters and pairs (Cowan and Shaw, 1954; Archer *et al.*, 2011; Vanderhaeghen *et al.*, 2015). The genus *Staphylococcus* consists of 47 species and 23 subspecies (Becker, Heilmann and Peters, 2014). It is further divided into coagulase positive *Staphylococcus* species (CoPS) and coagulase negative *Staphylococcus* species (CoNS) based on their ability to coagulate plasma (El-Jakee *et al.*, 2013). Of the 50 *Staphylococcus* species and subspecies that have been identified in mastitis (Jarløv *et al.*, 1996; Becker, Heilmann and Peters, 2014; Hosseinzadeh and Dastmalchi Saei, 2014), 38 are classified as coagulase negative *Staphylococcus* (Devriese *et al.*, 2002; Becker, Heilmann and Peters, 2014). Of these, 23 have been diagnosed in mastitis (Pyörälä and Taponen, 2009) with *S. chromogenes, S. epidermidis, S. simulans, S. haemolyticus,* and *S. xylosus* being the predominant species (De Visscher *et al.*, 2014; Vanderhaeghen *et al.*, 2015).

2.3.1 Aetiology and pathogenesis

Coagulase negative *Staphylococcus* are commensal of the skin and mucous membranes of humans and animals (Taponen *et al.*, 2006; Tomazi *et al.*, 2014; Karakullukçu *et al.*, 2017). They are frequently isolated from cow's hair coat, nares, and teat skin (Piessens *et al.*, 2011; De Visscher *et al.*, 2017; Tayyar *et al.*, 2017). They exist as a heterogeneous group of bacteria with species specific epidemiology and pathogenesis (Piessens *et al.*, 2011; Tayyar *et al.*, 2017). For example, *S. chromogenes* are classified as a bovine-adapted species, with most cases of mastitis being opportunistic (Taponen and Pyörälä, 2009). *Staphylococcus epidermidis* is a human adapted species, therefore, infections in dairy cattle are opportunistic (Jarp, 1991; Birgersson, Jonsson and Holmberg, 1992; Thorberg *et al.*, 2009; Becker, Heilmann and Peters, 2014; Vanderhaeghen *et al.*, 2015). While, *S. xylosus* appears to be versatile, common in the environment, and form part of the normal bovine skin flora (Piessens *et al.*, 2011).

Staphylococcus spp. including CoNS have virulence factors that play a significant role in the pathogenesis of the disease. The presence of these virulence factors enables CoNS to cause persistent and recurring infections in the mammary tissue of dairy cattle (Pyörälä and Taponen, 2009; Gomes, Saavedra and

Henriques, 2016; Schönborn *et al.*, 2017). Identified virulence factors include the secretion of cell surface-bound proteins (Lacey, Geoghegan and McLoughlin, 2016), evasion or inhibition of host defence mechanism, the degradation of host tissue, and biofilm formation (Foster, 1996).

2.3.2 Risk factors of infection

There are cow-specific factors that influence the risk of CoNS infection in dairy cattle (Oliveira *et al.*, 2015). These include parity (Kateete *et al.*, 2013; Abebe *et al.*, 2016), days in milk, SCC level, and a history of clinical mastitis (Abebe *et al.*, 2016; Alhussien and Dang, 2018). Cows in early lactation (Sawant, Gillespie and Oliver, 2009) and the end of the lactation compared to other cows are also at a higher risk of CoNS infection (Steeneveld *et al.*, 2008). The risk of CoNS infection also differs based on the type of herd (Piessens *et al.*, 2011; Tayyar *et al.*, 2017), the use of dry cow therapy, maintenance of milking machines, and the type of udder health management (Jashari, Piepers and De Vliegher, 2016; Santman-Berends *et al.*, 2016; Down *et al.*, 2017). Furthermore, CoNS mastitis is said to be higher in winter and spring compared to other seasons (Pyörälä and Taponen, 2009) as well as during the dry season compared to wet season (Østerås, Sølverød and Reksen, 2010).

2.3.3 Identification

Coagulase negative *Staphylococcus* species are identified based on their phenotypic characteristics, such as colony morphology, haemolysis patterns, and biochemical reactions including gram staining, catalase, and coagulase production (Cunha, Sinzato and Silveira, 2004; Bautista-Trujillo *et al.*, 2013). Analytical profile index kit (API) is also available for *Staphylococcus* species identification. However, they are mainly developed for human isolates (Thorberg, 2008; Schukken *et al.*, 2009; Taponen and Pyörälä, 2009). Molecular identification methods including PCR of the 16S rRNA gene, internal transcribed *Staphylococcus* species (**ITS**)-PCR (Hirotaki *et al.*, 2011), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) have also been used in CoNS identification (Cunha, Sinzato and Silveira, 2004). These methods provide more accurate results compared to biochemical tests (Heikens *et al.*, 2005). Recently, a new method has been validated for the identification of *Staphylococcus* species, the Matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis (Maldi-Tof) (Cunha, Sinzato and Silveira, 2004). The Maldi-Tof method is accurate, rapid, cost-effective, therefore, provides a valuable alternative to phenotypic and molecular methods (Schmidt, Kock and Ehlers, 2015; Singhal *et al.*, 2015; Marín *et al.*, 2017).

2.4 Biofilm formation

The presence of biofilm in CoNS is recognized as the most important virulent factor which enables attachment and persistence of the bacteria on foreign materials (Büttner, Mack and Rohde, 2015). In addition, biofilm production plays a significant role in the pathogenesis of CoNS infections and facilitates gene transfer among CoNS (Tremblay *et al.*, 2014). Organisms with biofilm formation compared to those without are known to cause persistent subclinical and clinical mastitis in dairy cattle. In addition, these organisms are able to persist on milking equipment's as well as on workers hands (Fey and Olson, 2011; Seng *et al.*, 2017). Their negative impact on treatment outcomes has also been noted in human and veterinary medicine (Pyörälä and Taponen, 2009; Płoneczka-Janeczko *et al.*, 2014; Seng *et al.*, 2017).

Biofilms are surface-associated bacterial communities that are embedded in a self-synthesized extracellular polymeric substance matrix (EPS). The extracellular polymeric substance contains proteins such as poly-N-acetylglucosamine encoded by *ica*ADBC gene cluster, cell-wall associated proteins, extracellular DNA, and teichoic acid (Olson *et al.*, 2002; Latimer, Forbes and McBain, 2012; May *et al.*, 2015; Veena *et al.*, 2015). These components facilitate the defence mechanism of bacteria against inimical agents such as antimicrobials and the host immune system (Felipe *et al.*, 2017; Zapotoczna *et al.*, 2018). Studies on biofilm formation in *Staphylococcus* species have largely focused on *S. aureus* and *S. epidermidis* (Silva *et al.*, 2002; Cassat, Lee and Smeltzer, 2007; Tremblay *et al.*, 2014). However, *S. chromogenes, S. hominis, S.kloosi, S. simulans,* and *S. xylosus* have also been reported to produce biofilm (Tremblay *et al.*, 2012).

2.4.1 Biofilm genes

The *ica* operon and *bap* gene are considered biofilm forming essential genes (Silva *et al.*, 2002; Szczuka, Jabłońska and Kaznowski, 2016). The presence of the *icaA* and *ica*D genes are associated with biofilm formation in *Staphylococcus* species (Sawant, Gillespie and Oliver, 2009; Tremblay *et al.*, 2012; Martini *et al.*, 2016; Felipe *et al.*, 2017). The *ica*A gene is responsible for regulating the polysaccharide intercellular adhesion (PIA) (Sawant, Gillespie and Oliver, 2009; Büttner *et al.*, 2015). This is mediated by a poly-B (1,6)-N-acetylglucosamine (PNAG) protein which mediates cell-to-cell adhesion and protects bacteria from the host immune response (Cucarella *et al.*, 2001; Büttner *et al.*, 2015; Elkhashab *et al.*, 2018). The *ica* operon has been isolated in *S. capitis, S. auricularis, S. lugdunensis, S. cohnii* and *S. caprae* (Silva *et al.*, 2002; Martini *et al.*, 2016; Seng *et al.*, 2017; Elkhashab *et al.*, 2018). Whereas, the *bap* gene encodes for surface proteins important

in biofilm formation (Cucarella *et al.*, 2001; Tormo, 2005; Trotonda *et al.*, 2005; Felipe *et al.*, 2017; Seng *et al.*, 2017)

2.4.2 Steps in biofilm formation

Activation of biofilm formation in the host can be due to environmental stress factors such as nutrition, temperature, osmolarity, pH, iron, and oxygen (Melchior, Vaarkamp and Fink-Gremmels, 2006; Fey and Olson, 2011; Crouzet *et al.*, 2014; Karimi *et al.*, 2015). Biofilm formation generally involves the adhesion of cells to a solid substrate, followed by the cell to cell adhesion, creating multiple layers of cells (Silva *et al.*, 2002). There are four steps in biofilm formation: (1) bacterial attachment to a surface, (2) micro-colony formation, (3) biofilm maturation and (4) detachment or dispersal of bacteria which may then colonize new areas (Dunne, 2002; Olson *et al.*, 2002; Melchior, Vaarkamp and Fink-Gremmels, 2006; Piessens *et al.*, 2012; Crouzet *et al.*, 2014; Karimi *et al.*, 2015) **(Figure 1).**

2.4.2.1 Attachment

In order to initiate biofilm formation, the bacteria should get close to a surface (Davey and O'toole, 2000; Kataky and Knowles, 2018). Bacterial flagella facilitate the initial attachment and type IV pili mediated motilities which allow for the initial interactions between cells and the surface (O'Toole and Kolter, 1998). This initial attachment process is weak and reversible (Nasr, Abushady and Hussein, 2012). The attached bacteria then excrete EPS allowing for the irreversible attachment of the bacteria to a surface (Donlan, 2001; Vu *et al.*, 2009).

2.4.2.2 Micro-colony formation

Following the irreversible attachment, the bacteria aggregate and form micro-colonies through the synthesis of a polysaccharide intercellular adhesion (PIA) molecule (Büttner, Mack and Rohde, 2015; Pönisch *et al.*, 2018). Bacteria rapidly reproduce and become sessile (De la Fuente-Núñez *et al.*, 2013). Upon bacterial reproduction, the first layer of biofilm is established leading to recruitment of cells into the biofilm matrix (Dunne, 2002).

2.4.2.3 Maturation

During maturation, the biofilm matrix forms a 'mushroom' shaped structure with over 100 layers, bacteria are arranged according to their metabolism and aerotolerance (Otto, 2004). Flat, two-dimensional micro-colonies eventually evolve into a mature biofilm featuring complex, three-dimensional structures containing cells

immobilized in the biofilm matrix (Jamal *et al.*, 2015; Suja *et al.*, 2017; Kataky and Knowles, 2018). The mature structure of the biofilm is heterogeneous with cells acting as a collective living system, with water channels that allow transport of essential nutrients and oxygen to the growing cells (Donlan, 2001, 2002; Kataky and Knowles, 2018). Typically, a mature biofilm is established after 48 hr. (Kataky and Knowles, 2018).

2.4.2.4 Detachment

Cells detach from the biofilm because of either cell growth and division or the removal of biofilm aggregates (Davey and O'toole, 2000). Some cells within the population can dissociate (disperse) from the sessile structure and colonize new surfaces (Dunne, 2002; Jefferson, 2004; Karimi *et al.*, 2015).

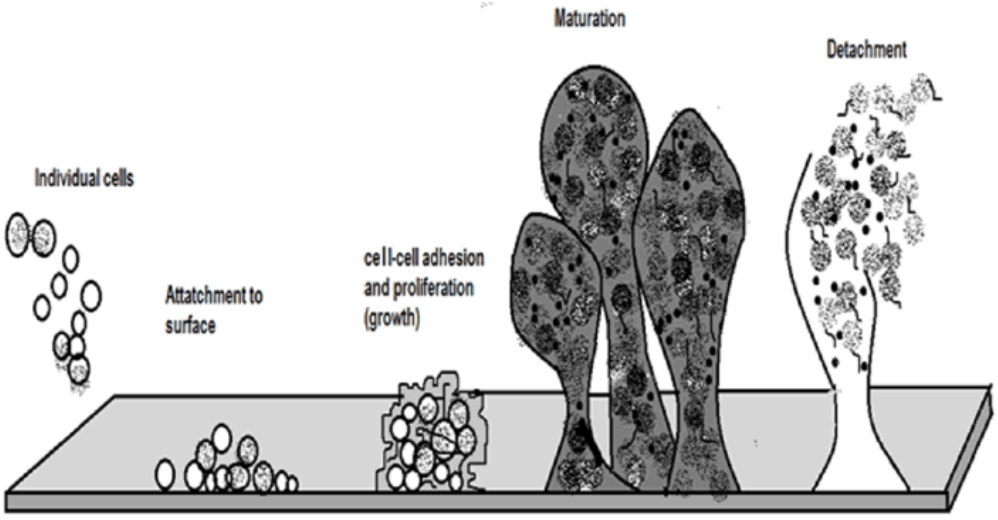

Figure 2. 1: The diagram indicates the mechanism of biofilm formation (Jamal *et al.*, 2015)

2.4.3 Significance of quorum sensing

Although there are conflicting reports on the role of quorum sensing in biofilm formation (Rasamiravaka *et al.*, 2015), autoinducers in quorum sensing have been reported to allow for the transduction of signals that leads to cell communication in a biofilm complex (De la Fuente-Núñez *et al.*, 2013; Kataky and Knowles, 2018). This process of quorum sensing is a signal peptide-mediated system **(Figure 2).** Scholars suggest that additional research is needed to understand the role of quorum sensing in biofilm formation, virulence, and development of antimicrobial resistance (Li and Tian, 2012; Castillo-Juárez *et al.*, 2015; Jamal *et al.*, 2015). Moreover, quorum sensing system regulates expression of biofilm genes, enhances access to nutrients, help in inactivation of competing bacteria and environmental stresses (Li and Nair, 2012; Li and Tian, 2012; Castillo-Juárez *et al.*, 2015). Quorum sensing can be unique for different pathogens, but generally Gram-negative bacteria will use

acylated homoserine lactones as autoinducers while Gram-positive use processed oligo-peptides to communicate.

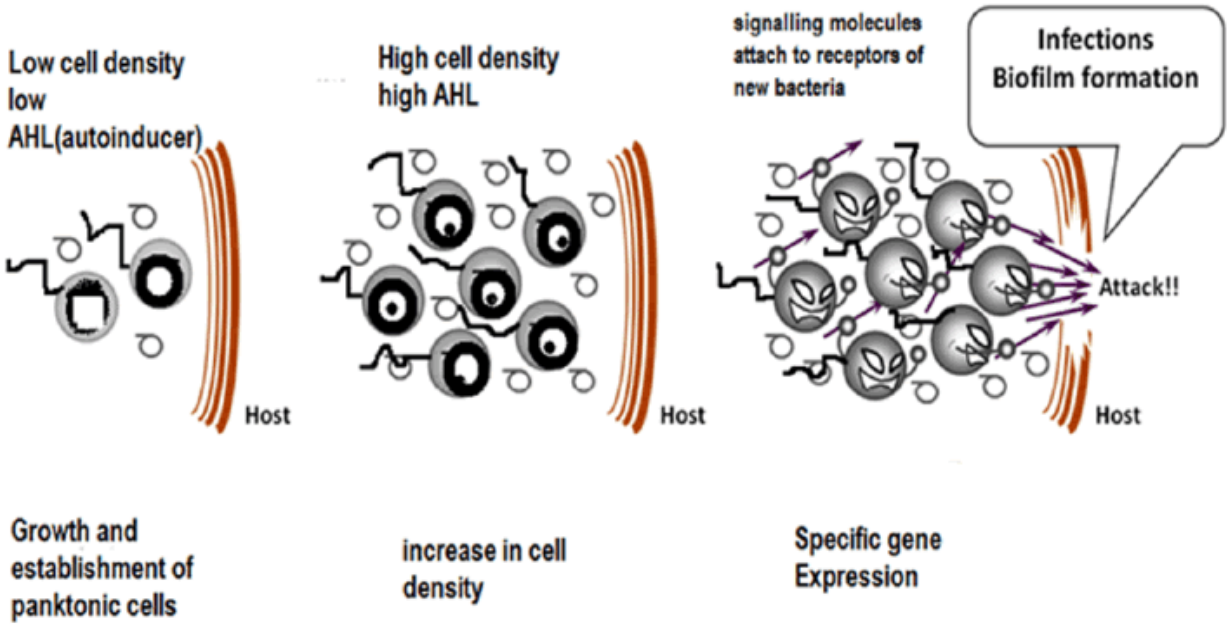

Figure 2. 2: The diagram is showing the mechanism of quorum sensing (Jamal *et al.*, 2015)

2.4.4 Identification of biofilm formation

There is no standardized method for the identification of biofilm formation in CoNS (Vukovic *et al.*, 2007). Phenotypic methods that have been used include Tissue Culture Plate (TCP), microtiter plate (MTP), test tube (TM), and Congo red agar (CRA) (Christensen *et al.*, 1982; Hassan *et al.*, 2011; Martini *et al.*, 2016). Molecular techniques such as Polymerase chain reaction (PCR) have been used in the detection of biofilm associated genes (Nasr, Abushady and Hussein, 2012; Lira *et al.*, 2016; Oliveira *et al.*, 2016; Elkhashab *et al.*, 2018)

2.4.4.1 Phenotypic method

Tissue Culture Plate method

The Tissue Culture Plate method is the most commonly used method for the identification of biofilm formation in *Staphylococcus* species (Hassan *et al.*, 2011; Simojoki *et al.*, 2012; Deka, 2014; Shrestha, Bhattarai and Khanal, 2017). It is regarded as the gold-standard due to its high specificity and accuracy. In the TCP method, the bacterial cells are grown in wells of polystyrene microtiter plates. The wells are washed, and the remaining bacteria are fixed and stained with crystal violet. Sugars may be added to assay media to increase further the ability of bacteria to form biofilm (Stepanović *et al.*, 2007). The optical density (OD) of each well

stained with crystal violet is measured at 570 nm using a microtiter-plate reader. A microtiter plate reader uses spectrophotometry to obtain the results. The analysis is performed in triplicate and repeated three times. The average optical density is calculated for all tested strains, including the negative controls (Stepanović *et al.*, 2007).

Tube Method

This is a qualitative assessment method described by Christensen et al. (1982). Microorganisms are incubated overnight, washed, and stained with crystal violet. Tubes are then put in an inverted position to dry. When a visible film lined the wall and bottom of the tube the tests are positive for biofilm formation. Experiments are also performed in triplicate and repeated three times. The presence of biofilm formation is scored from 0 to 3, 0 been absent and three strong. The Tube Method correlated well with the Tissue Culture Plate method in the identification of strong biofilm formation but differs in the identification of for weak and moderate biofilm formation (Hassan *et al.*, 2011; Deka, 2014).

Congo red method

The Congo red method is also a qualitative method for the identification of biofilm formation (Christensen *et al.*, 1982). It uses a specialized medium composed of Brain heart infusion broth and Congo red dye. Inoculated plates are incubated aerobically and black colonies with a dry crystalline consistency are indicative of biofilm. Whereas non-biofilm formation organisms remain pink. The Congo red method is the most commonly used method in the identification of biofilm formation in *Staphylococcus* species because it is easy to perform and less time consuming (Christensen *et al.*, 1982; Oliveira and Cunha, 2008; Koksal, Yasar and Samasti, 2009; Kenar, Kuyucuoğlu and Şeker, 2012). However, Congo red agar compared to the tube method, is less sensitive in identifying biofilm formation (Christensen *et al.*, 1982; Hassan *et al.*, 2011; Deka, 2014).

2.4.4.2 Molecular assessment

Molecular methods can also be used in the identification of biofilm-forming genes. Polymerase chain reaction (PCR) has been used in the detection of *ica*ADC genes associated with biofilm in *Staphylococcus* species (Lira *et al.*, 2016; Elkhashab *et al.*, 2018). The *ica*A gene is important in the production of polysaccharide intercellular adhesin (PIA). While *ica*D plays an important role in the phenotypic expression of the capsular polysaccharide (Wilkinson *et al.*, 2002; Nasr, Abushady and Hussein, 2012). Therefore, these genes

play an important role in the development of biofilm in *Staphylococcus* species (Nasr, Abushady and Hussein, 2012; Oliveira *et al.*, 2016)

2.5 Treatment and Antimicrobial Resistance

2.5.1 Antimicrobial treatment

Antimicrobial therapy is the treatment of choice for clinical mastitis in dairy cattle (Taponen *et al.*, 2017). The most commonly used method of application is intramammary unless there are systemic clinical signs (Gomes and Henriques, 2016; Santman-Berends *et al.*, 2016). Antimicrobial categories or classes that have been reported to be effective against CoNS infections include glycopeptides, aminoglycosides, macrolides, and β-lactam antimicrobials (Jain, Agarwal and Bansal, 2004; Ma *et al.*, 2011; Sujatha and Praharaj, 2012; El-Jakee *et al.*, 2013; Becker, Heilmann and Peters, 2014; Szczuka, Jabłońska and Kaznowski, 2016) with β-lactam antimicrobials been the most commonly used (Schaumburg *et al.*, 2015).

2.5.1.1 Antimicrobials mechanism of actions

Cell Wall Synthesis

A bacterial cells wall is made of peptidoglycans. The crossing linking of these peptidoglycans is by the action of transglycosidases. The D-alanyl-alanine portion of the peptide chain is said to be cross linked by glycine residues in the presence of penicillin binding proteins (PBPs) and this strengthens the cell wall. β-lactam antimicrobials target the PBPs by mimicking the D-alanyl D-alanine portion of peptide chain, making them unavailable for the synthesis of new peptidoglycan. The disruption of peptidoglycan layer leads to the lysis of bacterium. Similarly, Glycopeptides such as vancomycin inhibits the biding of the D-alanyl D-alanine, resulting in the inhibition of cell wall synthesis (Kapoor, Saigal and Elongavan, 2017).

DNA Synthesis

Antimicrobials such as fluoroquinolones inhibit the DNA gyrase in Gram-negative and Topoisomerase IV in Gram-positive (Vingopoulou *et al.*, 2018). By inhibiting Topoisomerase enzymes, these interfere with the splitting and resealing of DNA resulting in gaps in the DNA strands. The presence of these gaps induces synthesis of endonucleases leading to irreversible damage and cell death (Ghilarov and Shkundina, 2012).

Protein Synthesis

Ribosomes and cytoplasmic factors catalase protein biosynthesis in bacterial cells. The bacterial 70S ribosome consists of the 30S and 50S ribonucleoprotein subunits. Antimicrobials inhibit protein biosynthesis by

targeting either the 30S or 50S subunit of the bacterial ribosome. Aminoglycosides and tetracyclines target the 16S r-RNA of the 30S subunit resulting in misreading and premature termination of translation of mRNA. Whereas, chloramphenicol and macrolides interact with the 23S r-RNA of the 50S subunit, resulting in a premature detachment of incomplete peptide chains. Oxazolidinones inhibit protein synthesis by binding to 23Sr RNA of the 50S subunit and suppress 70S inhibition and interact with peptidyl-t-RNA (Bozdogan and Appelbaum, 2004).

Folic acid metabolism inhibitors

Sulphonamides and trimethoprim inhibit folic acid metabolism. Sulphonamides inhibit dihydropteroate synthase to it rather than the p-amino benzoic acid resulting in lack of synthesis of dihydrofolic acid (DHFA), Trimethoprim inhibits the enzyme dihydrofolate reductase resulting in lack of synthesis of tetrahydrofolic acid. These components are important in the synthesis of nucleic acid (DNA). The result will have a bacteriostatic effect due to no growth and cell division of the bacteria.

2.5.2 Antimicrobial resistance

Antimicrobial use in veterinary medicine has improved patient treatment and prognosis (Prestinaci, Pezzotti and Pantosti, 2015). However, the use of antimicrobials has been accompanied by an increase in the emergences of resistant microorganism (Davies and Davies, 2010; Laxminarayan *et al.*, 2013). Studies are reporting high proportions of antimicrobials resistance in CoNS compared to other mastitis causing pathogens (R.R. Marples, 1986; Lowy, 2003; Taponen *et al.*, 2006; Steeneveld *et al.*, 2008; Mao *et al.*, 2012; Tayyar *et al.*, 2017). For example, increased prevalence resistant to macrolides (Kenar, Kuyucuoğlu and Şeker, 2012; Szczuka, Jabłońska and Kaznowski, 2016), glycopeptides (Srinivasan, Dick and Perl, 2002; Sujatha and Praharaj, 2012; Bhattacharyya *et al.*, 2016; Blaskovich *et al.*, 2018), β-lactam (Raspanti *et al.*, 2016), and aminoglycosides have been reported in *Staphylococcus* species (Franco *et al.*, 2009; Davies and Davies, 2010; Fair and Tor, 2014). In addition, vancomycin resistance in *S. epidermidis* and *S. haemolyticus* has also been identified as a great concern for the treatment of MRSA infections (Srinivasan, Dick and Perl, 2002; Olufunmiso, Tolulope and Roger, 2017).

The antimicrobial resistance can either be intrinsic or adaptive (Sawant, Gillespie and Oliver, 2009; Fair and Tor, 2014; Prestinaci, Pezzotti and Pantosti, 2015). The adaptive resistance is mainly due to bacterial mutation, natural selection, transformation, and transduction or conjugation (Munita *et al.*, 2016). Intrinsic

resistance can be defined as the naturally occurring insensitivity in bacteria that predates antibiotic chemotherapy and is present in all bacterial species (Sirijan and Nitaya, 2006; Cox and Wright, 2013; Munita *et al.*, 2016). The mechanism of intrinsic resistance can be mediated by the bacterial outer membrane and active efflux activity (Tenover, 2006; Cox and Wright, 2013).

2.5.2.1 β-lactams

Coagulase negative *Staphylococcus* species resistant to all penicillinase-labile penicillins, including ampicillin, amoxicillin, piperacillin, and ticarcillin are said to be β-lactam resistant (Bard *et al.*, 2014). The β-lactam resistance mechanism in CoNS is mainly due to the expression of the *mec*A and *bla*Z genes (Jain, Agarwal and Bansal, 2004; Brakstad and A. Maeland, 2009). The *mec*A has been detected in various species of staphylococci including *S. intermedius, S. epidermidis, S. lentus, S. saprophyticus, S. xylosus, S. sciuri*, and *S. haemolyticus* (Devriese *et al.*, 2002; Lowy, 2003; Venkatesh, Placencia and Weisman, 2006; Sawant, Gillespie and Oliver, 2009). The *mec* genes are harboured by a staphylococcal cassette chromosome *mec* (SCC*mec*) mobile genetic element inserted into the chromosome (Becker, Heilmann and Peters, 2014).

The *mec*A encodes for penicillin-binding protein PBP2a and together with the *bla*Z gene have been reported in β-lactam resistant *Staphylococcus* species (Brakstad and A. Maeland, 2009; Becker, Heilmann and Peters, 2014; Osman *et al.*, 2017). In veterinary medicine, up to 84% of CoNS isolated from dairy cows with mastitis were β-lactam resistant (Archer and Scott, 1991; Gentilini *et al.*, 2010; Bansal *et al.*, 2015). In addition, the presence of *mec*A mediated oxacillin resistance is suggestive of methicillin resistant coagulase negative *Staphylococcus* species (Pitkälä *et al.*, 2010). All methicillin resistant CoNS contain a *mec*A gene or PBP2a (Hussain *et al.*, 2000; Koksal, Yasar and Samasti, 2009; Ibadin, Enabulele and Muinah, 2017). Methicillin-resistant *Staphylococcus* are resistant to all other penicillins, carbapenems, and cephems (CLSI, 2014). Both methicillin-resistant and β-lactams *Staphylococcus* species are said to be multidrug resistant (Taponen *et al.*, 2006; Koksal, Yasar and Samasti, 2009; Taponen and Pyörälä, 2009; Srednik *et al.*, 2017).

2.5.2.2 Antimicrobial resistance and biofilm formation

Biofilms have an intrinsic mechanism that is associated with antibiotic resistance (Bun Ng *et al.*, 2016; Hughes and Webber, 2017), including limited diffusion, enzyme causing neutralizations, heterogeneous functions, slow growth rate, efflux pump and membrane alteration (Hughes and Webber, 2017). Studies have shown a relationship between biofilm formation and increased prevalence of antimicrobial resistance (Olson *et*

al., 2002; Melchior, Fink-Gremmels and Gaastra, 2007; Jacques, Aragon and Tremblay, 2010; Tremblay *et al.*, 2012; Roy *et al.*, 2018). A study in Canada observed a decrease in CoNS susceptibility to antimicrobials for those CoNS isolates that formed biofilm (Tremblay *et al.*, 2014). Similarly, Castaneda et al (2016) observed that biofilm forming CoNS required up to 2048 higher antimicrobial concentration than CoNS without biofilms.

2.5.3 Antimicrobial stewardship

Antimicrobial stewardship is one of the most important pillars in combating antimicrobial resistance in human medicine (Schewe and Brock, 2018). Similarly, there are efforts to improve antimicrobial stewardship through the prudent use of antimicrobials in mastitis treatment (Kayitsinga *et al.*, 2017) in dairy cattle. The prudent use of antimicrobials includes correct diagnosis, use of narrow spectrum antibacterial, and correct dose (Ungemach, Müller-Bahrdt and Abraham, 2006; Silley and Stephan, 2017).

The correct antimicrobial and use of prescription for antimicrobial can help in combating antimicrobial resistance. Some studies have suggested that recording of medical records and prescriptions could help in allocating correct prescription and timely practice (Okeke and Lamikanra, 1995; Morgan *et al.*, 2011). In countries such as Europe and North America, outpatient antimicrobials are largely restricted to prescription use only, however over the counter medication is common in the rest of the world (Morgan *et al.*, 2011). The availability of over the counter medication can lead to an increase in antimicrobial resistance, a 90% increase in MDR was reported in *E. coli* isolates from children under the age of 5 years from Bolivia (Bartoloni *et al.*, 2006). Over the counter medication does not only lead to increased resistance but can also be a serious health concern and safety to patients. This drug can also be expired as a result of degradation and have decreased bioavailability which might predispose a patient to treatment failure and promote antimicrobial resistance (Okeke and Lamikanra, 1995).

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Chapter 3

Antimicrobial resistance patterns and biofilm formation of coagulase-negative *Staphylococcus* species isolated from subclinical mastitis milk samples submitted to the Onderstepoort Milk Laboratory.

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Abstract

Increased prevalence of antimicrobial resistance, treatment failure, and financial losses have been reported in dairy cows with coagulase-negative *Staphylococcus* (CoNS) clinical mastitis, however, studies on CoNS are limited in South Africa. Therefore, the objectives of this study were to investigate the antimicrobial resistance patterns and biofilm formation in CoNS isolated from cow milk samples submitted to the Onderstepoort Milk Laboratory. A total of 142 confirmed CoNS isolates were used for this study. Isolates were subjected to the tissue culture plate method for biofilm formation testing and antimicrobial susceptibility testing against a panel of 11 antimicrobials using the disk diffusion method. Biofilm formation was identified in 18% of CoNS tested. *Staphylococcus chromogenes* (11%) had the highest proportion of biofilm formation followed by *S. haemolyticus* 4.0%, *S. epidermidis*, *S. hominis, S. xylosus, and S. simulans* with 1% respectively. Ninety percent (90%) of CoNS isolates were resistant to at least one antimicrobial (AMR) and 51% were multidrug-resistant (MDR). Resistance among CoNS was the highest to ampicillin (90%) and penicillin (89%), few isolates resistant to cefoxitin and vancomycin, 9% respectively. Similarly, MDR-*S. haemolyticus* (44%), MDR-*S. epidermidis* (65%), and MDR-*S*. *chromogenes* (52%) were mainly resistant to penicillins. The most common resistance patterns observed were resistance to penicillin-ampicillin (16%) and penicillin-ampicillin-erythromycin (10%). Only 42% of biofilm positive CoNS were MDR. In conclusion, the majority of CoNS in this study were resistance to penicillins. In addition, most isolates were β-lactams resistant and MDR. Biofilm formation among the CoNS isolates in this study was uncommon and there was no significant difference in the proportion of MDR-CoNS based on the ability to form a biofilm, suggesting that biofilm formation is not a major contributing factor in MDR of CoNS in this study.

3.1 Introduction

Coagulase negative *Staphylococcus* (CoNS) are among the most frequently isolated bacteria from dairy cows with clinical and subclinical mastitis (Foster, 1996a; Schukken *et al.*, 2009; Pitkälä *et al.*, 2010). They are emerging as opportunistic pathogens in clinical mastitis in South Africa (Petzer *et al.*, 2009) and globally (Taponen *et al.*, 2007; Sampimon *et al.*, 2009; Kudinha and Simango, 2012; El-Jakee *et al.*, 2013; Becker, Heilmann and Peters, 2014; Fry *et al.*, 2014). The most commonly isolated CoNS in subclinical and clinical mastitis include *S. chromogenes, S. epidermidis, S. simulans, S. haemolyticus,* and *S. xylosus* (Foster, 1996b; Bexiga *et al.*, 2014; Xu *et al.*, 2015). Although intramammary infections caused by CoNS are usually self-limiting, there are clinical mastitis cases that often require antimicrobial treatment (Taponen *et al.*, 2006; Pieterse and Todorov, 2010). Penicillin antimicrobials have been reported to be effective against CoNS infections (Koksal, Yasar and Samasti, 2009; Becker, Heilmann and Peters, 2014; Bhattacharyya *et al.*, 2016). However, studies are reporting increasing prevalences of antimicrobial resistance in CoNS from clinical mastitis cases (Beuron *et al.*, 2014; Schmidt, Kock and Ehlers, 2015; Raspanti *et al.*, 2016) including resistance to penicillin, tetracycline, lincomycin, and streptomycin (Taponen *et al.*, 2006; Srednik *et al.*, 2017).

The increasing prevalence of resistance among CoNS could be due to the injudicious use of antimicrobials (Fair and Tor, 2014), the presence of penicillin binding protein 2a (PBP2a) (Brakstad and A. Maeland, 2009; Koksal, Yasar and Samasti, 2009; Silva *et al.*, 2014), and *mec*A mediated oxacillin resistance (Wilkinson *et al.*, 2002; Jain, Agarwal and Bansal, 2004; Szweda *et al.*, 2014; Mahato *et al.*, 2017). In addition, the high prevalence of antimicrobial resistance among CoNS could be due to their ability to form a biofilm which facilitates persistent infections (Becker, Heilmann and Peters, 2014; Yu *et al.*, 2017; Cepas *et al.*, 2019) and decreases susceptibility to commonly used antibiotics (Tremblay *et al.*, 2014). The ability of *Staphylococcus* species to form biofilm formation has been linked to the presence of biofilm-forming genes such as *ica*A and *bap* gene (Tremblay *et al.*, 2012, 2014; Srednik *et al.*, 2017) which have been isolated in *S. epidermidis, S. haemolyticus,* and *S. xylosus* compared to *S. chromogenes* and *S. simulans* (Tremblay *et al.*, 2012). To our knowledge, there are no studies that have reported an association between biofilm formation and high prevalence of MDR in CoNS isolated from subclinical mastitis cases in dairy cattle. In addition, no studies have been published on the antimicrobial resistance patterns of CoNS from dairy cattle in South Africa.

Thus, the aim of this study was to investigate the antimicrobial resistance patterns and biofilm formation of CoNS isolated from cow milk samples at the Onderstepoort milk laboratory. We hypothesize that

CoNS with biofilm formation isolated from subclinical mastitis cases have an increased prevalence of resistance to commonly used antimicrobials. In addition, it also possible that these isolates are β-lactam and multidrug-resistant (MDR).

3.2 Methods and materials

3.2.1 Data source

Coagulase negative *Staphylococci* isolated from composite milk samples of subclinical mastitis cases that were submitted to the Onderstepoort milk laboratory in 2017 were used. In total 142 pure CoNS isolates were included in this study.

3.2.2 Biofilm formation

The biofilm formation of CoNS isolates was investigated using the tissue culture plate method (Stephanovic *et al.*, 2007). Isolates were cultured in BTA (blood tryptose agar) for 24 hrs at 37 ^{o}C. A loopful of a colony was then inoculated into 5mL of trypticase soy broth (TSB) for 24 hrs at 37 ^{o}C. The inoculated broth was diluted using 1:100 to make a final volume of 2ml (1.98 ml TSB: 0.02 ml inoculum). Individual wells of sterile 96 well flat bottom polystyrene tissue culture-treated plates (Sigma-Aldrich, Costar, USA) were filled with 200 µL of the diluted broth, positive control and negative control in triplicate. The plates were incubated for 24 hrs at 37 ^{o}C. After incubation, the plates were read to obtain optical density (OD) before washing at a wavelength of 570 nm using a micro ELISA (enzyme-linked immunosorbent assay) auto-reader (model 680, Biorad, UK). The contents of each well were then removed by gently tapping. The wells were washed with 200-300 ul of phosphate buffer saline (pH 7.4) three times while gently flicking the plates after each wash and left to dry for about 15 min.

Biofilm formed and adhered to the wells were fixed using 150 ul of (96%) methanol for 20 min, where removed after the contents, and the plates were left to dry for 60 min. Each well was stained with 150 ul (0.2%) of crystal violet for 15 min, 150 ul of (96%) ethanol were then added into each well and covered for 30 min to elute the stain. The plates were read after washing at a wavelength of 570 nm using a micro ELISA auto-reader (model 680, Biorad, UK). This method was repeated 3 times and the OD (optical density) was averaged and subtracted from the cut off value to obtain the final OD for each isolate. A reference strain *S. epidermidis* ATCC 35984 was used as a control (Thermo Fischer).

The interpretation of the results was divided into the following categories; OD ≤ODc (Optical density cut-off value) =no biofilm producer; ODc <OD ≤2XODc=weak biofilm producer; 2XODc <OD

≤4XODc=moderate biofilm producer; 4XODc <OD=strong biofilm producer (Stephanovic *et al.*, 2007). For the purposes of analysis, weak, moderate, and strong biofilm were classified as biofilm positive.

3.2.3 Antibiotic susceptibility testing

Coagulase negative *Staphylococcus* isolates were subjected to antimicrobial susceptibility testing against a panel of 11 drugs using the disc diffusion method (Kirby-Bauer method) (Clinical Laboratory Standards Insitute, 2017) on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute guidelines. The antimicrobials investigated included 10 mcg ampicillin (AMP), 10 iu penicillin G (P), 30 µg oxytetracycline (OT), 15 µg erythromycin (E), 30 µg chloramphenicol (C), 10 µg streptomycin (S), 5 µg ciprofloxacin (CIP), 30 ug cefoxitin (FOX), 10 µg vancomycin (VAN), 10 mcg clindamycin (DA) and 5 mcg cloxacillin (OB) (Clinical Laboratory Standards Insitute, 2017). Based on the diameter of the zone of inhibition, isolates were classified as sensitive, intermediate or resistant (Clinical Laboratory Standards Insitute, 2017). For the purpose of analysis, the intermediate susceptibility was considered as resistant. Isolates that were resistant to at least one antimicrobial drug were defined as “resistant” while those resistant to three or more antimicrobial categories were defined as “multidrug resistant” (Magiorakos *et al.*, 2012). β-lactams resistance was classified as resistant to at least penicillins, cephalosporins or carbapenems (Oliver and Murinda, 2012; Becker, Heilmann and Peters, 2014). The interpretation of vancomycin was based on the criteria by Rezaeifar et al. (2016).

3.2.4 Data analysis

The proportions and frequencies of all the variables together with their 95% of confidence intervals (CI) were calculated and presented in table format. The 95% CI was used to assess independence of proportions.

3.3 Results

3.3.1 Coagulase negative *Staphylococcus* species

A total of 142 CoNS isolates were tested for biofilm formation and antimicrobial resistance, the majority of the isolates tested were *S. chromogenes* (70%; 100/142), followed by *S. epidermidis* (12%; 17/142), *S. haemolyticus* (11%; 16/142), *S. simulans* (2%; 3/142), *S. xylosus* (2%; 3/142), *S. hominis* (1%; 1/142), *S. hyicus* (1%; 1/142) and *S. scuiri* (1%; 1/142).

Of the isolates tested, 18% (26/142) formed biofilm. Among biofilm producing isolates, 11% were *S. chromogenes*, followed by *S. haemolyticus* (4%) and *S. epidermidis* (1%). No biofilm formation was identified in *S. scuiri* and *S. hyicus* **(Table 3.1)**.

Table 3. 1: Biofilm formation of coagulase-negative *Staphylococcus* species (n=142) isolated from cow milk samples at the Onderstepoort milk laboratory, 2017

Organism	Tested	Number of Biofilm forming Isolates			Total Biofilm formation			
		Weak	Moderate	Strong	Number	Percent	95% CI[a]	
S. chromogenes	100	6	7	3	16	11	7	18
S. epidermidis	17	1	0	0	1	1	0	4
S. haemolyticus	16	4	2	0	6	4	18	61
S. hominis	1	0	0	1	1	1	1	7
S. scuiri	1	0	0	0	-	-	-	-
S. xylosus	3	1	0	0	1	1	0	4
S. simulans	3	1	0	0	1	1	0	4
S. hyicus	1	0	0	0	-	-	-	-

[a]95% CI= 95 percent confidence interval

In total, 90% (128/142) of CoNS were resistant to at least one antimicrobial (AMR), with most isolates resistant to ampicillin (63%) and penicillin (63%). Few CoNS isolates were resistant to cloxacillin (16%), cefoxitin (9%), and vancomycin (9%). More than half (51%, 73/142) of CoNS were multidrug resistant (MDR). Multidrug resistant CoNS were mainly resistant to penicillin (88%), ampicillin (85%) and erythromycin (64%) **(Table 3.2)**. The most common resistant patterns identified among CoNS were penicillin-ampicillin (16%; 17/106) and penicillin-ampicillin-erythromycin (10%; 11/106).

Table 3. 2**:** Antimicrobial resistance patterns of coagulase-negative *Staphylococcus* isolated from cow milk samples at the Onderstepoort milk laboratory, 2017

Group	Antimicrobial	AMR-CoNS[b] (n=142)			MDR-CoNS[c] (n=73)		
		Percent	95% CI[a]		Percent	95% CI[a]	
Lincosamide	Clindamycin	11	7	17	19	12	30
Penicillins	Penicillin	63	55	70	88	78	93
	Ampicillin	63	55	71	85	75	91
	Cloxacillin	16	11	23	30	21	41
Tetracyclines	Oxytetracycline	11	7	17	21	13	31
Phenicols	Chloramphenicol	6	3	11	10	5	18
Fluoroquinolone	Ciprofloxacin	8	4	13	12	7	22
Cephalosporin	Cefoxitin	9	5	15	18	11	28
Glycopeptide	Vancomycin	9	5	15	16	10	27
Aminoglycoside	Streptomycin	30	23	38	47	36	58
Macrolide	Erythromycin	49	41	58	64	53	74

[a]95% CI= 95 percent confidence interval
[b]AMR-CoNS= Antimicrobial resistance of Coagulase negative *Staphylococcus* resistant to at least one antimicrobial
[c]MDR-CoNS= Multidrug resistance coagulase negative *Staphylococcus*.

Among biofilm positive CoNS, 92% (24/26) were resistant to at least one antimicrobial, half of the isolates were resistant to erythromycin (54%) and penicillin (50%). While 42% (11/26) of biofilm positive isolates were MDR. Biofilm positive isolates with MDR were resistant to penicillin (82%), erythromycin (73%), ampicillin (64%) and streptomycin (55%) (**Table 3.3**).

Table 3. 3: Antimicrobial resistance of coagulase-negative *Staphylococcus* species biofilm positive isolated from cow milk samples at the Onderstepoort milk laboratory, 2017

Group	Antimicrobial	AMR-CoNS[b] biofilm positive (n=24)			MDR-CoNS[c] biofilm positive (n=11)		
		Percent	95% CI[a]		Percent	95% CI[a]	
Lincosamide	Clindamycin	21	9	41	36	15	65
Penicillins	Penicillin	54	35	72	82	52	95
	Ampicillin	42	25	61	64	35	85
	Cloxacillin	4	1	20	9	2	38
Tetracyclines	Oxytetracycline	8	2	26	18	5	48
Phenicols	Chloramphenicol	8	2	26	18	5	48
Fluoroquinolone	Ciprofloxacin	13	4	31	18	5	48
Cephalosporin	Cefoxitin	8	2	26	18	5	48
Glycopeptide	Vancomycin	8	2	26	9	2	38
Aminoglycoside	Streptomycin	46	28	65	55	28	79
Macrolide	Erythromycin	58	39	76	73	43	90

[a]95% CI= 95 percent confidence interval
[b]AMR-CoNS= Antimicrobial resistance of Coagulase negative to at least one antimicrobial
[c]MDR-CoNS= Multidrug resistance of coagulase negative staphylococcus.

3.3.2 *Staphylococcus chromogenes* species

Ninety-three percent (93\100) of *S. chromogenes* were resistant to at least one antimicrobial. Isolates were mainly resistant to ampicillin (66%), penicillin (63%) and erythromycin (54%). Low resistance was observed to vancomycin (11%) and cefoxitin (6%). Multidrug resistant *S. chromogenes* (52%; 52/100)

exhibited a high prevalence of resistant to penicillin (87%), ampicillin (87%), erythromycin (69%) and streptomycin (54%) **(Table 3.4).** The most common resistant patterns among *S. chromogenes* were the penicillin-ampicillin-erythromycin (91%) and penicillin-ampicillin pattern (71%). Among biofilm positive *S. chromogenes,* 50% (8/16) were MDR.

Table 3. 4: Antimicrobial resistance of *Staphylococcus chromogenes* isolated from cow milk samples at the Onderstepoort milk laboratory, 2017

		AMR-*S. chromogenes*[b] (n=100)			MDR-*S. chromogenes*[c] (n=52)		
Group	**Antimicrobial**	**Percentage**	**95% CI[a]**		**Percentage**	**95% CI[a]**	
Lincosamide	Clindamycin	14	9	22	25	15	38
Penicillins	Penicillin	63	53	72	87	75	93
	Ampicillin	66	56	75	87	75	93
	Cloxacillin	14	9	22	25	15	38
Tetracyclines	Oxytetracycline	6	3	12	12	5	23
Phenicols	Chloramphenicol	4	2	10	8	3	18
fluoroquinolone	Ciprofloxacin	4	2	10	6	2	16
Cephalosporin	Cefoxitin	6	3	12	12	5	23
Glycopeptide	Vancomycin	11	6	19	19	11	32
Aminoglycoside	Streptomycin	34	25	44	54	41	67
Macrolide	Erythromycin	54	44	63	69	56	80

[a]95% CI= 95 percent confidence interval
[b]AMR- *S. chromogenes* = *S. chromogenes* resistant to at least one antimicrobial
[c]MDR- *S. chromogenes* = Multidrug resistance *S. chromogenes*.

3.3.3 *Staphylococcus epidermidis* species

Overall, 94% (16/17) of *S. epidermidis* were resistant to at least one antimicrobial while 65% (11/17) were MDR. The resistance was high to penicillin (82%) and ampicillin (77%). Few isolates were resistant to vancomycin (12%), cloxacillin (35%) and cefoxitin (29%). Multidrug resistant *S. epidermidis* showed an increased prevalence of resistance to penicillin (91%) and ampicillin (91%) **(Table 3.5)**. Twenty-five percent (1/4) of the biofilm positive *S. epidermidis* were resistant to at least one antimicrobial while none of the biofilm positive *S. epidermidis* were MDR.

Table 3. 5: Antimicrobial resistance patterns of *Staphylococcus epidermidis* isolated from cow milk samples at the Onderstepoort milk laboratory, 2017

		AMR-*S. epidermidis*[b] (n=16)			MDR-*S. epidermidis*[c] (n=11)		
Group	**Antimicrobial**	**Percent**	**95% CI[a]**		**Percent**	**95% CI[a]**	
Lincosamide	Clindamycin	0	0	18	0	0	26
Penicillins	Penicillin	82	59	94	91	62	98
	Ampicillin	77	53	90	91	62	98
	Cloxacillin	35	17	59	55	28	79
Tetracyclines	Oxytetracycline	41	22	64	64	35	85
Phenicols	Chloramphenicol	17	6	41	18	5	48
fluoroquinolone	Ciprofloxacin	17	6	41	27	10	57
Cephalosporin	Cefoxitin	29	13	53	45	21	72
Glycopeptide	Vancomycin	12	3	34	18	5	48
Aminoglycoside	Streptomycin	12	3	34	18	5	48
Macrolide	Erythromycin	41	22	64	45	21	72

[a]95% CI= 95 percent confidence interval
[b]AMR- *S. epidermidis* = *S. epidermidis* to at least one antimicrobial
[c]MDR- *S. epidermidis* = Multidrug resistance *S. epidermidis*.

3.3.4 *Staphylococcus haemolyticus* species

Eighty-one percent (13/16) of *S. haemolyticus* were resistant to at least one antimicrobial, while 44% (7/16) were MDR. The highest prevalence of resistance observed was to penicillin (56%) and few isolates were resistant to cloxacillin (13%) and cefoxitin (13%). Multidrug resistant *S. haemolyticus* were mainly resistant to penicillin (100%), ampicillin (71%), and erythromycin (57%) **(Table 3.6)**. Among *S. haemolyticus* biofilm positive isolates, 100% (6/6) were resistant to at least one antimicrobial while 50% (3/6) of the isolates were MDR.

Table 3. 6: Antimicrobial resistance of *Staphylococcus haemolyticus*, isolated from cow milk samples at the Onderstepoort milk laboratory, 2017

		AMR-*S. haemolyticus*[b] (n=13)			MDR-*S. haemolyticus*[c] (n=7)		
Group	**Antimicrobial**	**Percent**	**95% CI[a]**		**Percent**	**95% CI[a]**	
Lincosamide	Clindamycin	6	1	28	14	3	51
Penicillins	Penicillin	56	33	77	100	65	100
	Ampicillin	44	23	67	71	36	92
	Cloxacillin	13	3	36	29	8	64
Tetracyclines	Oxytetracycline	13	3	36	29	8	64
Phenicols	Chloramphenicol	6	1	28	14	3	51
fluoroquinolone	Ciprofloxacin	19	7	43	29	8	64
Cephalosporin	Cefoxitin	13	3	36	29	8	64
Glycopeptide	Vancomycin	0	0	19	0	0	35
Aminoglycoside	Streptomycin	31	14	56	43	16	75
Macrolide	Erythromycin	25	10	50	57	25	84

[a]95% CI= 95 percent confidence interval
[b]AMR- *S. haemolyticus* = *S. haemolyticus* resistant to at least one antimicrobial
[c]MDR- *S. haemolyticus* = Multidrug resistance of *S. haemolyticus*.

3.4 Discussion

Coagulase negative *Staphylococcus* species (CoNS) have been reported as a cause of mastitis in dairy cattle (Taponen *et al.*, 2006) with prognosis in affected patients dependent on antimicrobial resistance profile of the isolate, the presence of virulence factors, and biofilm formation (Cepas *et al.*, 2019). In this study, we investigated antimicrobial resistance patterns and biofilm formation of CoNS isolated from cow milk samples submitted to the Onderstepoort milk laboratory.

3.4.1 Biofilm formation of CoNS

We observed a low proportion of biofilm-forming CoNS compared to other studies (Simojoki *et al.*, 2012; Tremblay *et al.*, 2012; Srednik *et al.*, 2017). For example, Tremblay et al (2012) in Canada reported 96.7% proportion of biofilm formation in CoNS from dairy cattle with mastitis. Similarly, 85.1% of CoNS from subclinical and clinical mastitis cases of dairy cattle in Argentina formed biofilm (Srednik *et al.*, 2017). Simojoki et al (2012) in Finland also reported a high (31.3%) proportion of biofilm-forming CoNS from clinical mastitis cases in dairy cattle. The low proportion of biofilm formation of CoNS in this study compared to the

above-mentioned studies may be attributed to the difference in the study population. In this study, we investigated subclinical mastitis cases while Tremblay et al (2012) and Simojoki et al (2012) investigated clinical mastitis cases. In addition, the type of growth media used for biofilm formation assay could have resulted in the low proportion of biofilm identified in this study (Fabres-Klein *et al.*, 2015). Nonetheless, the low proportion of biofilm formation in this study suggests that biofilm formation is not common in CoNS subclinical mastitis. Therefore, the role played by biofilm formation in the prognosis of CoNS subclinical mastitis in this study is limited. However, more studies need to be done to further explore the molecular epidemiology of biofilm formation CoNS from subclinical and clinical mastitis cases in dairy cattle, South Africa.

3.4.2 Antimicrobial resistance of CoNS

A high (90%) proportion of CoNS in this study were resistant to at least one antimicrobial and no significant difference was observed in the proportion of resistance among CoNS species. Antimicrobial resistance in this study was higher than 21.4% of the 56 CoNS isolates tested reported in clinical mastitis cases of lactating cows in Sweden (Bengtsson *et al.*, 2009). The reason for the high proportion of resistant CoNS isolates in this study is not clear. However, this could be due to selection pressure associated with injudicious use of antimicrobials for the treatment of clinical mastitis in dairy cattle from South Africa (Fair and Tor, 2014). Moreover, antimicrobial drugs are easily available for farmers as over the counter medication (Oguttu, Qekwana and Odoi, 2017). Studies investigating antimicrobial use among farmers in the dairy industry in South Africa will be beneficial in understanding their role in antimicrobial stewardship. In addition, findings of this study suggest that interventions in the use of antimicrobial treatment among farmers in South Africa including restriction of antimicrobial use, limitation of over the counter antimicrobial, veterinary consultation, and the improvement of knowledge on antimicrobial resistance must be considered.

3.4.3 Penicillin resistance

A high (63%) proportion of penicillins resistant CoNS was observed in this study compared to that reported in clinical mastitis of dairy cattle in Finland (32%) (Simojoki *et al.*, 2012), Estonia (38.5 %) (Pitkälä *et al.*, 2007), and Zimbabwe (8%) (Kudinha and Simango, 2012). In contrast to other studies (Gentilini *et al.*, 2010; Kenar, Kuyucuoğlu and Şeker, 2012), we observed no difference in the proportion of penicillin resistance among CoNS species. The high proportion of penicillin resistance in this study could be due to the low affinity associated with penicillin-binding protein 2a (PBP2a) produced by *Staphylococcus* species (Brakstad and A. Maeland, 2009; Koksal, Yasar and Samasti, 2009; Silva *et al.*, 2014). In addition, this could

also be due to overuse of these antimicrobials as they are readily available as over the counter antimicrobials for treatment of mastitis in dairy cattle in South Africa (Henton *et al.*, 2011) mainly due to their narrow-spectrum activity (Bengtsson *et al.*, 2009; Persson Waller *et al.*, 2011; Szweda *et al.*, 2014).

3.4.4 Erythromycin resistance

The resistance to erythromycin among CoNS was higher (49%) in this study than reported in subclinical mastitis of dairy cattle in Argentina (29%) (Raspanti *et al.*, 2016) and in Germany (22%) (Lüthje and Schwarz, 2006). In contrast, a higher (73.2%) proportion in 67 isolates of erythromycin resistant CoNS has been reported in a study done on subclinical mastitis cases of dairy cattle in Turkey (Kenar, Kuyucuoğlu and Şeker, 2012). The presence of erythromycin resistance in this study may be attributed to the presence of a ribosomal methylase based resistance, encoded by *msr*A and *erm*C (Sawant, Gillespie and Oliver, 2009). Furthermore, Luthje et al (Lüthje and Schwarz, 2006) suggest that alteration of the ribosomal methylase activity in *Staphylococcus* spp. could lead to horizontal gene transfer. Although erythromycin is one of the antimicrobial drugs used for control of CoNS isolates in bovine mastitis, it is not used for the treatment of CoNS in South Africa. Therefore, the high prevalence of resistance observed in this study needs further investigation.

3.4.5 Vancomycin resistance

Vancomycin resistance among CoNS in this study was uncommon (9%) compared to the 58.2% in 67 CoNS samples reported in subclinical bovine mastitis cases in Turkey (Kenar, Kuyucuoğlu and Şeker, 2012). In contrast, Bengtsson et al (2009) in Sweden reported no vancomycin resistance among CoNS isolated from mastitis cases in dairy cattle. Although vancomycin is not currently used for the treatment of clinical mastitis in South Africa, other peptides antimicrobial drugs such as bacitracin are used in combination intramammary applications. The presence of vancomycin resistance CoNS is of public health significance as vancomycin is the drug of choice for treatment of MRSA in human medicine (Foster, 1996a). Therefore, measures must be implemented including restriction on the use of peptides antimicrobial drugs in the treatment of mastitis in the dairy industry to curb the potential development of vancomycin-resistant CoNS.

3.4.6 Cefoxitin and β-lactam resistance

The cefoxitin test is the preferred method for testing the CoNS for *mec*A mediated oxacillin resistance (Hussain *et al.*, 2000; Clinical Laboratory Standards Insitute, 2017; Suja *et al.*, 2017). We observed lower (9%) proportion of cefoxitin resistant CoNS compared to the 29.41% of 68 CoNS isolates reported in clinical

mastitis cases from dairy cattle in Tunisia (Klibi *et al.*, 2018) and 40% reported in subclinical mastitis from dairy cattle in Switzerland (Sakwinska *et al.*, 2011). In addition, there was no significant difference in the proportion of cefoxitin resistant isolates within CoNS species. The presence of *mec*A mediated oxacillin resistance is suggestive of methicillin-resistant coagulase negative *Staphylococcus* species (Pitkälä *et al.*, 2010) and also encodes for penicillin-binding protein PBP2a. Together with the *bla*Z gene have been reported in β-lactam resistance among *Staphylococcus* species (Becker, Heilmann and Peters, 2014; Osman *et al.*, 2017). Antimicrobial resistance of β-lactams is attributed to the hydrolysis and alteration of the β-lactam ring in bacteria (Becker, Heilmann and Peters, 2014) and is a common resistance mechanism to penicillins (Tremblay *et al.*, 2012). In this study, we observed a higher proportion of β-lactam resistant CoNS compared to the 23% reported in 65 isolates with subclinical mastitis cases of dairy cows in Finland (Taponen *et al.*, 2006). In contrast, all (100%) CoNS isolates from clinical mastitis cases of dairy cattle in Argentina were β-lactam resistant (Gentilini *et al.*, 2010). The high presence of β-lactam resistant isolates and the potential presence of methicillin resistance among CoNS are likely to result in the poor clinical outcome as these isolates are likely to be resistant to other antimicrobial groups including tetracyclines, lincosamides, aminoglycosides, and macrolides (May *et al.*, 2014; Silva *et al.*, 2014; Ibadin, Enabulele and Muinah, 2017; Suja *et al.*, 2017).

3.4.7 Multidrug resistance and biofilm formation

We observed a high (51%) proportion of MDR-CoNS compared to 45% reported in clinical mastitis cases in India (Mahato *et al.*, 2017). The high proportion of MDR-CoNS in this study could be attributed to the presence of *mec*A mediated oxacillin resistance and a high proportion of β-lactams resistant CoNS isolates (Wilkinson *et al.*, 2002; Jain, Agarwal and Bansal, 2004; Szweda *et al.*, 2014; Mahato *et al.*, 2017). Nonetheless, the high occurrence of MDR-CoNS further emphasizes the need for judicious use of antimicrobial drugs in the dairy industry in South Africa.

There was no significant difference in the presence of MDR among CoNS with biofilm formation compared to those without. To our knowledge, this is the first study to compare antimicrobial resistance patterns and biofilm formation in subclinical mastitis in dairy cattle. In contrast, a study done in clinical patients in human medicine reported a high prevalence of multidrug resistance in biofilm positive CoNS compared to biofilm negative CoNS (Shrestha, Bhattarai and Khanal, 2017). In addition, multidrug resistance among *S. aureus* from human clinical isolates in Korea was more common in isolates with biofilm formation compared to those without (Kwon *et al.*, 2008). Oliveira et al (2016) in human medicine reported that biofilm-forming organisms are up to 1000 times more resistant compared to non-biofilm forming organisms. The

results of this study suggest that biofilm formation is not a major contributing factor in multidrug resistance in this study.

3.5 Limitation of the study

The type of growth media used in the study to assay biofilm formation may have played a role in the low proportion of biofilm identified in this study as the chemical composition of growth media have been shown to influence the expression of biofilm-forming genes in bacteria (Fabres-Klein *et al.*, 2015). In addition, vancomycin resistance was assessed using the disk diffusion method, however, the MIC antimicrobial test is the preferred method for analysis of vancomycin resistance (Clinical Laboratory Standards Insitute, 2017). The population of isolates used in this study came from samples submitted to the one laboratory. Therefore, results of this study should not be generalized to the entire dairy industry in South Africa.

3.6 Conclusion

Biofilm formation among the CoNS isolates in this study was uncommon and there was no significant different proportion of MDR-CoNS based on the ability to form a biofilm. Suggesting that biofilm formation is not a major contributing factor in antimicrobial resistance in this study. The majority of CoNS in this study were resistance to penicillins. In addition, most isolates were β-lactams resistant and MDR.

The presence of high antimicrobial resistance in this study is a clinical concern and urgent actions should be taken to address the situation. Farmers in South Africa need to be made aware of the high MDR among CoNS and the need for judicious use of antimicrobials in the treatment of CoNS subclinical mastitis. The role of antimicrobial use practise in the development of resistance in subclinical mastitis in the dairy industry should be investigated. The relationship between antimicrobial resistance and biofilm formation in CoNS biofilm formation in the dairy industry is not clear and this concept needs further investigation.

3.7 References

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Chapter 4

4.1 Summary, discussions and conclusions

This chapter summarizes the key findings, provide conclusions and recommendations for future research on biofilm formation and antimicrobial resistance patterns in coagulase negative *Staphylococcus* species (CoNS). The first objective of the study was to investigate biofilm formation in CoNS isolated from subclinical bovine mastitis. The proportion of biofilm-forming CoNS isolates in this study was lower than previously reported in Argentina (Srednik *et al.*, 2017), Canada (Tremblay *et al.*, 2012) and Finland (Simojoki *et al.*, 2012). Although the results were not statistically significant, biofilm formation was more common in *S. haemolyticus* compared to other CoNS species. This may not be surprising as intraspecies variations in biofilm formation among CoNS have been previously reported (Simojoki *et al.*, 2012; Srednik *et al.*, 2017). Nonetheless, the low proportion of biofilm formation in this study suggests that it is not a major contributing factor in the prognosis of bovine subclinical mastitis cases. In addition, more studies need to be done to further explore the molecular epidemiology of biofilm formation in CoNS from subclinical and clinical mastitis cases in dairy cattle, South Africa.

The second objective of the study was to describe antimicrobial resistant patterns among CoNS isolated from subclinical bovine mastitis cases. Resistance to at least one antimicrobial among CoNS in this study was high compared to other studies (Bengtsson *et al.*, 2009; Pitkälä *et al.*, 2010; Kudinha and Simango, 2012; Raspanti *et al.*, 2016). There was also no significant difference in the proportion of antimicrobial resistance among the CoNS. The majority of CoNS in this study had an increased proportion of resistance to penicillins and erythromycin contrast to previous studies (Pyörälä and Taponen, 2009; Kudinha and Simango, 2012; Srednik *et al.*, 2017). The high proportion of penicillins resistant CoNS may be attributed to intrinsic resistance to β-lactam antimicrobials among *Staphylococcus* spp. (Hartman and Tomasz, 1984; Bengtsson *et al.*, 2009; Gentilini *et al.*, 2010) or the overuse of these antimicrobials in the treatment of subclinical and clinical mastitis in dairy cattle in South Africa. In view of this, further research needs to be done to investigate the role of antimicrobial use practice in the development of resistance in subclinical mastitis cases in the dairy industry.

Vancomycin resistance among CoNS was detected in this study based on the disk diffusion methods as described by Rezaeifar *et al*(2016). Although vancomycin is not currently used for the treatment of clinical mastitis, other peptides antimicrobials are used in South Africa. Therefore, there should be a restriction on the

use of peptides antimicrobial drugs in the treatment of mastitis. Moreover, vancomycin is currently the drug of choice for treatment of MRSA in human medicine (Foster, 1996). The results of the disk diffusion must be interpreted with caution as minimum inhibitory concentration (MIC) is considered a gold standard for detection of vancomycin resistance in *Staphylococcus* spp. (CLSI, 2017). Therefore, it possible that the occurrence of vancomycin resistant in this study could have been overestimated.

Multidrug resistance among CoNS in this study was higher compared to other studies done in Tunisia (Kenar, Kuyucuoğl, and Şeker, 2012) and the USA (Sawant, Gillespie and Oliver, 2009). This could have been as a result of high proportion β-lactam resistance and *mec*A mediated oxacillin resistance among CoNS in this study. The *mec*A encodes for penicillin-binding protein PBP2a in *Staphylococcus* spp. resulting in β-lactam resistance (Becker, Heilmann and Peters, 2014; Osman *et al.*, 2017) and isolates that are cefoxitin resistant are often multidrug-resistant (May *et al.*, 2014; Silva *et al.*, 2014; Ibadin, Enabulele and Muinah, 2017; Suja *et al.*, 2017). Since certain intramammary applications are available as over the counter medications, farmers must be made aware of the risk of MDR among CoNS and the need for judicious use of antimicrobials in the treatment of CoNS subclinical mastitis.

There was no statistically significant difference in the presence of MDR among CoNS with biofilm formation compared to those without. To our knowledge, there are no studies that have investigated biofilm formation MDR in CoNS from bovine subclinical mastitis cases. However, human studies have reported higher proportions of MDR in biofilm-forming compared to non-biofilm forming CoNS (Kwon *et al.*, 2008; Oliveira *et al.*, 2016; Shrestha, Bhattarai and Khanal, 2017). The results of this study suggest that biofilm formation is not a major contributing factor in multidrug resistance among CoNS in this study. However, more studies are needed to further investigate the relationship between antimicrobial resistance and biofilm formation in clinical and subclinical mastitis cases in dairy cattle.

Antimicrobial resistance genes are fundamental in the study of resistance, studies in South Africa dairy industry should focus on the isolation of resistance genes that might be responsible for the increase in antimicrobial resistance. Such is the case for biofilm formation, with its complex nature leading to successful colonization, the genes responsible for formation should be further isolated and studied.

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