UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ANTIBIOTIC RESISTANCE OF COAGULASE POSITIVE STAPHYLOCOCCI ISOLATED FROM MILK OF SOUTH AFRICAN DAIRY HERDS

Joanne Karzis

UNIVERSITY OF PRETORIA

Antibiotic resistance of coagulase positive staphylococci isolated from milk of South African dairy herds

by

Joanne Karzis

Submitted in partial fulfilment of the requirements for the PhD degree

In the Faculty of Veterinary Sciences

University of Pretoria

Onderstepoort

Pretoria

2020

Supervisor	: Prof Edward F. Donkin
Co-supervisors	: Prof Inge-Marié Petzer
	: Prof Vinny Naidoo

Table of Contents

Publications arising from this Thesis

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., 2018, Proactive udder health management in South Africa and monitoring of antibiotic resistance of *Staphylococcus aureus* in dairy herds from 2001 to 2010. Journal of South African veterinary Association 89, a1490. doi:https://doi.org/10.4102/jsava.v89i0.1490

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., Etter, E.M.C., 2019, Climatic and regional antibiotic resistance patterns of *S. aureus* in South African dairy herds. Onderstepoort Journal of Veterinary Research 86(1), a1674. https://doi.org/10.4102/ojvr.v86i1.1674

Conference presentations and posters arising from this Thesis

Van der Schans, T.J., Karzis, J., Petzer, I.M., 2014, Eleven year Antibiotic Resistance profile of *Staphylococcus aureus* in Dairy herds across Southern Africa. Faculty day, Faculty of Veterinary Science, Onderstepoort, 4 September, 2014. (Poster)

Karzis, J., Petzer, I.M., Donkin, E.F., Etter, E.M.C, Naidoo, V., 2016, Change in antibiotic resistance of *Staphylococcus aureus* in dairy herds in southern Africa between 2000 and 2010 due to management practice. IDF Mastitis Congress, Nantes, France, 7-9 September 2016. (Presentation)

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., 2017, The Influence of proactive udder health management on antimicrobial resistance patterns for *Staphylococcus aureus* in Southern Africa dairy herds (2000 to 2010). RuVASA, Muldersdrift, 31 May –2 June 2017. (Presentation)

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., 2017, Possible influence of management on antibiotic susceptibility patterns for *Staphylococcus aureus* in South African dairy herds (2000 to 2010). S.A. Large Herd Conference, Champagne Sports Resort, Drakensberg, 5-7 June 2017. (Presentation)

Etter, E.M.C., Karzis, J., Petzer, I.M., Theobald, S., Donkin, E.F., Naidoo, V., Gerber, D., Abolnik, C., 2018, Antimicrobial resistance in intensive poultry and milk reduction in South Africa: from historical data analysis to the monitoring of the use of antibiotics. 15th International

Symposium of Veterinary Epidemiology and Economics ISVEE congress in Chiang Mai. 12 - 16 Nov 2018. (Presentation)

Etter, E.M.C., Karzis, J., Petzer, I.M., Theobald, S., Donkin, E.F., Naidoo, V., Gerber, D., Abolnik, C., 2018, Antimicrobial resistance in intensive poultry and milk reduction in South Africa: from historical data analysis to the monitoring of the use of antibiotics. One Health Symposium, Pretoria, 27 November, 2018. (Poster)

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., Etter, E.C., 2018, Antimicrobial resistance of *Staphylococcus aureus* isolated from milk in South Africa differs between seasons and regions. RuVASA, Birchwood – Gauteng, 18-20 June 2018. (Presentation)

Karzis, J., Petzer, I.M., 2019, Development in diagnostics and antibiotic resistance of *Staphylococcus pseudintermedius* from milk of dairy cows. RuVasa Congress, Coastlands Hotel, Umhlanga Rocks, 20-22 May 2019. (Presentation)

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., Etter, E.M.C., 2019, Antibiotic resistance and diagnosis of *Staphylococcus pseudintermedius* from milk of dairy cows. 7th IDF Mastitis Congress in Copenhagen, Denmark, 14-16 May 2019. (Poster)

Declaration

I, Joanne Karzis declare that the thesis, which I hereby submit for the PhD degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: ……

Date: ……

Acknowledgements

The research presented in this dissertation is the product of a wide-ranging collaboration. I am grateful for many people; not only for their contributions to scientific knowledge, but also for my personal benefit from the privilege of working alongside them. The mentorship and guidance of these colleagues has been the best material from which my graduate education could be made.

I would like to express my sincere appreciation to:

First and foremost to the good Lord for giving me strength, courage and wisdom and for granting me the greatest blessing of all, my health without which nothing is possible.

To my wonderful family and friends for all the love and support. To my supportive and caring parents who have always been my pillar of strength. To my loving husband and son for your unconditional love, support, patience, motivation and faith in me, without which this would not have been possible. Also a special word of thanks to my husband for proof reading this thesis for me.

A word of sincere appreciation to all my supervisors and colleagues, I am truly privileged to have been able to learn from the best. I would like to thank Prof Edward Donkin for all his help, guidance, motivation, unselfish assistance, mentorship and perseverance throughout the years, for always being my biggest fan and continuing to work so hard to help me achieve this goal even after his retirement.

Prof Vinny Naidoo, for agreeing to become a supervisor, for adding value and persevering with the study despite added workload and commitments.

A huge thank you to Prof Inge-Marié Petzer for all her help, guidance, motivation and support over the years and in contributing significantly to my development and experience in the udder health field and as a whole.

Prof Eric Etter for his tireless efforts with the statistical evaluations, and for all his assistance, despite a high workload.

Dr Christiaan Labuschagne of Inqaba Biotec, for going the extra mile and for sharing his vast knowledge of genetics with me despite added work pressures. I would also like to thank Erika Viljoen and all the other staff at Inqaba Biotec for the molecular testing and analysis.

A special word of thanks to the staff of the Milk Laboratory of the Department of Production Animal Studies, especially Mrs JC Watermeyer, Mrs R Badenhorst, Mrs EF Konaite and Mr KS Malekane, for all their help and support with the laboratory work.

To the milk producers of South Africa for providing me with the opportunity to do such research by allowing me to use the data from the routine sampling at the Milk laboratory for this study.

Prof Olga Perovic and Marshagne Smith of the NICD-NHLS Sandringham for their guidance and for the MALDI-TOF analysis and for their valuable advice on MIC testing. I would like to thank Thomas Rafundisani of Beckman Coulter for all his assistance with the MIC testing and the Microscan testing and data analysis. I would like to thank Prof Ntombi Mbelle, Dina Pombo and all the staff and post graduate students of the NHLS, Tshwane, for allowing me to use the facilities in their laboratory and for their assistance with the MIC testing.

To the National Research Foundation (NRF) for partially supporting this study.

List of Tables

List of Figures

List of Abbreviations

AMIR	Antibody-mediated immune responses
AMP	Ampicillin
AMR	Antimicrobial Resistance
ATCC	American Type Culture Collection
AUC	Area Under Curve
BMSCC	Bulk Milk Cell Count
BRICS	Brazil, Russia, India, China, South Africa
bp	Base pair
C	Cytosine (nucleotide base pair of DNA strand)
CA	Community Acquired
CE	Competitive Exclusion
CLSI	Clinical and Laboratory Standard Institute
CL	Cephalonium
CMIR	Cell-mediated immune responses
CPS	Coagulase Positive Staphylococci
CXM	Cefuroxime
CNS	Coagulase Negative Staphylococci
DA	Clindamycin
DAFF	Department of Agriculture, Forestry and Fisheries (Erstwhile)
DNA	Deoxyribonucleic acid
EC	Eastern Cape
ESBL	Extended spectrum Beta-Lactamase
ESKAPE	*Enterococcus faecalis* & *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* & *Escherichia coli*
EMA	European Medicines Agency
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FS	Free State
FVS-UP	Faculty of Veterinary Science, University of Pretoria
G	Gauteng
GLMM	General Linear Mixed Model
HGT	Horizontal Gene Transfer

IDF	International Dairy Federation
IHC	Immunohistochemistry
IMI	Intramammary Infection
KZN	KwaZulu Natal
LAB	Lactic Acid Bacteria
L	Limpopo
LL	Lower Limit
LOESS	Local regression
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization- Time of Flight
MIC	Minimum Inhibitory Concentration
MIC 50	the Minimum Inhibitory Concentration which inhibits 50% of your isolates.
MIC 90	the Minimum Inhibitory Concentration which inhibits 90% of the isolates of the species, tested.
MLST	Multi Locus Sequence Typing
MLVA	Multiple-Locus Variable number tandem repeat Analysis
MP	Mpumalanga
MRSA	Methicillin resistant *Staphylococcus aureus*
MRSP	Methicillin resistant *Staphylococcus pseudintermedius*
NARSF	National Antimicrobial Resistance Strategy Framework
NAS	Non-aureus Staphylococci
NC	Northern Cape
NMC	National Mastitis Council of the USA
NK cells	Natural Killer Cells
NW	North West
OB	Cloxacillin
OIE	World Organization for Animal Health
OR	Odds Ratio
OT	Oxy-tetracycline
P	Penicillin G
PBP	Penicillin binding protein
PCR	Polymerase Chain Reaction
PJP	*Pneumocystis jiroveci* pneumonia
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

ROS	Reactive Oxygen Species
SA	South Africa
SAAHA	South African Animal Health Association
SANVAD	South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs
SCC	Somatic Cell Count
SIG	Staphylococcus intermedius group
STH	*S. aureus* (lytic group III) (STH)
T	Thymine (nucleotide base of DNA strand)
TMR	Total Mixed Rations
TY	Tylosin
UL	Upper Limit
URT	Upper respiratory tract
US	United States
USA	United States of America
USFDA	United States Food and Drug Administration
WC	Western Cape
WHO	World Health Organisation

Thesis Abstract

Antibiotic resistance profiles of coagulase positive staphylococci isolated from milk of South African dairy herds

The discovery and the subsequent global use of antibiotics has led to the survival of resistant microorganisms and suppression of susceptible species. This has caused a worldwide interest in antibiotic resistance and its threat to human and animal health. World-wide and particularly in South Africa there is a lack of antibiotic resistance surveillance data specifically for dairy cattle. The routine sampling of the Milk Laboratory Faculty of Veterinary Science at the University of Pretoria, as part of the pro-active udder health programme, has generated such data which needed to be analysed, interpreted and applied in practice. *Staphylococcus aureus (S. aureus),* one of the biggest problems in the dairy industry, was chosen as the organism to be used as the starting point for this ongoing project.

The retrospective antibiotic resistance data (Kirby Bauer) were analysed for *S. aureus* (n= 2532) to eight commonly used antibiotics available as intramammary remedies for specific mastitis treatments in Southern Africa from 2000 to 2010. While overall antibiotic resistance was generally increasing over time as shown worldwide, antibiotic resistance was in fact decreasing over time for twenty well-managed herds (nineteen in South Africa and one in Zambia). This was attributed mostly to the effects of good management in the herds that were regularly tested as part of the pro-active udder health programme.

There were also significant effects of seasons and regions on antibiotic resistance in tested isolates. All of the antibiotics tested, barring cephalosporins, showed a predicted prevalence of resistance of above 50% in most provinces. This is a concern. The lowest prevalence of resistance to the majority of the categories of antibiotics tested was in KwaZulu-Natal Province during spring. The reasons for the differences in antimicrobial rsistance between seasons and provinces are obscure. These differences may be a secondary effect related to the amount of antibiotic usage. The cephalosporins had the lowest levels of prevalence of bacterial resistance in Gauteng Province during winter. Although, mostly unexplained, such effects on antibiotic resistance could possibly be attributed to the different weather conditions in different regions of the country during different seasons.

The conventional procedures for the identification of *S. aureus* led to the identification of coagulase positive and maltose negative staphylococci with doubtful identification of species. This research aimed at confirming the identification of this organism (conventional microbiology), which seemed to be an emerging pathogen, using molecular methods (MALDI-TOF MS, and 16s rRNA sequencing). The isolates of the maltose negative *Staphylococcus* sp. tested, were confirmed as being *S. aureus* by both molecular methods (100% correlation). However, it is also important to differentiate between maltose negative and maltose positive *S. aureus* isolates during routine diagnostics because these organisms react differently and thus need to be treated differently in practice. Also, maltose negative *S. aureus* tested positive for both *malA* and *malR* genes. A stop codon was discovered at position 844 of the *malA* gene caused by a cytosine to thymine transition which resulted in early termination of the α-glucosidase protein which would most likely be inactivated. This truncated protein may be the cause of the maltose negative phenotype. The discovery of this stop codon proves that maltose negative *S. aureus* ST 2992 is indeed different to conventionally identified maltose positive *S. aureus.*

Antibiotic resistance of maltose negative *S. aureus* was analysed using retrospective data of this pathogen (n = 271), from milk samples of 117 farms between 2010 and 2017 (Kirby Bauer). The analysis was done using both the previous system (intermediate grouped with resistant) and more recent system (intermediate grouped with susceptible) CLSI breakpoints. The results between the previous and more recent analysis differed for tylosin, cefalonium, oxy-tetracycline and cloxacillin. Neither the previous system nor more recent system of analysis showed any difference between provinces for the maltose negative *S. aureus*. Strains of *S. aureus* which differed on phenotypic identification with the maltose test, also differed in antibiotic resistance patterns over time, per province, per season and SCC category.

Further antibiotic susceptibility testing (MIC) was carried out, using the automated broth microdilution method for both maltose positive (n= 57) and maltose negative (n = 57) *S. aureus* from 34 farms. The MIC results for maltose negative *S. aureus* confirmed the results of the Kirby Bauer for the products tested. MIC 50 and MIC 90 were susceptible for both maltose negative and maltose positive *S. aureus*, except for MIC 90 of maltose negative *S. aureus*. This MIC analysis indicated more resistance in general seen in the maltose negative *S. aureus,* than in the maltose positive strains. Uncommon resistance patterns such as the resistance to vancomycin, oxacillin and carpapenems were shown for maltose negative *S. aureus* isolates, implying a possible anthroponosis (previously known as reverse zoonosis or

zooanthroponosis). It is still unclear why this is found and how this might be linked to the difference in the phenotypic identification of this organism.

The surveillance and monitoring of antibiotic resistance is important in order to assist decision makers, influence legislation, control antibiotic resistance, preserve human and animal health and to promote food security.

Chapter 1: Introduction

Antibiotic resistance of coagulase positive staphylococci isolated from milk of South African dairy herds

1. Mastitis

Mastitis is the inflammation of the mammary gland and udder tissue, and is a major endemic disease of dairy cattle. Bovine mastitis remains a major challenge and the disease responsible for most economic losses in dairy cows in developed countries despite improvements in management of subclinical mastitis over the past decade (Geary et al. 2012). Quarter milk samples with a Somatic Cell Count (SCC) of ≤ 100.000 cells/mL from which no microorganisms were isolated and without a history of recent infection are considered to be normal (Harmon 2001, Smith et al. 2001).

In 2001, the International Mastitis Council defined subclinical mastitis as an infected quarter with a SCC ≥ 200.000 cells/ml milk and in composite milk samples 150 000 cells/ml milk (Petzer et al. 2017) in the absence of clinical changes to milk (Petzer et al. 2017) based on findings by DeGraves and Fetrow (1993), Harmon (1994) and Hillerton (1999). A SCC of ≥ 400.000 cells/ml milk was selected for practical reasons. The data used for this study originate from routine herd examinations and antibiograms were performed for producer / veterinary use in the field. We anticipated that bacteria isolated from milk with increased SCC may be potentially more pathogenic and that these isolates could provide a better indication for antibiotic selection of the particular herd.

1.1 Mastitis in South African dairy herds

Knowledge of mastitogenic pathogens is important. Their categorisation reflects their basic epidemiology and can guide proactive management of dairy herds. Monitoring of udder health has been promoted in South Africa to enable the establishment of meaningful goals for effective udder health herd management (Petzer et al. 2009). The National Milk Recording Scheme has helped substantially by monitoring somatic cell count, protein, milk fat and milk urea nitrogen (MUN) on an individual cow basis, but, this scheme is no longer as effective as it used to be.

2. Causes of mastitis

2.1 Bacterial species and occurrence

The degree of importance of a specific bacterial agent as a cause of mastitis in dairy cows is largely dependent on its pathogenicity and the interaction of the environment, the host and agent.

Contagious mastitis is defined as intramammary infections (IMI) transmitted from cow to cow, and there are many mastitis pathogens for which the primary reservoir is the cow and the prevalence of IMI due to the pathogen is significant. Mastitis pathogens that conform to this description include *Streptococcus agalactiae* (*S. agalactiae*), *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Staphylococcus aureus* (*S. aureus*), *Corynebacterium bovis*, multiple *Mycoplasma spp.* (Fox & Gay 1993) and *Prototheca zopfii* (Corbellini et al. 2001).

Historically, mastitis in general, and contagious mastitis specifically, were regarded as caused primarily by *S. agalactiae*, formerly known as *Streptococcus* mastitis. During the first half of the 20th century, it was not unusual to find 50% to 60% of cows in a dairy herd infected with that pathogen (Schalm et al. 1971). Concerted regional efforts eradicated *S. agalactiae* IMI using a programme that included antibiotic therapy, milking time hygiene, and post milking teat disinfection. As a result, *S. agalactiae* is less of a scourge of the dairy industry and although it still has been reported to be present in a significant proportion in most of dairy herds (Goldberg et al. 1991), it responds well to treatment. However, more recently there is an increase in prevalence of *S. agalactiae* shown in the Scandanavian countries and Columbia (Cobo-Ángel et al. 2018, Pang et al. 2017, Reyes et al. 2017)

Some studies between 1975 and 2018 show that *S. aureus* at that time may have been the most prevalent cause of mastitis with estimates that 7% to 40% of all cows were infected (Bakken et al. 1981, Elliott et al. 1975, Fox & Gay 1993, Gommers et al. 1985, Holmes & Zadocks 2011, Mørk et al. 2005, Sol et al. 2000, Wilson & Richards 1980, Wang et al. 2018). These same investigators reported a range of 1% to 8% of cows with IMI caused by streptococci including *S. agalactiae* and other species (Fox & Gay 1993).

In South Africa, *S. aureus* remains the principle organism which causes mastitis (Petzer et al. 2009, Petzer et al. 2012). An increase in the average herd size over time increases the risk of spread of mastitis and other diseases, mainly due to the merge of herds (Lactodata 2013).

Bacterial isolates from lactating and dry cows were cultured from dry cow udder quarter secretion samples and from lactating cow samples in a study undertaken from 1996-2007. Identification of bacterial isolates from the dry cow secretions (n= 11 946) revealed that coagulase-negative staphylococci (CNS) also now called non-aureus staphylococci (NAS) were by far the most numerous at 61.71 %, followed by *S. aureus* (17.28 %), αβ haemolytic *S. aureus* (STH) (7.81 %), *Enterococcus faecalis (E. faecalis)* (4.49 %), *Streptococcus dysgalactiae (S. dysgalactiae)* (2.51 %), *Streptococcus uberis* (*S. uberis*) (1.21 %), *Streptococcus agalactiae (S. agalactiae)* (1.21 %) and other bacteria (3.69 %). Similar percentages of NAS, *S. aureus*, STH and *S. dysgalactiae* were isolated from dry and lactating milk samples. Higher percentages of *S. agalactiae* and *S. uberis* were isolated from lactating cows, while *E. faecalis* was more prevalent in dry cows (Petzer et al. 2009).

2.1.1 *Staphylococcus aureus*

Staphylococcus aureus is the most common contagious mastitis pathogen in many dairies, even though the prevalence, in general, has decreased due to improved milking hygiene and widely implemented mastitis control strategies (Hillerton et al. 1999). The infected udder is considered the primary reservoir of *S. aureus* and the organism is believed to be transmitted during milking. Despite this, a proportion of heifers enter the milking herd already infected with *S. aureus* (Nickerson et al. 1995). This suggests routes of transmission other than milking equipment. A good understanding of *S. aureus* reservoirs and transmission is essential for effective control of the organism in a herd (Abede et al. 2016, Rainard et al. 2017). Modern molecular techniques have recently been used to identify different strains of the organism (Dingwell et al. 2006) and these tools are likely to improve our understanding of *S. aureus* epidemiology in dairy herds (Leuenberger et al. 2019, Rajala-Schultz et al. 2010).

Over the last 10 years, the number of milk producers has decreased (from 15.000 producers to <2.000), with an increase in average herd size (from 167 to 293 cows per herd) (Lactodata 2013). The udder health management system as a whole has improved as these larger herds consist mainly of wellmanaged large commercial dairy herds (Lactodata 2014). But during the process of expanding of herds, there was an increased risk of spread of contagious mastitis-causing pathogens through herds which might have led to outbreaks of mastitis (Lactodata 2014).

Staphylococcus aureus is highly contagious (Gram-positive cocci) and a common cause of mastitis and therefore often the focus of mastitis research. *Staphylococcus aureus* can cause both sub-clinical and clinical mastitis, but it is mostly identified in chronic mastitis with progressive udder parenchyma damage (Bannerman et al. 2004). Chronic carrier cows are the major source of infection, shedding the *S. aureus* bacteria intermittently. *S. aureus* IMI are usually transmitted from infected cows to non-infected cows during milking via contaminated teat liners, milkers' hands and communal cloths (Leslie & Schukken 1999). The design and function of the milking machine and the milking parlour and the resultant milking routine can also predispose cows to mastitis (Dodd & Neave 1970). Flies have also been implicated in the transfer of *S. aureus* from one animal to another (Mellenberger & Kirk 2001).

Once *S. aureus* infects the udder it may cause fibroses and abscessation, which may cause a re-appearance of clinical signs or elevated somatic cell counts and may permanently limit an infected quarter's ability to produce milk and its ability to respond to treatment (Mellenberger & Kirk 2001). *Staphylococcus aureus* is particularly difficult to deal with, because it may secrete α-haemolysin which can lead to gangrenous mastitis, which can be fatal to the cow (Mellenberger & Kirk 2001). These bacteria can avoid phagocytosis by producing a polysaccharide mucous biofilm, around themselves, leading to poor penetration of the antibiotic during treatment (Parul et al. 2019). They are further shielded from the body's defences inside the cell, making it difficult for the extra-cellular defence mechanism of the host to attack it. All these factors make *S. aureus* difficult to treat and historically resistant to many antibiotics (Mellenberger & Kirk 2001, Silva & Silva 2005). As with other bacterial infections, the epidemiology of IMI caused by *S. aureus* depends on both bacterial characteristics and cow susceptibility (Bannerman et al. 2004, Barkema et al. 2006, Petzl et al. 2008).

The management of *S. aureus* clinical mastitis in South African dairy herds differs from that of other many countries. In South Africa a pro-active udder health management approach is followed which includes milk sampling for microbiology and cytology of all lactating cows on a routine basis (Petzer et al. 2012). These routine examinations allow for the identification of all the IMI in a specific herd in order to be able to apply management strategies such as separating the *S. aureus* cows for life, milking them last, checking for prognosis and treating the cows that can be treated and culling those that need to be culled. In essence this system involves managing the cows that are free from *S. aureus* so that they remain free of this infection; and in due course, culling those cows that have incurable *S. aureus* infections.

2.1.2 αβ haemolytic *Staphylococcus aureus* lytic group III (STH)

During 1989 the Faculty of Veterinary Science at Onderstepoort isolated a distinct αβ haemolytic *S. aureus* from milk samples from a large commercial dairy herd for the first time. Phage typing identified it as belonging to lytic group III (with variation in its phage pattern) where all the other *S. aureus* isolated from the milk of cows, belonged to lytic groups I and II. The owner, who suffered from chronic sinusitis, tested positive on a nasal swab for *S. aureus* (lytic group III) (STH) (Petzer at al. 2009). A South African study of *S. aureus* from dairy herds in Bloemfontein in 1985 also isolated *S. aureus* (lytic group III) (STH), believed to be of human origin (Swartz et al. 1985).

Since that time, the STH has been isolated from large numbers of South African dairy herds, as well as from the people in close contact with those dairy cows (Petzer at al. 2009). Since identifying the STH in herds and taking preventive measures, a decline in the percentage of isolates has been noticed. The presence of anthroponosis as shown by these examples therefore warrants further investigation (Petzer at al. 2009). The percentage of STH isolated increased from low numbers during 1996 and 1997 (0%) to (20%) which exceeded the percentage of the other *S. aureus* isolates (13%) in 1999 (Petzer at al. 2009). What is of concern is also the finding of a higher pathogenicity of the STH compared with the other *S. aureus* bacteria. For example, 52.4% of *S. aureus* isolated were from mastitic quarters, while 67.1 % of STH were mastitic (Petzer at al. 2009). Out of the quarters infected with *S. aureus* (including STH), 57.7 % had clinical mastitis (Petzer at al. 2009). *S. aureus* (including STH) (25.10% of isolates) was the second most abundant and *S. agalactiae* (5.92 % of isolates) the third. These three organisms accounted for almost 92 % of isolates (Petzer at al. 2009). The possible effect of anthroponosis of pathogens from immunosuppressed individuals to cows should not be ignored (Petzer et al. 2009, Schmidt et al. 2015).

2.1.3 MRSA (Methicillin resistant *Staphylococcus aureus*)

Milk samples from dairy cattle in South Africa tested negative for the mecA gene in the Polymerase Chain Reaction (PCR), but showed phenotypic MRSA, using the cefoxitin disc (Badenhorst et al. 2014). This could have been the strain of *S. aureus* now found to carry a homologue of the mecA gene now known as mecC gene (García-Álvarez et al. 2011).

Methicillin-resistant *S. aureus* is a major cause of healthcare-associated, community-associated and livestock associated infections. Recently, the discovery of human and bovine

MRSA isolates carrying a new mecA gene homologue, mecA LGA251 (now designated mecC), has caused concern because they are not detected by conventional, confirmatory tests for MRSA. Samples (from dairy cattle and human origin) show phenotypic Methicillin resistance but test negative for mecA gene on PCR. Very little is known about their frequency, epidemiology and possible transmission between livestock and humans. In a study reported by Petersen et al. (2013), the epidemiology of the mecC isolates in Denmark was investigated by screening the national collections of MRSA cases (from 1988 onwards) and *S. aureus* bacteraemia cases (from 1958 onwards). Isolates carrying mecC were only recovered infrequently before 2003 (n = 2) but now seem to be increasing, with 110 cases in 2003 – 2011. Clinical data on mecC-carrying MRSA demonstrated that mecC-MRSA were primarily community acquired (CA-MRSA) and affected persons typically living in rural areas, who were older than other CA-MRSA patients. Among 22 cases in Region Zealand of Denmark, four reported contact with cattle and sheep. Two of these lived on farms with livestock positive for mecC-carrying MRSA, sharing spa type (t843), MLVA (MT429) and PFGE pattern with the human isolates. These observations indicated that mecC-carrying MRSA can be exchanged between humans and ruminants (Petersen et al. 2013, García-Álvarez et al. 2011).

Staphylococcus aureus is an opportunistic pathogen often carried asymptomatically on the human and animal body. Methicillin-resistant *S. aureus* are strains that have acquired a gene rendering them resistant to methicillin and essentially all other beta-lactam antibiotics (Marais et al. 2009). Methicillin resistant *S. aureus* was first reported in 1961, soon after methicillin was introduced into human medicine to treat penicillin-resistant staphylococci (Jevons 1961). Methicillin resistant *S. aureus* is of serious concern in human medicine and an emerging concern in veterinary medicine. It was originally identified in humans that had been admitted to hospital (Pavillard et al. 1982) but since 1990 community-acquired MRSA strains, not originating in hospitals, were also isolated (David & Daum 2010, Purcell & Fergie 2005). At first community- acquired MRSA appeared in high-risk populations such as intravenous drug users, people in nursing homes and people who were chronically ill, but lately it is reported even in healthy children. In a survey performed at two academic hospitals in Johannesburg, MRSA was identified in 23% of *S. aureus* cases (Marais et al. 2009). South African based literature, (Perovic et al. 2006), has showed that the bacteraemia rate was significantly higher ($P < 0.0001$) among South African patients with hospital-acquired MRSA than those with the community-acquired strain.

2.1.3.1 Means of transmission of MRSA

Staphylococcus aureus is transmitted between humans, between animals, from human to animals and animals to humans when in close contact (Juhász-Kaszanyitzky et al. 2007, Leonard et al. 2006, Seguin et al. 1999). Horizontal transmission between humans usually occurs by direct contact, often via the hands from colonized or infected people or from contaminated food (Pavillard et al. 1982, Perovic et al. 2006, Kaszanyitzky et al. 2004). Low-degree contamination with *S. aureus* has been reported in retail meat, raw chicken, turkey, pork, veal, beef, mutton / lamb, rabbit and game and in cheese (Kaszanyitzky et al. 2004). Numerous studies indicate MRSA as a anthroponosis (Juhász-Kaszanyitzky et al. 2007, Leonard et al. 2006, Seguin et al. 1999, Van den Broeket al. 2009, Cuny et al. 2006, Fessler et al. 2010, Vintov et al. 2003). Approximately 25-50% of the human population are nasal carriers of *S. aureus* and of these 20% carry one strain persistently while up to 60% are intermittent carriers (David & Daum 2010, Leonard & Markey 2008). Methicillin resistant *S. aureus* carriage rates in the general population vary from less than 1% to 5% (David & Daum 2010, Leonard & Markey 2008). Methicillin resistant *S. aureus* strains causing mastitis in cattle have been shown to be of human origin, although bovine-associated strains have been suggested (Van den Broek et al. 2009, Spohr et al. 2011).

Many studies indicate MRSA that has originated in animals (David & Daum 2010, Seguin et al. 1999, Youn et al. 2010). The pig-associated lineage MRSA CC398 strain is of particular concern and was first recognized as a zoonosis in the Netherlands. In Belgium people in contact with veal calves have a significant higher risk for colonization with the MRSA CC398 strain. Almost 10% of the Belgium farms have mastitis problems and 4-7% of their cattle were MRSA positive (Devriese & Hommez 1975). Similar MRSA strains were isolated from mastitis cows and milkers in a herd (Juhász-Kaszanyitzky et al. 2007) while other authors identified MRSA from mastitis cases in dairy cattle (Van den Broek et al. 2009, Fessler et al. 2010, Spohr et al. 2011). In South Korea, where MRSA is common among people, the quarter-level prevalence of MRSA in milk of dairy cows was reported to be less than 0.5% (Youn et al. 2010) while in a study from Switzerland, MRSA was detected in 1% of calves and 0.3% of cattle (Van den Broek et al. 2009). Genetic analysis of methicillin-resistant *S. aureus* strain CC97 was found to have originated from cattle (Price et al. 2013).

Most strains in pets seem to have originated from humans (Seguin et al. 1999) but there are concerns that they may be transmitted back to people, particularly those who are immuno-

suppressed, chronically ill, or unusually susceptible for other reasons (Juhász-Kaszanyitzky et al. 2007, Leonard et al. 2006, Seguin et al. 1999).

2.1.4 *Staphylococcus pseudintermedius*: Emerging udder pathogen

The Genus Staphylococcus consists of a variety of opportunistic pathogens of variable relevance in veterinary medicine. The most clinically relevant staphylococci in veterinary medicine are the coagulase positive, namely *S. aureus* and members of the *Staphylococcus intermedius* (*S. intermedius*) group, particularly *Staphylococcus pseudintermedius* (*S. pseudintermedius*). Lately *Staphylococcus xylosus* (*S. xylosus*) was identified as expressing a versatile coagulase factor and can from time to time test coagulase positive.

A noted property of staphylococci is their ability to become resistant to antimicrobials. Methicillin resistance is of particular relevance because it is conferred by the presence of the mecA gene, which encodes for production of an altered penicillin binding protein (PBP) (PBP2a or PBP2') that has a low affinity for the beta-lactam antimicrobials (penicillins, cephalosporins, carbapenems) (Kwon et al. 2006). The mecA gene resides on a staphylococcal chromosomal cassette (SCCmec). Other resistance genes can also be located on this chromosomal cassette or elsewhere in the genome, further limiting treatment options. Methicillin-resistant *S. aureus* (MRSA) and methicillin- resistant *S. pseudintermedius* (MRSP) have emerged as significant problems in veterinary medicine, from both animal and human public health perspectives.

Staphylococcus intermedius was described as a species in 1976 based on G+C content and phenotypic tests (Hajek 1976). It is part of the normal microflora of the skin and mucosa of dogs and cats (Cox et al. 1988, Cox et al. 1985, Talan et al. 1989) and has also been found in a wide range of other animals including horses, goats, minks, foxes, raccoons and pigeons. During the past few years, there has been confusion about the classification of *S. intermedius*. In 2005, a *S. pseudintermedius* was described based on 16S rRNA gene sequence analysis of isolates from a cat, a dog, a horse and a parrot (Devriese et al. 2005). Recently isolates formerly identified as *S. intermedius* by phenotypic characteristics were reclassified (Sasaki et al. 2007), into three clusters: *S. intermedius, S. pseudintermedius* and *S. delphini* based on the nucleotide sequence analysis of the sodA and hsp60 genes (Sasaki et al. 2007). Bannoehr et al. (2007) investigated 105 isolates of the *S. intermedius* group from 10 countries and three continents by multi-locus sequence typing and found a population structure consistent with that reported by Sasaki et al. (2007). All canine strains examined in both studies were

classified as *S. pseudintermedius*. Therefore, it has been proposed to report all strains from dogs as *S. pseudintermedius*, unless genomic investigations prove that the strain belongs to another related species (Devriese et al. 2009).

While there have been studies on *S. pseudintermedius* and multi-drug resistant *S. pseudintermedius* isolated from dogs, cats and horses, little work has been undertaken on *S. pseudintermedius* causing mastitis in dairy cattle. *Staphylococcus pseudintermedius* was initially isolated from one large commercial dairy herd in South Africa in 2005 / 2006, which had been found to have a large number of *S. aureus* with low SCC < 100 000 cells/ml milk. After further diagnosis on maltose agar, it was found that in fact most of these coagulase positive staphylococci were indeed *S. pseudintermedius*. From 2008 *S. pseudintermedius* was isolated from dairy herds in South Africa, but mostly these organisms were susceptible to antibiotics when tested routinely and isolated from milk with low SCC. However in more recent years 2016/2017/2018, *S. pseudintermedius* were isolated showing resistance (MRSA, cefoxitin disc) and associated with high SCC > 600 000 cells/ml milk (Field et al. 2015, Pilla et al. 2013).

The present study has been intended to confirm identification of this organism using phenotypic and genotypic methods and to determine the genetic relatedness between the bacterial isolates of *Staphylococcus pseudintermedius* isolated from dairy cattle in SA over time using PFGE (Barrett et al. 2006, Tenover et al. 1995). Isolates can be grouped into three categories defined as (1) closely related, (2) possibly related, and (3) different/unrelated (Tenover et al. 1995). Closely related isolates are those that differ by a single genetic event, indicated by 2-3 band differences, whereas isolates defined as possibly related or unrelated differ by two genetic events (4-6 band differences) and >3 genetic events (>7 band differences), respectively. However, these criteria are most appropriate in studies with limited genetic variability among isolates, typically modest regional studies (Olive & Bean 1999), as the criteria inadequately address strain variations related to genetic events such as horizontal gene transfer (Barrett et al. 2006).

2.1.5 Non-aureus Staphylococci (NAS) (previously coagulase negative staphylococci)

Staphylococcus aureus is still the principal mastitogenic pathogen in South Africa due to its chronic and destructive nature (Petzer at al. 2009). However, currently NAS are the main cause of bovine IMI in many countries and not *S. aureus* (De Visscher et al. 2014, De Visscher et al. 2016, Petzer at al. 2009). These microorganisms most frequently isolated from bovine

milk worldwide, are a heterogeneous group of numerous species. The distribution differs considerably among NAS species IMI; therefore, accurate identification (species level) is essential for studying NAS epidemiology (Condas et al. 2017).

A study by Supré et al. (2011) found the distribution of NAS causing IMI was highly herd-dependent, but overall, *S. chromogenes*, *S. xylosus*, *S. cohnii*, and *S. simulans* were the most prevalent. In general, much less NAS species than *S. aureus* were found to cause clinical mastitis. However, this differs between the various NAS species. The effect of the most prevalent species on the quarter milk somatic cell count (SCC) was analyzed using a linear mixed model, showing that *Staphylococcus chromogenes* (*S. chromogenes*), *Staphylococcus simulans* (*S. simulans*) and *Staphylococcus xylosus (S. xylosus)* induced an increase in the SCC that was comparable with that of *S. aureus*. Almost all NAS species examined in the study by Supré et al. (2011) were able to cause persistent IMI, with *S. chromogenes* causing the most persistent infections, whereas other NAS species do not cause increased SCC or persistent infections. In conclusion, accurate species identification cannot be ignored when studying the effect of NAS on udder health, as the effect on SCC differs between species and species distribution is herd-specific. *S. chromogenes*, *S. simulans*, and S. xylosus seem to be the more important species and deserve special attention in further studies (Supré et al. 2011).

However, this trait appears to be quite complicated, being partly strain dependent and partly dependent on the host's immunity. The factors explaining the anticipated differences in pathogenic behaviour appear to be more difficult to evaluate. Biofilm formation and the presence of various staphylococcal virulence factors do not seem to (directly) influence the effect of NAS on IMI but the available information is indirect or insufficient to draw consistent conclusions. Within-species variation should also be investigated (De Visscher et al. 2015).

Since phenotypic methods to identify NAS from the milk of ruminants often yield unreliable results, methods for molecular identification based on gene sequencing or fingerprinting techniques have been developed (Vanderhaeghen et al. 2015). In addition to culture-based detection of isolates, culture-independent methods may be of interest. On the basis of molecular work in the study by Vanderhaeghen et al. (2015), the five NAS species most commonly seen to cause IMI were *S. chromogenes*, *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus haemolyticus (S. haemolyticus)*, *S. simulans* and *S. xylosus* . Current knowledge suggests that *S. chromogenes* is a bovine-adapted species, with most cases of IMI due to this bacterium being opportunistic. *Staphylococcus haemolyticus* also appears to be an opportunistic pathogen, but this bacterium occupies a variety of habitats, the

importance of which as a source of IMI remains to be elucidated. *Staphylococcus xylosus* appears to be a versatile species, of which we are still unsure. *Staphylococcus epidermidis* is considered to be a human-adapted species and most cases of IMI appear to arise from human sources, but the organism is capable of residing in other habitats. Anthroponosis should therefore be considered as a mechanism of transmission in future when working with this organism. *Staphylococcus simulans* typically causes contagious IMI, but opportunistic cases also occur and the ecology of this bacterium requires further study (Vanderhaeghen et al. 2015).

A study by Piessens et al. (2011) found that the NAS species predominating in the environment were *S. equorum, S. sciuri, S. haemolyticus* and *S. fleurettii*. There is conflicting literature on the origin of *S. haemolyticus* and according to Vanderhaeghen et al. (2015) it is an opportunistic organism. Herd-to-herd differences in distribution of NAS species were observed in both milk and the environment, suggesting that herd-level factors are involved in the establishment of particular species in a dairy herd (Piessens et al. 2011). Primary reservoirs of the species causing IMI were found to vary. *Staphylococcus chromogenes* and *S. epidermidis* were rarely found in the environment, indicating that other reservoirs were more important in their epidemiology. For *S. haemolyticus* and *S. simulans*, the environment was found as a reservoir, suggesting that IMI with these species were possibly environmental in origin (Piessens et al. 2011).

2.2. Infectious agents other than bacteria

2.2.1 The role of viruses

Bovine herpesvirus 1 (Gourlay et al. 1974, Muylkens et al. 2007), bovine herpesvirus 4 (Wellenberg et al. 2000), foot-and-mouth disease virus (Burrows et al. 1971), and parainfluenza 3 virus (Kawakami et al. 1966) have been isolated from milk from cows with clinical mastitis. Intramammary inoculations of bovine herpesvirus 1 or parainfluenza 3 virusinduced clinical mastitis, while an intramammary inoculation of foot and mouth disease virus resulted in necrosis of the mammary gland. Subclinical mastitis has been induced after a simultaneous intramammary and intranasal inoculation of lactating cows with bovine herpesvirus 4. Bovine leukaemia virus has been detected in mammary tissue of cows with subclinical mastitis, but whether this virus was able to induce bovine mastitis has not been reported (Yoshikawa et al. 1997). Bovine herpesvirus 2, vaccinia, cowpox, pseudocowpox, vesicular stomatitis, foot-and-mouth disease viruses (Fuchs 1994) and bovine papilloma-

viruses can play an indirect role in the aetiology of bovine mastitis. These viruses can induce teat lesions, for instance in the ductus papillaris, which result in a reduction of the natural defence mechanisms of the udder and indirectly in bovine mastitis due to bacterial pathogens. Bovine herpesvirus 1, bovine viral diarrhoea virus, bovine immunodeficiency virus (Snider et al. 1996) and bovine leukaemia virus infections may play an indirect role in bovine mastitis, due to their immunosuppressive properties, although more research is warranted to underline their indirect role in bovine mastitis. Viral infections can play a direct or indirect role in the aetiology of bovine mastitis; therefore, the importance of this in the aetiology of bovine mastitis and the economic impact of these agents needs further attention (Wellenberg et al. 2002).

2.2.2 *Mycoplasma bovis*

Mycoplasma bovis (*M. bovis*) is a pathogen causing respiratory disease, otitis media, arthritis, mastitis, and a variety of other diseases in cattle worldwide (Maunsell et al. 2011). It is increasingly recognized by the veterinary and livestock communities as having an important impact on the health, welfare, and productivity of dairy and beef cattle. *Mycoplasma bovis* diseases can be difficult to diagnose and control because of inconsistent disease shedding and response to treatments and vaccines, and large gaps in our understanding of the epidemiology and pathophysiology of these diseases (Maunsell et al. 2011).

The ability of *M. bovis* to cause mastitis (Bennett & Jasper 1977), respiratory disease (Nicholas et al. 2002) and arthritis (Stalheim & Page 1975) has been demonstrated in experimental infection studies, although variation in disease severity is common. In natural infections, *M. bovis* has been isolated in pure culture from the mammary gland of cows with mastitis (Gonzalez et al. 1992a) and from the joints, tendon sheaths, or periarticular tissues of cattle with arthritis, tenosynovitis, or chronic pneumonia and polyarthritis syndrome (CPPS) (Stalheim & Page 1975, Adegboyes et al. 1996, Stipkovits et al. 1993, Butler et al. 2000, Gagea et al. 2006). *Mycoplasma bovis* is the predominant pathogen isolated from the middle ear of calves with otitis media (Lamm et al. 2004, Francoz et al. 2004). The role of *M. bovis* in the multifactorial bovine respiratory disease (BRD) complex is not as easily defined. At the group level, seroconversion to *M. bovis* is associated with increased risk of being treated for BRD (Martin et al. 1990). *Mycoplasma bovis* is often isolated from the lungs of cattle with pneumonia, (Adegboye et al. 1996, Thomas et al. 2002) and identified within lesions using immunohistochemistry (IHC) (Gagea et al. 2006). However, *M. bovis* can also be isolated from the lungs of some cattle without clinical disease or lesions and so variable disease expression appears to be a key feature of both natural and experimental infections. *Mycoplasma bovis* is

often present in the upper respiratory tract (URT) of cattle without clinical disease. Therefore, although *M. bovis* alone can cause natural and experimentally induced clinical disease, the presence of *M. bovis* does not always result in disease, and clinical disease does not appear necessary for the maintenance and dissemination of *M. bovis* in the cattle population (Maunsell et al. 2011).

Bramley and Dodd (1984) commented that mycoplasma mastitis may be most prevalent in California (Bushnell 1984, Fox & Gay 1993, Gonzalez et al. 1992b), although it clearly is a problem in other states of the USA and worldwide (Boughton 1979, Jasper 1981, Jasper 1987, Ter Laak 1992, Fox & Gay 1993). In South Africa there is limited research done up to date on Mycoplasma mastitis (Motaung et al. 2017).

3. Factors affecting the incidence of mastitis in cows: Cow factors

3.1 The cows' anatomy: e.g. udder suspension, teat canal, internal blood supply, lymphatic system

Mastitis is a major cause of economic loss in the dairy industry with approximately 60% of the loss estimated to be because of decreased milk production (Akers & Nickerson 2011). The external signs of clinical mastitis are change in milk (floccules, blood, watery), changes in the udder (heat, swelling and hardness). While these changes are useful for diagnosing the disease, it is the internal effects of mastitis on the udder that have a detrimental effect on milk quality and yield. Normal milk production depends on both the mammary vascular and secretory systems. Milk synthesis relies on the proliferation of epithelial (udder tissue) cells and it is estimated that 400L of blood must flow through the blood vessels of the udder to produce just 1L of milk (Agricultural & Horticulture Development Board 2016). Pathogen infection and immune response can damage these internal vascular and secretory structures, negatively impacting their function which leads to decreased milk quality and production.

3.2 Previous udder damage: e.g. from physical trauma, or damaged parenchyma, previous infections

Mastitis usually occurs primarily in response to intramammary bacterial infection, but also to intramammary mycoplasmal, fungal, or algal infections. Mechanical trauma, thermal trauma, and chemical changes predispose the mammary gland to intramammary infection (IMI) (Zhao

& Lacasse 2007). Mastitis is the most costly infectious disease of dairy cattle in first world countries, with a large cause being the irreversible damage to the mammary tissue (Oliver & Calvinho 1995). Although antibiotics may be useful to treat the infection, they do not directly protect the gland from being damaged (Zhao & Lacasse 2007).
Dealing with clinical mastitis cases remains important but damage to the udder parenchyma may lower a cow's lifetime production potential and increase the risk of infecting fellow cows when infected with host adapted udder pathogens by the shedding of pathogens (DeGraves & Fetrow 1993, White 2010).

The reliability of records of clinical mastitis cases depends on accurate detection of mastitis on a day-to-day basis on farms, and these records are often questionable (Petzer et al. 2016). This is when the evaluation of the status of the udder parenchyma (assessed by palpation) and teat canal scores is necessary to confirm clinical cases and or udder damage (Petzer et al. 2016).

3.3 High yields

There are numerous genetic, physiological, and environmental factors that can compromise host defence mechanisms of the mammary gland. For example, emphasis on genetic selection to maximize milk production has increased metabolic stresses associated with milk synthesis and secretion and a negative correlation exists between milk production capacity and resistance to mastitis (Sordillo 2009, Sordillo & Aitken 2009).

The concept that immunocompromised animals are more susceptible to disease is well established. It is unlikely that disease susceptibility caused by increased production demands on food-producing animals will decrease as farmers strive to compete within a global economy. Intensity of dairy cattle management and genetic selection to increase milk production will continue and most likely result in additional immunological stresses being placed on dairy cows. Such stresses are likely to lead to increased risk associated with mastitis.

3.4 The immune system: past and present

Antibody-mediated immune responses (AMIR) and cell-mediated immune responses (CMIR) have been used as indicator traits of adaptive immune responses of livestock (Biozzi et al. 1979, Heriazon et al. 2009, Mallard et al. 1992, Sarker et al. 2000). The cell-mediated immune

responses are based on T-cell interactions with bacterial antigens resulting in the production and release of cytokines which lead to inflammatory responses as indicated below. The immune system generally responds to extracellular pathogens by mounting type 2 immune responses, which are typically characterized by production of antibody of a particular isotype, immunoglobulin (Ig) G1. The immune system, however, generally responds to intracellular pathogens by a type 1 immune response, typically characterized as CMIR and dominated by production of IgG2. Both CMIR and AMIR are essential for host protection against a variety of infectious diseases (Thompson-Crispi & Mallard 2012).

Intramammary infections induce an inflammatory reaction which causes an increase of SCC and activation of bacteriostatic enzymes and proteins in the milk. It has been demonstrated that during spontaneously occurring subclinical mastitis the SCC, mainly macrophages, secrete cytokines, eicosanoids, acute phase proteins and other immunomediators. In contrast, the bacteriostatic protein lactoferrin is mainly secreted by mammary epithelial tissue, while major milk proteins like α-lactalbumin and κ-casein are already down-regulated during subclinical infection (Bruckmaier & Meyer, 2005).

Good health of dairy cows is influenced by the interaction between the innate adaptive components of the immune system and other factors, such as the local and systemic inflammatory response, which can sometimes be more harmful than useful. Therefore, for dairy cows, particularly those in the peri-parturient period, it is important to avoid, or reduce any kind of infectious, parasitic or metabolic disease and the associated inflammation as much as possible. Such inflammation can impair cow performance by lowering milk yield, dry matter intake, fertility, and energy efficiency and can reduce liver function (Bertoni et al. 2015).

Mastitis is a significant disease of adult dairy cattle affecting up to 40 percent of cows within a herd at any given time. Recent surveys show that udder health problems are consistently the most frequent cause of morbidity with the US dairy cattle population (McConnel et al. 2018, Sordillo 2009).The incidence of mastitis is directly related to changes in the composition, magnitude, and efficiency of the mammary gland defence system. However, many different aspects of bovine mammary gland defences are sub-optimal during distinct periods of the lactation cycle, particularly around the transition period (3 weeks before and 3 weeks after calving) (Sordillo & Streicher 2002). Most notably, this three-week period prior to calving and through to the first three weeks of lactation have long been recognized as a period when defence mechanisms of the cow alter dramatically. Therefore, dairy cattle are more susceptible to mastitis during the peri-parturient period and through peak milk production. New

intramammary infections occurring during the perinatal period are especially problematic as they may greatly impact the productive efficiency of dairy cattle in the ensuing lactation.

The mammary gland is protected by a variety of defence mechanisms that can be separated into two distinct categories: innate immunity and specific immunity. Innate immunity, also known as nonspecific responsiveness, is the predominant defence during the early stages of infection (Rainard & Riollet 2006). Nonspecific responses are present or are activated quickly at the site of infection by numerous stimuli. Nonspecific or innate responses of the mammary gland are mediated by the physical barrier of the teat end, macrophages, neutrophils, natural killer (NK) cells, and by certain soluble factors (Sordillo 2009). The specific or acquired mammary immune system recognizes specific determinants of a pathogen. Activation of specific mammary immune defences results in the selective elimination of mastitis-causing pathogens (Sordillo & Streicher 2002). Recognition of pathogenic factors is mediated by several lymphoid populations, macrophages, and antibody molecules (Sordillo & Aitken 2009). This may contribute to the spontaneous cure of mastitis which is seen in some cases. Because of the “memory” of certain lymphocytes, specific immune responses can be supplemented by repeated exposure to a pathogen. Vaccination of dairy cattle against certain pathogens can occur if specific mammary immune mechanisms are effectively activated (See paragraph 5.4.1).

Optimal protection of the mammary gland from new IMI requires that both innate and acquired protective factors interact in a highly coordinated fashion. Completely eliminating any form of stress is impractical, and an alternative approach to reduce the influence of stress on disease susceptibility is to modify the host response to the stressor. If stress-induced changes in host immunity predispose dairy cattle to disease, then methods of improving the immune response during distinct periods of stress should increase disease resistance. The challenge that confronts researchers now is to gain a better understanding of the complex interactions between the pathogenesis of bacteria, host responses needed to eliminate the pathogens from the mammary gland, and ways to enhance the immune potential of these factors before disease is established. Oxidation and the production of free radicals are an integral part of aerobic metabolism. Considerable evidence supports the contention, however, that oxidative stress during the peri-parturient and early lactation period may contribute to several health disorders in dairy cattle (Sordillo & Aitken 2009). The antioxidant requirements of cows will likely increase as production demands continue to escalate within the dairy industry (Sordillo 2009).

3.5 Stage of lactation and parity

"Heifer mastitis" is a disease that threatens production and udder health in the first and subsequent lactations. In general, NAS are the predominant cause of IMI and subclinical mastitis in heifers around parturition, whereas *S. aureus* (contagious or host adapted pathogen) and other environmental pathogens cause a minority of the cases (McDougall et al. 2007a, McDougall et al. 2007b). Clinical heifer mastitis is typically caused by the major pathogens. The variation in proportions of causative pathogens between studies, herds, and countries is considerable. The magnitude of the effect of heifer mastitis on an individual animal is influenced by the form of mastitis (clinical versus subclinical), the virulence of the causative pathogen(s) (major versus minor pathogens), the time of onset of infection relative to calving, cure or persistence of the infection when milk production has started, and the host's immunity (Nyman et al. 2007, Olde Riekerink et al. 2008).

Intramammary infection in early lactation caused by NAS does not necessarily have a negative effect on subsequent productivity (Andersen et al. 2010). At the herd level, the impact will depend on the prevalence and incidence of the disease, the nature of the problem (clinical, subclinical, non-functional quarters), the causative pathogens involved (major versus minor pathogens), the ability of the animals to cope with the disease, and the response of the dairy manager to control the disease through management changes.

Specific recommendations to prevent and control mastitis in late gestation in peri-parturient heifers are not part of the current National Mastitis Council (NMC) mastitis and prevention program (Vliegher et al. 2012). Control and prevention is currently based on avoidance of inter-sucking among young stock, fly control, optimal nutrition, and implementation of hygiene control and comfort measures, especially around calving. More risk factors for subclinical and clinical heifer mastitis have been identified (e.g., season, location of herd, stage of pregnancy) although they do not lend themselves to the development of specific intervention strategies designed to prevent the disease. Pathogen-specific risk factors and associated control measures need to be identified due to the pathogen-related variation in epidemiology and effect on future performance. Pre-partum intramammary treatment with antibiotics has been proposed as a simple and effective way of controlling heifer mastitis in the past. However, positive long-lasting effects on somatic cell count and milk yield do not always occur, thus ruling out universal recommendation of this practice which many are against, unless the IMI has been identified (Vliegher et al. 2012). Moreover, use of antibiotics in this manner is "off-label" (relating to the prescription of a drug for a condition other than that for which it has been

officially approved) and results in an increased risk of antibiotic residues in milk. Pre-partum treatment can be implemented only as a short-term measure to assist in the control of a significant heifer mastitis problem under supervision of the herd veterinarian (Vliegher et al. 2012). When NAS are the major cause of intramammary infection in heifers, productivity is not affected, making pre-partum treatment redundant and even unwanted (Supré et al. 2009, Piessens et al. 2010, Braem et al. 2011, Park et al. 2011). In conclusion, heifer mastitis can affect the profitability of dairy farming because of a potential long-term negative effect on udder health and milk production and an associated culling risk, specifically when major pathogens are involved. Prevention and control is not easy but are possible through changes in the management of young stock and heifers. However, the pathogenesis and epidemiology of the disease remain largely unknown and more pathogen-specific risk factors should be identified to optimize current prevention programs (Vliegher et al. 2012).

4. Factors affecting the incidence of mastitis in cows: Environmental and management factors

There have been many research studies and reports over decades concerning mastitis in dairy cows. Only a few relevant and selected reports are discussed here. An example is the early study by Gill et al. (1990), which evaluated the economics of mastitis control programmes in the USA.

4.1 General environment: e.g. cleanliness

Although contagious pathogens can be controlled by pre- and post-milking disinfection of teat ends and dry cow therapy, these control measures have been less effective against environmental streptococci (Dodd 1983, Ebehart et al. 1983, King et al. 1981, Natzke 1981) and ineffective against coliform bacteria (Ebehart & Buckalew 1972, Eberhart 1975, Eberhart 1977, Ebehart et al. 1979).

It is clear that a poor environment should result in dirtier udders. There is a relationship between teat or udder contamination and occurrence of mastitis (de Pinho et al. 2012).
Total confinement housing, increased cow densities per unit area, and use of bedding materials that support bacterial growth also can have a marked impact on the susceptibility of dairy cattle to new IMI by overwhelming important local defence mechanisms (Sordillo 2009).

A thorough study of a dairy herd in total confinement in the United States, as reported by Smith et al. (1985), has given a detailed record of the rate of IMI by environmental pathogens (coliform bacteria and species of streptococci other than *S. agalactiae*). As reported by Smith et al. (1985) in Ohio, USA, the rate of IMI was higher during the dry period than during lactation and increased progressively as parity increased, and was maximal during summer, which coincided with exposure to coliforms in bedding. *Streptococcal spp.* infections lasted longer than coliform infections, and 59% of streptococcal infections and 69% of coliform infections were present for 30 days of lactation or less (Smith et al. 1985). Clinical cases were highest during the first 76 days of lactation and during summer (summer rainfall region). Only 6.7% of coliform infections resulted in acute coliform mastitis, and all acute cases were during summer or early lactation (Smith et al. 1985). Dry cow therapy reduced the rate of streptococcal infection during the early dry period but was without effect during the pre-partum period. There was no effect of dry cow therapy on coliform infection rate during the dry period (Smith et al. 1985). A study by Ward et al. (2002), found that any of the early lactation cows were heavily and persistently contaminated with faeces, while the dry cows were much cleaner. Groups of cows with firmer faeces were also cleaner. The farm with the lowest incidence of mastitis had the cleanest cows and the most satisfactory beds.

4.2 Specific environment: e.g. milking hygiene

Hygiene essentially is part of preventive medicine. Therefore milking hygiene in its broader sense is defined as the sum of all methods to prevent diseases of the cow and related influences on the milking composition (Hamman 1991).

Many management practices have shown consistent associations with herd-level SCC when used in usual dairy settings. These practices should be the cornerstone of udder health recommendations to dairy producers (Dufour et al. 2011). Simple hygiene routines combined with antibiotic therapy for clinical mastitis and for all cows at drying-off, have been shown to reduce new udder infection from as far back as 1970 (Kingwill et al. 1970). After two years of these procedures, the levels of infection decreased from 55 to 22% of cows and from 28 to 9% of quarters in 30 herds. Clinical mastitis had declined by at least 40%. The level of infection had been reduced in every herd except one, which was the only herd with <5% of quarters infected at each herd test, which was a very low level throughout the experiment. The experiment revealed wide differences between herds in the rate of new infection, occurrence of clinical mastitis, and response to therapy of staphylococcal infections. The geometric mean bulk milk cell count of the 30 herds was 730 000 cells/ml at the start of the study and fell to

400 000 cells/ml. Within the same period 100 untreated herds had a geometric SCC mean of 790 000 cells/ml bulk milk, with no apparent trend.

Dodd & Neave (1970) and Kingwill et al. (1970) described Mastitis Field Experiments 3 (MF3), from which the original 5 point plan was developed, of which three points were teat disinfection and wearing gloves, drying off and lactation therapy, and machine testing and maintenance, but did not include culling. Dodd (1983), consistently reported a three-point plan until at least 1983. The pro-active udder health programme (Petzer 2018), was developed by modifying the original 5-point mastitis plan (Dodd & Neave 1970).

4.3 Climate and seasonal variations, such as rainfall and temperatures

Heat stress can have a great effect on dairy cows in South Africa. Heat stress occurs in the current climatic situation where very hot environmental temperatures are normal in summer; or these hot conditions might occur as a predicted result of global warming in the future. A study by Williams et al. (2016), used current milk production data of Holstein dairy herds on pasture in South Africa, together with climate variables related to heat stress, to model and identify geographical areas for optimal milk production under current and future climatic conditions. The modelled map indicated optimal milk production areas in the eastern parts of South Africa, which correlates well with the geographical influence of heat stress as represented by the temperature humidity index for the country (Williams et al., 2016). Future climate change hypothetical projections for many decades ahead were used to predict optimal milk-producing areas for the future. This indicated progressive decreasing of current suitable areas and a geographical shift towards the southern parts of the east coast of South Africa. Possible long-term viable alternatives suggested by Williams et al. (2016), included changes in nutrition and replacing existing breeds with more heat tolerant genotypes. According to the results of Hammami et al. (2013), in Luxembourg all temperature and humidity indices had identified heat stress thresholds for the production and SCC in Holstein cows. The thresholds were lower compared with those of tropical, subtropical, and Mediterranean climate conditions. The low threshold values could be potentially attributed to a reduced adaptability of Holsteins to heat stress under temperate conditions (Hammami et al. 2013). A study by Smith et al. (2013) in Mississippi State which compared heat stress between Jersey and Holstein cows, showed that Jersey cows appeared to be more heat tolerant than Holstein cows. However, Holstein cows still produced larger volumes of milk (Smith et al. 2013).

Extreme/ intense weather conditions and climatic differences may cause bacteria to form protective mechanisms such as biofilm (genetic virulence factor) which can cause an increase in the prevalence of antibiotic resistance (da Silva-Meira et al. 2012, Melo et al. 2014). Thus climatic and seasonal variations may influence intramammary infections and in turn antibiotic resistance of mastitis causing pathogens. According to Wingfield & Kenagy (1991) and Blank (1992) seasonal changes are cyclic, largely predictable, and represent the strongest and most abundant source of external variation influencing human and natural systems. Although generalizations can be made about the climate in the various provinces, there are considerable variations within each province of South Africa (Smith 2006).

Observed trends in seasonal and annual total rainfall, number of rain days and daily maximum and minimum temperature have been calculated for several stations in South Africa for the period 1960–2010 (MacKeller et al. 2004). Statistically significant decreases in rainfall and the number of rain days have been shown over the central and North Eastern parts of the country in the autumn months and significant increases in the number of rain days around the southern Drakensberg were evident in spring and summer. Maximum temperatures were shown to have increased significantly throughout the country for all seasons and increases in minimum temperatures for most of the country. A notable exception was the central interior, where minimum temperatures decreased significantly. No regionally aggregated rainfall trends for six water management zones covering the entire country were evident for total rainfall, but there were some significant trends for the number of rain days (MacKellar et al. 2014). How these changes might affect the incidence of mastitis is not clear, as no studies are known to be investigating these trends at present.

4.4 Physical damage: e.g. from poorly designed or maintained milking machines, or incorrect hand milking

Three main types of milking systems are used in dairy operations: the stanchion (portable individual cow milking system), the parlour systems and robotics: In stanchion systems, hand milking may be carried out, or milking units are brought to the tethered dairy cow for milking. The workers typically stand in between tethered cows where they kneel or squat to perform milking tasks. Stanchion systems are common among smaller dairy operations (<100 head). In contrast, parlour systems involve cows moving into stalls where cows are milked simultaneously by workers located in a pit adjacent to the milking stalls (Douphrate et al. 2009). The number of cows milked at one time varies according to parlour design.

Parlour systems are used in commercial dairy herds in South Africa and abroad as they can accommodate large numbers of dairy cattle (Donkin 1981, NAHMS 2003). Three parlour configurations are commonly used in South Africa: parallel (quick exit), herringbone (tandem parlours with side opening gates) and rotary (usually rotary abreast) parlours. These configurations present different workstation designs and may create different demands on the cow and milkers, affecting both the management and the physical well-being of the cow. For example, in the herringbone configuration, the cows enter and stand next to each other, facing away at an angle from the milking operator's pit. The milkers access the udder from the side of the animal (Douphrate 2009). The limitation is that cows enter and leave in batches, which might not make sufficient allowance for the differences in milking time within a group (Donkin 1981). Parallel milking parlours are similar to herringbone milking; however, in a parallel parlour cows stand perpendicular to the milking operator pit rather than at an angle, and the cows are milked from the rear between the cow's hind legs rather than from the side. The advantage of the parallel parlour design is that the cows stand closer together so the walking distance for milkers' is shorter than in a herringbone design. The disadvantage is that cows must be milked from behind, so the tail will often be in the way, and the reach distance may be longer (Douphrate 2009).

Rotary parlours are gaining in popularity among large dairy farms with herds in excess of 500 cows. Although three designs have been developed (rotary abreast, rotary tandem and rotary herringbone), the rotary abreast parlour is most favoured in South Africa. With the rotary milking parlour design, cows enter a carousel that rotates in a turntable-like fashion while milkers' stand in one location. The rotary milking parlour design is considered to be advantageous because of its high efficiency in throughput of cows per hour. Ideally one operator should be able to run the entire milking operation from the same position while cows enter and exit at a constant rate with little or no pause in milking tasks between cows; but this can only be done if there is automated cluster removal and teat dipping, which is done in New Zealand but not in South Africa. From a milking efficiency perspective, the rotary milking parlour design is preferred as more cows can be milked per unit time compared to other parlour designs (Reinemann 2005 & Sorenson 2015). However, rotary parlours cost a great deal more to install compared to the parallel and herringbone parlour designs (Douphrate et al. 2009). The disadvantage of rotary parlours is that the speed at which the animals are moved from the entrance to the exit is preselected and may be determined by the animal of the herd requiring the longest time to be connected and to be milked. This could lead to the other animals being moved unnecessarily slowly from the entrance to the exit, or in contrast a faster

milking routine not allowing enough time for all the recommended procedures during the milking routine (washing the udder, drying the udder and stripping the foremilk) (Van Der Lely 2000). Because of these considerations, rotary parlours might place the cows at a greater risk of mastitis than those milked in simpler systems (Donkin 1981).

Teat end damage is caused by many factors that include the milking machine layout, setting, maintenance and use. As far as the layout is concerned, the height of the milk line in relation to the height of the cows' udder will determine the level of the system vacuum. The higher the system vacuum the greater the risk for high vacuum at teat end. The latter is a major cause for teat canal damage. In addition, there are many swing-over parlours in South Africa with high milk lines. The teat canal forms the cow's first line of defence against the bacteria which cause mastitis (Petzer & Swan 2018). The infrequency of new infection suggests that the teat canal is normally a very effective barrier. It has a single opening which is surrounded with muscle fibres. These close the teat canal tightly, retaining the milk within the udder and preventing the entry of microorganisms. The teat canal is lined with keratin which aids the physical seal and has antibacterial properties. Machine milking can alter the integrity of the teat orifice/canal to a varying extent and so may reduce the resistance to bacterial invasion. Ideal machine milking conditions would minimize congestion of the teat end, conserve the keratin layer and leave the muscles with the ability to close rapidly when the milking cluster is removed. The milking conditions should provide adequate stimulation and rapid milk extraction consistent with least damage and minimal handling of the teats. Transmission of mastitis pathogens between the teats of a cow and through the teat canal during milking can be minimized by modifying the design of the cluster, e.g. by having a continuous flow of milk from each teat, with no surging of the milk in the claw piece (Grindal 1988).

4.5 Nutrition: type of feeding system; adequacy of diet

Dairy cows undergo substantial metabolic and physiological adaptations during the transition from pregnancy to lactation. Coordinated shifts in nutrient partitioning must occur in order to meet the increased demand for energy and other nutrients necessary for foetal growth and lactation (Campbell & Miller 1998, Drackley 1999). Good nutrition is essential in maintaining a functional immune system, while also avoiding other causes of inflammation, such as tissue damage and digestive and metabolic syndrome-related disorders. Provision of appropriate nutrients, such as antioxidants, omega-3 polyunsaturated fatty acids, conjugated linoleic acid and vitamin D can have anti-inflammatory effects (Bertoni et al. 2015).

In South Africa there was a shift from herds predominantly fed on total mixed rations (TMR) in 1997 to pasture- based herds up to 2013. Pasture based herds have an increased exposure to environmental pathogens while grazing and are under less production stress due to being milked only twice daily and are less likely to develop clinical mastitis. They therefore do not need to be treated as often for clinical mastitis, and thus are also less likely to develop antibiotic resistance (Crompton et al. 2007). In TMR dairy operations, in addition to treatment for mastitis, antibiotics have several other uses, including prophylaxis and can also be used in milk replacers for calves (WHO 2002).

However, bacteria such as *S. uberis* is the main organism causing IMI in New Zealand (pasture based farms) dairy herds and they are difficult to treat successfully. One of the main reasons is perceived to be the use of faecal slurry on pastures in New Zealand. Cows excrete *S. uberis* in their faeces and some may become chronic carriers. Pasture based dairy herds have increased in South Africa and the same land has now been used over decades. In some cases, in South Africa, farmers have also started to use slurry on their pastures. A recent South African study, Blignaut et al. (2018) indicated that the *S. uberis* IMI increased in pasture-based herds in South Africa over time.

Oxidation and the production of free radicals are an integral part of aerobic metabolism. A variety of reactive oxygen species (ROS) are produced by normal metabolic processes and by certain leukocyte populations during defence against disease. Accumulated scientific evidence supports the concept that oxidative damage of tissues and cellular components are either a primary or secondary cause of many human diseases. Unfortunately, considerably less is known about how oxidative stress can affect veterinary health and well-being, particularly during times of high metabolic activity. The performance of high producing dairy cattle can be optimized to a certain extent by supplementing diets with optimal levels of micronutrients with antioxidant capabilities. However, oxidative stress continues to be a problem in transition cows. Innovative approaches are needed to enhance the antioxidant defence mechanisms of dairy cattle during times of increased metabolic demands (Sordillo & Aitken 2009).

4.6 Human management and personal hygiene

The influence of management practices on bulk milk SCC (BMSCC) has been evaluated in several studies (Barkema et al. 1998, Bartlett et al. 1992, Erskine et al. 1987, Faye et al. 1997, Goodger et al. 1993, Hutton et al. 1990, Kingwill et al. 1970, Moxley et al. 1978, Neave et al. 1969). The influence of management style on disease, production, and culling has been described in several studies (Beaudeau et al. 1996, Bigras-Poulin et al. 1985a, Bigras-Poulin et al. 1985b, Dohoo et al. 1984, Enevoldsen et al. 1996, Faye 1991, Kiernan & Heindrichs 1994). Management style and its association with bulk milk somatic cell count (SCC) and the incidence rate of clinical mastitis were studied by Barkema et al. (1999) in 300 Dutch dairy herds. The most striking difference between farmers of herds with low and high bulk milk SCC was that the first group worked precisely rather than fast; the latter group of farmers worked quickly rather than precisely. As a result, the farms with herds that had a low bulk milk SCC had better hygienic conditions than those farms with herds that had a high bulk milk SCC (Barkema et al. 1999).

4.7 Mastitis management in South Africa and *Staphylococcus aureus* antibiotic resistance

The management of *S. aureus* IMI in South African dairy herds differs from most other countries. In South Africa a pro-active udder health management approach is followed which includes whole herd microbiological and cytological examinations on a routine basis (Petzer et al. 2012). In essence this is to identify *S. aureus* IMI, or cows with a less pathogenic strain of *S. aureus*, and to facilitate the control with a view to eradication of *S. aureus* from positive herds.

The pro-active udder health management programme includes the following procedures:

a) *S. aureus* is routinely identified from whole herd by examinations of microbiology and cytology. Whole herd investigations are done during which all the lactating animals are tested (microbiology and cytology) every 2 to 3 months. Such monitoring ensures the isolation of all intramammary *S. aureus* infections.

b) *S. aureus* cows are separated for life and milked last.

c) Early responsible treatment using probability of cure calculations (Sol et al. 1997)

d) Strict culling programmes

e) Decreasing *S. aureus* prevalence also by using vaccination

f) Resulting in decreased antimicrobial resistance of *S. aureus*

g) Re-training of staff and re-evaluating of microbiology and cytology of whole herd on a routine basis (ideally every 2 to 3 months).

5. Antimicrobials

Antimicrobials play a vital role in the management of bacterial infections, reducing morbidity and preventing mortality. However, the extensive use of antimicrobials in animal and human health, agricultural, and environmental sectors has resulted in increased drug resistance that threatens to reverse the life-saving power of these medicines (Department of Health and Department of Agriculture, Forestry and Fisheries (DAFF) 2018). The first commercially available antibacterial was Prontosil, a sulfonamide developed by the German biochemist Gerhard Domagk in the 1930s (Sneader 2001). In the case of Prontosil (sulfa drugs), it was discovered in 1932, introduced in 1936 and resistance was first observed in 1942 (Lewis 2013).

The discovery of penicillin in 1928 by Alexander Fleming (Fleming 1929), is recognized by many people as the first true antibiotic, a term coined by Selman Waksman as a compound produced by or derived from microorganisms that in dilute concentration, effectively inhibit the growth of or effectively kill other organism (Waksman 1947). The active compound of penicillin was isolated and set in production thanks to the work of Howard Florey and Ernst Boris Chain (Ligon 2004), for which they alongside Fleming received the Nobel Prize in 1945. Penicillin was discovered in 1928, introduced in 1938 and resistance was first observed in 1945 (Lewis 2013).Previously untreatable, devastating diseases, such as streptococcal and chlamydial infections became treatable with the introduction of penicillin. The discovery of antibiotics inspired a new era in the treatment of infectious diseases and paved the way for modern medicine, through the golden era of antibiotic drug discovery from the 1940’s to the 1960´s (Lewis 2013).

Commonly used antibiotics available for use in South Africa as intramammary remedies are limited mostly to ampicillin, cloxacillin and combinations of these, with only very few cephalosporins, lincosamides, tetracyclines available in both lactating and dry cow intramammary remedies , due to the relatively small market in South Africa (Carrington et al. 2016). See Appendix A, for the complete list of intramammary remedies (dry cow and lactating cow), available over the time of the study. Also not all of the products on Appendix A were available for the full study period. Since then some of these remedies are no longer available

specifically since 2019 and very few new remedies (same active ingredients) have come onto the market, making the current selection available even smaller (only 3 dry cow and 4 lactating cow remedies currently available on South African market in January 2020).

5.1 Antibiotic classification

5.1.1 Antibiotic classes

Antibiotics are divided into classes and then further into subclasses. The main antibiotic classes are: beta lactams, tetracyclines, quinolones, sulphonamides, aminoglycosides, lincosamides, macrolides, amphenicols, pleuromutilins and polypeptides (OIE 2007, Gualerzi et al. 2014). Within each class, the antibiotics are further divided by characteristics such as activity against specific organisms, ability to work under anaerobic conditions or being beta-lactamase stable. As an example, the penicilions which fall under the greater beta lactams grouping is further divided into the natural penicillins (Penicillin G), aminopenicillins (amoxicillin), beta-lactamase stable/resistant (oxacillin and methicillin), beta-llactamase inhibitor combinations (Amoxycillin and clavulanic acid)(OIE 2007, Gualerzi et al. 2014).

5.1.2 Antibiotic action

The difference between bactericidal (kills the organism) and bacteriostatic (inhibits growth temporarily) agents appears to be clear according to the in vitro definition, but this only applies under strict laboratory conditions and is inconsistent for a particular agent against all bacteria (Pankey & Sabath 2004). Although bacteriostatic/bactericidal data may provide valuable information on the potential action of antibacterial agents in vitro, it is necessary to combine this information with pharmacokinetic and pharmacodynamics data to provide a more meaningful prediction of efficacy in vivo, to achieve a clinical outcome (Pankey & Sabath 2004).

Pharmacokinetics is the mathematical description of the relationship of antibiotic concentration in the body relative to the time of administration. Pharmacodynamics describe the relationship of antibiotic concentration in relation to the pharmacologic effect or microorganism death (van Bambeke & Tulkens 2001). When these two principles are taken into consideration in combination, the effect of a specific antimicrobial can be better described. Two commonly used principles are used to describe the action of the antimicrobial drugs viz. time and concentration dependent

The term,"time dependent" means that the rate and extent of microorganism killing remains unchanged regardless of maximum antimicrobial concentration achieved, on condition that the minimum inhibitory concentration is maintained for a specified period of time. The pharmacodynamic parameter that is most often predictive of outcome for concentration independent drugs is temperature (T)> minimum inhibitory concentration (MIC), although the area under the curve (AUC)/MIC can be used because the AUC takes both the antimicrobial concentration and time into account. Examples of concentration independent antimicrobials include: vancomycin, macrolides, aztreonam, carbapenems, clindamycin, tetracyclines, quinupristin/dalfopristin, flucytosine, and azole antifungals (van Bambeke & Tulkens 2001).

The term "concentration dependent" (time independent) means that the rate and extent of microorganism killing is a function of the antimicrobial concentration (increase as the concentration increases) achieved in relation to the minimum inhibitory concentration independently of the time of this said concentration being maintained. The pharmacodynamic parameter that is most often predictive of outcome for concentration dependent drugs is Cmax/MIC, although the AUC/MIC can be used because the AUC takes both the antimicrobial concentration and time into account. Examples of concentration dependent antimicrobials include: fluoroquinolones, aminoglycosides, and amphotericin B (van Bambeke & Tulkens 2001).

5.1.3 Antibiotic spectrum

Antibiotics are in use against bacterial as well as some parasitic infections and have either a bacteriostatic or a bactericidal effect and can be effective against a small group of bacteria (narrow spectrum) or a wide range of pathogens (broad spectrum) (Apua glossary 2019).
The term broad-spectrum antibiotic can refer to an antibiotic that acts on the two major bacterial groups, Gram-positive and Gram-negative, or any antibiotic that acts against a wide range of disease-causing bacteria (Apua glossary 2019), e.g. the aminoglycosides, the 2nd and 3rd generation cephalosporins, the quinolones and some synthetic penicillins, some of these are narrower than the very broad ones (e.g. tetracyclines are broader than tazocillin, which is broader than cephalosporins) (Acar 1997) sometimes referred to as moderate spectrum antibiotics, e.g. macrolides.

Narrow-spectrum antibiotics are active against a selected group of bacterial types and are used mostly for treatment of a specific infection when the causative organism is known (Apua

glossary 2019); for example, considering the older penicillins (penicillin G), the macrolides and vancomycin, some of these have a narrower spectrum than others (e.g. macrolides versus metronidazole) (Acar 1997). These will not kill as many of the normal microorganisms in the body as the broad-spectrum antibiotics.

5.1.4 Intrinsic and acquired resistance

Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance to antibiotics via mutations in chromosomal genes and by horizontal gene transfer. The intrinsic resistance of a bacterial species to a particular antibiotic is the ability to resist the action of that antibiotic as a result of inherent structural or functional characteristics, which can be predicted from an organism's identity (Blair et al. 2014, Cox & Wright 2013). Knowledge of the intrinsic resistance of a pathogen of concern is important in practice to avoid inappropriate and ineffective therapies (Cox & Wright 2013). For bacterial pathogens which are naturally insensitive to a large number of classes of antimicrobials, such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*, this consideration can pose a limitation in the range of options for treatment and thus consequently further increase the risk for emergence of acquired resistance (Cox & Wright 2013).

5.2 Legislation and the use of antibiotics in South Africa

Antibiotics, including intramammary mastitis remedies, are registered in South Africa under two Acts namely: Medicines and Related Substances Control Act as amended (Act 101 of 1965) for scheduled medicine that are only available on prescription from veterinarians; and the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947) where antibiotics and stock remedies are made available over the counter. There is much evidence to suggest that dairy producers often treat cows with antibiotics symptomatically or without confirmation. Such treatment may contribute to the emergence and/or persistence of antibiotic resistant strains in humans (Burgos et al. 2005), and may lead to a similar scenario in animals. However, the focus worldwide and in South Africa, is now on prudent use which should have a greater effect on the effectiveness, antibiotic use and any consequent resistance. Any type of regulatory system which offers easy availability of antibiotic products may be a contributing factor to an increase in antibiotic resistance of mastitis causing organisms, even though in South Africa the emphasis for many years has been on the prudent use of antibiotics regardless of the availability of the products.

5.2.1 Antibiotic use in animals

The previous Department of Agriculture, Forestry and Fisheries (DAFF) in partnership with the South African Animal Health Association (SAAHA) has been reporting antimicrobial consumption in animals in accordance with the World Organisation for Animal Health (OIE) requirements (National Department of Health 2018). From 2014 to 2015 the predominant antibiotic group used in animal health have been the growth promoters (62%), followed by tetracyclines (17%) and macrolides (11%). The growth promoter group includes antibiotics not used in human health such as ionophores, flavophospholipol (flavomycin), olaquindox, zinc bacitracin and tylosin (National Department of Health 2018). Only tetracycline and tylosin are registered as antibiotics for growth promotion by The Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act 36 of 1947) whilst ionophores are classified as antiparasitics but interpreted by the pharmaceutical industry as “growth promoters” and therefore are being reported as such (National Department of Health 2018). In South Africa at present antibiotics such as, monensin, an ionophore (Erasmus et al. 2005), is still allowed to be used in feed of dairy cattle and is available over the counter under Act 36, as a stock remedy (Henton et al. 2011).

5.2.2 Antibiotic use in humans

South Africa’s antibiotic use in 2015 was 21 149 standard units per 1000/population (IMS Health 2015, 1 standard unit is equivalent to 1 tablet, or injection), significantly higher than most other countries in the world. Broad-spectrum penicillin usage was 1.3 to 3.3 time more than that used in other BRICS countries (Brazil, India, Russia, China and South Africa) and 0.8 time that used in the United Kingdom or the USA (National Department of Health 2018).

The consumption of antimicrobials by humans in the public sector, sourced from procurement data, shows that cotrimoxazole made up almost 50% of all antibiotics procured with a decreasing trend over time as the contribution of the antiretroviral programme started to take greater effect and there was a decreased need for *Pneumocystis jiroveci* pneumonia (PJP) prophylaxis (National Department of Health 2018). There is minimal use of narrow-spectrum penicillins as compared to broad-spectrum penicillins, however the proportionate consumption of broad-spectrum penicillins has remained between 19.6% to 18.2%, for 2016 to 2017 (National Department of Health 2018).

5.3 Antibiotic resistance

In recent years, the Infectious Diseases Society of America has highlighted a group of antibiotic resistant bacteria which comprises of six pathogens commonly associated with antimicrobial resistance: *Enterococcus faecalis* and *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Escherichia coli* (*E.coli*) (ESKAPE) (National Department of Health 2018, Santajit & Indrawattana 2016). The ESKAPE pathogens are the leading cause of nosocomial infections throughout the world. Most of them are multidrug resistant isolates, which is one of the greatest challenges in clinical practice. Multidrug resistance is amongst the top three threats to global public health and is usually caused by excessive drug usage or prescription, inappropriate use of antimicrobials, and substandard pharmaceuticals (Santajit & Indrawattana 2016).

Information has become available about antimicrobial resistance from surveillance data in blood culture of humans for ESKAPE pathogens in South Africa (National Department of Health 2018). Resistance levels for *S. aureus* have declined from 36% to 23% (of which 1 in 4 are MRSA) over the past 6 years, and resistance varies across the provinces of South Africa (National Department of Health 2018). Ampicillin remains the drug of choice for *Enterococcus faecalis*, but ampicillin resistance of *Enterococcus faecium* is greater than 90% with the added growing concern of vancomycin resistance (a last resort antibiotic), especially in the Free State (National Department of Health 2018). In South Africa for *E. coli* one in four blood stream infections are resistant to 3rd generation cephalosporins (ESBL). For *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, one in twelve, one in four and eight in ten blood stream infections are resistant to carpapenems respectively (National Department of Health 2018).

Certain erythromycin-resistant strains of *S. aureus* are sensitive to other macrolide antibiotics. If these strains are exposed to low levels of erythromycin, resistance to other antibiotics is induced. The antibiotics to which resistance is induced by erythromycin include: other macrolides as well as lincosaminide, streptogramin (group B) antibiotics but not chloramphenicol, amicetin, streptogramin (group A) antibiotics, tetracyclines, and aminoglycosides. Hence erythromycin induces resistance exclusively towards inhibitors of 50*S* ribosomal subunit function and, thus far, only with respect to three of six known classes of inhibitors which act on this subunit (Weisblum & Demohn 1969).

Resistance patterns to commonly used antibiotics in dairy herds are often not known, nor for different seasons and regions. Limited data are available in South Africa on antibiotic resistance surveillance in dairy cattle (Petzer et al. 2012, Schmidt et al. 2015, van Vuuren et al. 2007) which highlighted the need for more detailed information of resistance patterns. This knowledge will be valuable to veterinarians and producers when using antibiotics and for reactive treatment of dairy cattle in various regions. This information will also be valuable to pharmaceutical companies, to know which antibiotic active ingredients are likely to be effective in dairy cattle, as there is not much variety in intramammary products available in the South African market. This information can also assist in development of policy on access and use of antibiotics and inform users on some general aspects and trends of effective antibiotics.

There is only a small selection of intramammary antibiotics available for use on the South African market, which are mainly ampicillin and/or cloxacillin based products. Antibiotic usage has been shown to correlate with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains in ruminants (Ben Zakour et al. 2008). Dairy cattle producers are particularly affected because of the negative impact resistant strains have on milk production (Bean et al. 2004). Programmes for the control of contagious mastitis involve improvements in hygiene and disinfection aimed at disrupting the cow-to-cow mode of transmission as well as the elimination of infected cows via antibiotic treatment or culling (Barkema et al. 2006). The ability to treat mastitis effectively depends not only on the efficacy of the active ingredient of the antibiotic (Bradley & Green 2009), but also on many aspects of management, hygiene, cow immunity, application of the intramammary product and other factors. Bacterial susceptibility to an antibiotic as determined by susceptibility testing can aid in the appropriate selection of an antibiotic for treatment of mastitis in dairy cows (Brînda 2009). The knowledge that a particular pathogen is resistant to an antibiotic should reduce the incidence of unnecessary or incorrect selection of antibiotics.

The dairy industry is a large global industry and mastitis (clinical and sub-clinical) has been estimated to be the single largest contributor to loss of revenue worldwide (Awale et al. 2012). Worldwide losses due to mastitis have been estimated at approximately 35 billion US dollars (Modi et al. 2012).Estimated milk production losses in a specific herd associated with elevated quarter milk somatic cell counts (SCC) of all lactating cows in the herd have been estimated as an annual milk loss of 46 190 L valued at ZAR 205 544 (Petzer et al. 2016). Such estimations differ between countries and between management systems. As most mastitogenic pathogens are ubiquitous in the environment, mastitis can be managed but not eliminated, and one of the primary management strategies is treatment with antibiotics. The

discovery of antibiotics has revolutionized medicine in many respects and many lives have been saved. However, the use of these potential wonder drugs has been accompanied by the rapid appearance of resistant strains of bacteria. A data base has listed more than 20 000 potential resistance genes (r genes) of nearly 400 different types, predicted from the main bacterial genome sequences available (Davies & Davies 2010). A recent World Health Organization (WHO) health report has warned that resistance to antibiotics in general is a 'global' threat (WHO 2014), and one that impacts on both human health care and the agricultural industry. The exposure of humans to antibiotic resistant pathogens through agricultural products and contact with animals has increased from the turn of this century (Kluytmans 2010), compared to exposure through hospitals and human medicine-based pathways (Witte 1998). Antibiotics used in feed for commercial livestock production increased by 50% between 1985 and 2001 (Emanuele 2010). In the United State of America (USA), antibiotics are routinely fed to livestock, poultry, and fish on industrial farms to promote faster growth and allegedly in some cases to compensate for poor management (Gerber et al. 2007). According to a report by the Food and Drug Association (FDA), approximately 80% of all antibiotics used in the USA went to livestock production (USFDA 2009). Several studies in the USA have found that health problems are more commonly reported in workers on farms than in the general population (Von Essen & Auvermann 2005). Despite the recent efforts of many international health organizations (EUTAG 2006, Shryock & Richwine 2010) to control and withdraw antibiotic use in animal husbandry, new antibiotic resistance continues to emerge, as there have been many factors causing increased antibiotic resistance over the past 60 years (Levy 2002). In Germany, 1734 tons of antimicrobial agents were used for animals in 2011, compared to 800 tons for humans (Meyer et al. 2013). Sweden banned the use of antibiotics in food animals in 1986 and Denmark started limiting use from 1994, so that its use is now 60% less (Koch 2013). Contrary to the above findings, in the Netherlands, the use of antibiotics to treat diseases increased after the ban on its use for growth purposes in 2006 (Maron et al. 2013). In 2011, alarmed at the signs that this general overuse of antibiotics is hindering the value of antibiotics for humans, the European Union (EU) voted to ban the prophylactic use of antibiotics (EU 2005). Overall, the dairy industry is one of the major contributors to worldwide antibiotic usage and information as to the trends of susceptibility and resistance in host adapted pathogens such as *S. aureus* is critical.

The European Medicines Agency (EMA) and the European Food Safety Authority (EFSA) published a joint opinion in which they argue it is time to reduce, replace and re-think the use of antimicrobials in animals (International Dairy Federation Annual Report 2016-2017). They have reviewed measures taken in the EU to reduce the need for and use of antimicrobials in

food-producing animals, and the resultant impacts on antimicrobial resistance (AMR). They conclude that due to the multiplicity of factors contributing to AMR, the impact of any single measure is difficult to quantify, although there is evidence of an association between reduction in antimicrobial use and reduced AMR.

The European Food Safety Authority (EFSA) have argued that the use of antimicrobials in animals should be reduced to the minimum that is necessary to treat infectious diseases. Other than in exceptional cases, use of antimicrobials to prevent such diseases should be phased out in favour of alternative measures. Critically important antimicrobials for human medicine should only be used in animals as a last resort. Alternatives to antimicrobials that reduce the need to use antimicrobials include vaccines, probiotics, prebiotics, bacteriophages and competitive exclusion cultures.

The European Food Safety Authority (EFSA) note, however, that reducing the use of antimicrobials and finding alternatives is not enough. There is a need to re-think the livestock system by implementing farming practices that prevent the introduction and spread of the disease into farms and by considering alternative farming systems which are viable with reduced use of antimicrobials. Education and awareness of antimicrobial resistance (AMR) should be addressed to all levels of society but in particular to veterinarians and farmers. These experts concluded that it is reasonable to assume that reducing antimicrobial use in food-producing animals would result in a general decrease in antimicrobial resistance in the bacteria that they carry and the food products derived from them.

The most frequently used antibiotic class in China were cephalosporins which accounted for 28.6% of total consumption, followed by beta-lactam-beta-lactamase inhibitor combinations at 20.0%, macrolides at 17.4%, and fluoroquinolones at 10.5% (all figures from 2015) (Wushouer et al. 2017).

Until 2011, data on the volume of antibiotics used in livestock production were scarce in South Africa, and information was lacking about the patterns of antibiotic consumption in food animals. Because antibiotic use in animals is controlled by two very different Acts (Stock Remedies Act (Act 36 of 1947) and Medicines and Related Substances Control Act (Act 101)), and because pharmaceutical companies protect sensitive information, it was very difficult to obtain an accurate estimate of the amount of antibiotics used in livestock production in South Africa (Henton et al. 2011).

However, a more recent ministerial report showed import data for antimicrobials between 2014 and 2015 estimated procurement of antibiotics for animal health at 23-36% and for human use at 74-77%. Humans consumed the majority of penicillins and streptomycins (National Department of Health 2018). This ratio of animal (26%) to human use (74%) was in contrast to reports from the USA, China and India where animal consumption is far larger in proportion compared to use in humans (National Department of Health 2018).
Resistance of *S. aureus* to some antibiotics has shown seasonal patterns. In particular, a study by Alvarado et al. (2009) found the highest resistance of *S. aureus* to ampicillin and penicillin occurred during autumn (southern USA). The causes of the variation were not clear but could possibly be attributable to on site storage of feed materials. In this study of *S. aureus* and other species of *Staphylococcus* isolated from mastitis in cattle, >60% were resistant to penicillin and amoxicillin, and >40% were resistant to tetracyclines.

Levels of resistance were far lower in the South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD) (van Vuuren et al. 2007) surveillance, where only 10% resistance to the three antibiotics was found. Far less resistance was noted to other commonly used mastitis remedies. In contrast, about 80% of *S. pseudintermedius* isolates from pyoderma and other infections in dogs were resistant to amoxicillin, and about 20% were resistant to first-generation cephalosporins. Petzer et al. (2007) found resistance rates of 45% for penicillin, 37% for ampicillin, and 23% of tetracyclines in *S. aureus* isolates from milk samples. *Pasteurella, Mannheimia, Histophilus* and related bacteria usually isolated from cattle respiratory infections showed a <20% resistance rate to commonly used antibiotics for such infections, such as penicillin, amoxicillin, ceftiofur, florfenicol (related to chloramphenicol) and tetracycline (Henton et al. 2011).

By undergoing a simple mating process called "conjugation," bacteria can transfer genetic material, including genes encoding resistance to antibiotics (found on plasmids and transposons) from one bacterium to another. Viruses are another mechanism for passing resistance traits between bacteria. Bacteria have a remarkable genetic plasticity that allows them to respond to a wide array of environmental threats, including the presence of antibiotic molecules that may jeopardize their existence. As mentioned, bacteria sharing the same ecological niche with antimicrobial-producing organisms have evolved mechanisms to withstand the effect of the harmful antibiotic molecule and, consequently, their intrinsic resistance permits them to thrive in its presence.

From an evolutionary perspective, bacteria use two major genetic strategies to adapt to the antibiotic “attack”:

a) mutations in gene(s) often associated with the mechanism of action of the compound and

b) acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT).

5.4 Alternatives to antibiotics

The widespread development of antibiotic drug-resistant strains has accentuated the importance of the use of alternative antimicrobials in the medical and veterinary fields (Pieterse & Todorov 2010, Vanderhaeghen et al. 2010, Francoz et al. 2017). Although, it will be difficult to eliminate the use of antibiotics, the effective application of the following alternatives will help to reduce antibiotic usage dramatically.

5.4.1 Vaccines

Historically mastitis vaccinations have been used with limited efficacy as a preventative measure against bovine mastitis (Mamo et al. 1994, Giraudo et al. 1997). There are newer vaccines that are effective not at preventing IMI but at decreasing the severity and the prevalence of IMI. Such vaccines work on the biofilm of specific bacteria (Bradley et al. 2015). An example of a successful vaccine is the *E. coli* J5 vaccine, which reduced the incidence of infection by 70-80% in the targeted herd (Middleton et al. 2009) and the Startvac vaccine decreasing clinical signs and duration of mastitis caused by *S. aureus*, NAS, *Escherichia coli* and other coliforms and lowering shedding of bacteria and in that way reducing new IMI.

5.4.2 Probiotics, prebiotics and competitive exclusion cultures

Utilization of this native or artificially introduced microflora population to improve animal health and productivity has been termed a ‘probiotic’, or competitive enhancement strategy (Fuller 1989). Competitive enhancement strategies include probiotics, prebiotics and competitive exclusion (CE) cultures and utilize the activities of the native microbial ecosystem against pathogens by capitalizing on the natural microbial competition (Callaway et al. 2008).

Data are scarce but there is some evidence of a link between using biocides in veterinary products, and increased resistance to antibiotics (European Commission 2009).

The prospects of oral probiotics are not promising for ruminants and those for intramammary probiotics should be considered with caution, but teat apex probiotics deserve further research (Rainard & Foucras 2018). Probiotics may elicit immunomodulatory effects through interactions with the epithelium, especially in organs that are not densely populated by a commensal microbiota, whereas indirect interactions through modulation of massive endogenous microbiota (as in the colon) may be more important (Rainard & Foucras 2018).

5.4.3 Bacteriocins and bacteriophages

Bacteriocins are a group of proteins secreted by bacteria that kill or inhibit competing strains. Klaenhammer (1993) and Topley & Wilson (2010) classified bacteriocins based on the structure and mode of action of the peptide and including mainly those produced by lactic acid bacteria (LAB). Bacteriocin-producing organisms could be considered as an important source of antimicrobial agents in the medical and veterinary fields (Pieterse & Todorov 2010). Bacteriocins, by definition usually only target closely related species; they could offer an advantage over antibiotics in that treatment could be targeted against specific pathogenic organisms. Bacteriocins, identified for potential use as antimicrobials include lantibiotics produced by Gram-positive lactic acid bacteria, and colicins and microcins, produced by Gram-negative bacteria (Gillor et al. 2005). Some examples include: Gram-positive bacteria Lacticin 3147, produced *by Lactococcus lactis* subsp. *lactis*which has the potential of being used to treat mastitis in cattle (Ryan et al. 1998); Epidermin, produced by *Staphylococcusepidermidis (S. epidermidis)* which can be potentially used to treat skin infections such as acne (Allgaier et al. 1986). Microcins J25 and 24 produced by *E. coli* have potential of being used to treat *E. coli* and *Salmonella* infections in chickens (Sable et al. 2000, Wooley et al. 1999).

Bacteriophages are viruses that infect specific bacteria without harming the plant or animal host of the bacterium (Topley & Wilson 2010). Bacteriophage therapy has been used by Basdew & Laing (2011), to treat mastitis on an experimental basis. Both bacteriocins and bacteriophages have anti- microbial activity and they share some features, such as the uptake site on their host bacterium (Chan 2019; Topley & Wilson 2010). In a study by O'Flaherty et al. (2005), a lytic bacteriophage (phage K) was assessed in vitro for its ability to inhibit emerging resistant *Staphylococcus aureus* strains from hospitals and other *Staphylococcus* spp. isolated from bovine infections. The results enforce the principle that, while certain target bacteria may be relatively insensitive to lytic phage, this can be overcome by obtaining

modified phage variants from passage of the phage through the insensitive strains (O'Flaherty et al. 2005).

5.4.4 Gene therapy

Gene therapy is an experimental technique that uses genes to treat or prevent disease (Biffi et al. 2013). This may perhaps be applied to inactivate resistance genes in antibiotic resistant mastitis causing organisms in future. Research has been done in an attempt to transfect (infect a cell with free nucleic acid) the udders of dairy cattle with cercropin B, a lytic peptide found in Cercropia moths, that has a broad spectrum of bactericidal properties. This technology has been applied to other species through different experimental procedures and has yielded favourable outcomes and a decrease in targeted infectious diseases. This research with dairy cattle has not yet yielded favourable results, but with some experimental modifications, could be proven effective in preventing mastitis (Bordeaux 2016).

5.5 Antibiotic sales and use

5.5.1 Antibiotic sales in Zambia 2015

The pattern of resistance detected (Mainda et al. 2015) reflected the relative levels of sales of antibiotics, with the majority use of the tetracycline class matching the high prevalence of resistance found. A total of 41,280.87 kg of active ingredient of antibiotics was dispensed from the major supplier during the period under review (January 2013–February 2014). Of the total, 63.6% were parenteral antibiotics and 36.4% were for oral use. Tetracyclines were the most sold class of antibiotic during the period reviewed for both parenteral and oral sales and contributed 68% and 59% respectively (by weight). Penicillins were the second most abundant class of antibiotics sold by weight accounting for 27.2% of parenteral and 9.6% of oral sales. These were followed by the sulphonamides and then other classes of antibiotics (Mainda et al. 2015).

5.5.2 Antibiotic sales in the animal sector in South Africa, 2000-2010 and recently.

Antibiotic sales in the animal sector in South Africa for 2014 were: sulphonamides 3%, penicillins 3%, aminoglycosides 2%, fluoroquinolones 1%, pleuromutillins 1%, macrolides 6%, tetracylines 28% and others 56% (National Department of Health 2018). These differed from the antibiotic sales in the animal sector in South Africa for 2015, which were: sulphonamides

11%, penicillins 1%, pleuromutillins 1%, macrolides 8%, tetracyclines 17% and other 62% (National Department of Health 2018). Estimated consumption by animals of tetracyclines makes up about 27% of total antimicrobial sales, compared to the OIE reported 63% for most African countries over the same period (OIE 2016, OIE 2017).

Between 2000 and 2010, consumption of antibiotic drugs overall (human and animal), increased worldwide by 35% (from 52 057 163 835 standard units to 70 440 786 553). Brazil, Russia, India, China, and South Africa accounted for 76% of this increase. In most countries, antibiotic consumption varied significantly with season. There was increased consumption of carbapenems (45%) and polymixins (13%), two last-resort classes of antibiotic drugs (van Boeckel et al. 2014).

5.5.3 Patterns of Antibiotic use by sector (South Africa)

The greatest volume of antibiotic use is in intensively farmed poultry (including broilers for meat and layers for eggs) and pigs. These animals are kept indoors at a high density, which promotes the rapid transmission of bacterial infections, primarily affecting the respiratory and intestinal tracts.

Feedlot cattle and dairy cows are the next group in terms of the amount of antibiotics used. Slaughter cattle are generally raised under extensive conditions on farms, and then sent to a feedlot for rounding off before going to the abattoir. Feedlot cattle are prone to respiratory disease, caused by *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* and *Mycoplasma,* and mastitis, usually caused by *S. aureus.*
Other ruminants (sheep and goats) are extensively farmed, together with the bulk of the population of cattle in South Africa. The main source of food is veld grass, and the density levels are low. Extensively kept ruminants are far healthier than those kept under intensive conditions, and suffer from far fewer bacterial infections.

South Africa is drought-prone and there are few aquaculture ventures. Fresh water farms for trout are only found in the Lydenberg, Drakensberg and Western Cape areas. Suitable rivers are scarce and, where a river can support farmed fish, there may be more than one farm on the river. Downstream farms can become infected with bacteria from fish farms in the upper reaches. Marine aquaculture ventures are also scarce, considering the extensive coastline of South Africa. There are a few abalone farms in the Hermanus area, and along the West Atlantic coast a total of eight at present. The water flow rate in an abalone farm is too rapid for

antibiotic administration. Ornamental fish are mostly imported, and little breeding is carried out in South Africa (Henton et al. 2011)

5.5.4 Patterns of use by purpose

The most frequent uses of antibiotics by weight (as measured by sales) were for treating and preventing diseases in poultry and pigs, and as growth promoters generally. The antibiotic tylosin, one of 4 growth promoters banned in Europe, was the most extensively sold antibiotic in the survey. It is primarily administered through animal feed at sub-therapeutic levels and is available as an over-the-counter stock remedy. About two-thirds of the antibiotics surveyed were administered in feed. The second-, third- and fourth-largest groups of antibiotics sold in the study (tetracyclines, sulphonamides and penicillins) are also readily available and have a wide spectrum of antimicrobial activity against common infections (Henton et al. 2011).

The volume of antibiotics used for treating and preventing disease is unknown and difficult to assess. Intensive farming systems have a rapid turnover rate, and profit margins are generally low. Infectious diseases have a negative effect on profitability, but the high cost of administering antibiotics to all the animals in the barn (metaphylaxis, i.e. sick as well as healthy animals, or prophylaxis, where antibiotics are given to prevent disease before it occurs) also affects profitability. Chronically ill animals are usually culled and not treated (Henton et al. 2011).

5.6 The importance of antimicrobial resistance in One Health

The One Health approach is a global strategy that encourages interdisciplinary collaboration and communication on health between the human, animal and environmental sectors (Essack 2018). It is based on the view that such an interdepedence will advance future health care by speeding up biomedical research and innovation, improving public health, expanding the scientific knowledge base, improving medical and veterinary education and clinical care, and positively impacting on longevity and quality of life (http://www.onehealthinitiative.com/about.php).

Thus a One Health approach is required in order to understand and control antimicrobial resistance, which is a direct consequence of the selection pressure resulting from indiscriminate antibiotic use in humans, animals and the environment (WHO 2014).

The complexity and diversity of AMR poses a further challenge (Essack 2018). Bacteria are becoming more resistant to multiple antibiotics classes and single isolates or strains exhibit multiple resistance mechanisms such as: enzymatic degradation, target alteration, impermeability and efflux to single antibiotic classes in different permutation and combinations. Resistance genes as well as virulence factors are carried on different mobile genetic elements such as plasmids, transposons, insertion sequence common regions, integrative and conjugative elements, gene cassetes and integrons (Stokes & Gillings 2011) which are capable of exchange amongst and between bacteria in humans, animals and the environment (Essack 2018).

Despite a series of WHO strategies and resolutions since 1998 there was no meaningful global action on AMR until after the 2011 World Health Day campaign (Shallcross & Davies 2014). This is when the WHO launched the 6-point AMR policy package followed by the report which revealed high resistance rates in bacteria frequently implicated in hospital, community and food-chain-related infections in all WHO regions (WHO 2014).

5.7 General conclusion

Mastitis is a major cause of economic loss in the dairy industry (Akers & Nickerson 2011), and the incidence is affected by various cow, management and environmental factors. Mastitis caused by *S. aureus* is still a problem in udder health in South African dairy herds. Treatment of mastitis by itself, as the only response to the diagnosis of clinical mastitis is not successful in managing the problem of mastitis in a herd. Therefore, the pro-active udder health programme has been developed and is used widely in South Africa. It involves a complete herd approach (routine microbiology and cytology) in order to identify causative agents and early warning signs for appropriate action (Petzer et al. 2009). However, of course clinical mastitis should always be treated (Petzer et al. 2009).

In South Africa there have been limited data available on antibiotic resistance surveillance in dairy cattle (Petzer et al. 2012, Schmidt et al. 2015, van Vuuren et al. 2007), which has stimulated the need for more detailed information to be generated through appropriate research.

Antimicrobial resistance is a major threat to the long-term security of public health and has the potential to impact society negatively. This is a multifaceted public health problem and a direct threat to human and animal health, food security and the continued use of available

antimicrobials. It is a serious and growing global health risk, which needs to be prioritised at local and international levels (Department of Health and Department of Agriculture, Forestry and Fisheries (DAFF) 2018). The financial costs of treating antimicrobial resistant infections in humans and animals will place a significant economic burden on society and compromise food security (Department of Health and Department of Agriculture, Forestry and Fisheries (DAFF) 2018).

The development and implementation of a National Antimicrobial Resistance Strategy Framework (NARSF) that complements international efforts is a major step towards containment of the growing threat of antimicrobial resistance in human and animal health in South Africa (Department of Health and Department of Agriculture, Forestry and Fisheries (DAFF) 2018). The responsibility for reducing antibiotic resistance is a shared one, thus global partnerships need to be strengthened. This responsibility is not only limited to the health care sector, but calls for a “One Health” approach with collaboration from human, animal and agricultural sectors (Department of Health and Department of Agriculture, Forestry and Fisheries (DAFF) 2018).

Little is known about the antibiotic resistance and clinical implications of *S. pseudintermedius* from milk of dairy cattle, and this will be examined and the diagnosis will be verified using genotyping, in future research.

5.8 Aims and objectives

The objective of this study was to establish the trend of resistance of *S. aureus* to antibiotics in dairy cows with sub-clinical mastitis in South Africa over an extended time period in the various regions and seasons. *Staphylococcus aureus* is the pathogen on which this dissertation focuses as it is one of the six ESKAPE pathogens commonly associated with antibiotic resistance (National Department of Health 2018). This thesis will investigate the surveillance of antibiotic resistance of *S. aureus* isolated from milk of dairy cattle over an eleven-year period in different seasons and regions of South Africa.

References

Acar, J., 1997. Broad- and narrow-spectrum antibiotics: an unhelpful categorization. Clinical Microbiology and Infection 3(4), 395-396. doi.org/10.1111/j.1469-0691

Abede, R., Hatiya, H., Abera, M., Megersa, B., Asmare, K., 2016. Bovine mastitis: prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. BMC Veterinary Research 12(270), (2016). doi:10.1186/s12917-016-0905-3

Adegboye, D.S., Halbur, P.G., Nutsch, R.G., Kadlec, R.G., Rosenbusch, R.F., 1996. *Mycoplasma bovis*-associated pneumonia and arthritis complicated with pyogranulomatous tenosynovitis in calves. Journal of the American Veterinary Medical Association 209, 647-649.

Agricultural & Horticulture Development board. (2016, June 07). Udder Physiology. Retrieved from Agriculture and Horticulture Development Board: Dairy: http://dairy.ahdb.org.uk/technical-information/animal-health-welfare/mastitis/symptoms-of-mastitis/udder -physiology/#.V1b0ACnmqM8

Akers, R.M., Nickerson, S.C., 2011. Mastitis and its impact on structure and function in the ruminant mammary gland. Journal of Mammary Gland Biology Neoplasia 16(4), 275-289. doi:10.1007/s10911-011-9231-3

Allgaier, H., Jung, G., Werner, R.G., Schneider, U., Zahner, H., 1986. Epidermin: sequencing of a heterodetic tetracyclic 21-peptide amide antibiotic. European Journal of Biochemistry 160, 9-22.

Alvarado, C.S., Gandara, A., Flores, C., Perez, H.R., Green, C.F., Hurd, W.W., Gibbs, S.G., 2009. Seasonal changes in airborne fungi and bacteria at a dairy cattle concentrated animal feeding operation in the southwest United States. Journal of Environmental Health 71(9), 40-44.

Andersen, S., Dohoo, I.R., Olde Riekerink, R., Stryhn, H., 2010. Diagnosing intramammary infections: Evaluating expert opinions on the definition of intramammary infection using conjoint analysis. Journal of Dairy Science 93, 2966–2975.

Apua Glossary, Glossary- Alliance for the prudent use of antibiotics. https://apua.org/glossary (accessed 2 July 2019).

Awale, M.M., Dudhatra, G.B., Avinash, K., Chauhan, B.N., Kamani, D.R., Modi, C.M., Patel, H.B., Mody, S.K., 2012. Bovine Mastitis: A Threat to Economy. Open Access Scientific Reports 1, 295.

Badenhorst, R., Karzis, J., Petzer, I.M., 2014. Methicillin resistance in staphylococci isolated from milk samples of South African Dairy Cows – a Pilot Study. Faculty day, Faculty of Veterinary Science, Onderstepoort, 4 September, 2014.

Bakken, G., 1981. Subclinical mastitis in Norwegian dairy cows: prevalence rates and epidemiological assessment. Acta Agricultura Scandanavica 31, 273-286.

Bannerman, D.D., Paape, M.J., Lee, J.W., Zhao, X., Hope, J.C., Rainard, P., 2004. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. Clinical Diagnosis of Laboratory Immunology 11, 463-472.

Bannoehr, J., Ben Zakour, N., Waller, A., Guardabassi, L., van den Broek, A., Thoday, K., Fitzgerald, J., 2007. Population genetic structure of the Staphylococcus intermedius group: insights into agr diversification and the emergence of methicillin-resistant strains. Journal of Bacteriology 189, 8685–8692.

Barkema, H. W., Schukken, Y.H., Lam, T.J.G.M., Beiboer, M.L., Benedictus, G., Brand, A., 1998. Management practices associated with low, mid, and high bulk milk somatic cell count. Journal of Dairy Science 81, 1917–1927.

Barkema, H.W., Van Der Ploeg, J.D., Schukken, Y.H., Lam, T.J.G.M., Benedictus, G., Brand, A., 1999. Management Style and Its Association with Bulk Milk Somatic Cell Count and Incidence Rate of Clinical Mastitis. Journal of Dairy Science 82, 1655–1663.

Barkema, H.W., Schukken, Y.H., Zadoks, R.N., 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. Journal of Dairy Science 89, 1877–1895.

Barrett, T.J., Gerner-Smidt, P., Swaminathan, B., 2006. Interpretation of pulsed-field gel electrophoresis patterns in foodbourne disease investigations and surveillance. Foodbourne Pathogens and Disease 3(1), 20-31.

Bartlett, P. C., Miller, G.Y., Lance, S.E., Heider, L.E., 1992. Environmental and managerial determinants of somatic cell counts and clinical mastitis incidence in Ohio dairy herds. Preventive Veterinary Medicine 14, 195–207.

Basdew, I.H., Laing, M.D., 2011. Mini-Review: Biological control of bovine mastitis using bacteriophage therapy. Science against microbial pathogens: communicating current research and technological advances. World Scientific, Singapore pp, 386-393.

Bean, A., Williamson, J., Cursons, R.T., 2004. Virulence genes of Escherichia coli strains isolated from mastitic milk. Journal of Veterinary Medicine B. Infectious Diseases and Veterinary Public Health 51, 285–287.

Beaudeau, F., van der Ploeg, J.D., Boileau, B., Seegers, H., Noordhuizen, J.P.T.M., 1996. Relationships between culling in dairy herds and farmers' management styles. Preventive Veterinary Medicine 25, 327–342.

Ben Zakour, N.L., Sturdevant, D.E., Even, S., Guinane, C.M., Barbey, C., Alves, P.D., 2008. Genome-wide analysis of ruminant *Staphylococcus aureus* reveals diversification of the core genome. Journal of Bacteriology 190, 6302–6317.

Bennett, R.H., Jasper, D.E., 1977. Bovine mycoplasma mastitis from intra-mammary inoculation of small numbers of *Mycoplasma bovis*. Veterinary Microbiology. 2(4):341–355.

Bertoni, G., Minuti, A., Trevisi, E., 2015. Immune system, inflammation and nutrition in dairy cattle. Animal Production Science 55(7), 943-948. https://doi.org/10.1071/AN14863

Biffi, A., Montini, E., Lorioli, L., Cesani, M., Fumagalli, F., Plati, T., Baldoli, C., Martino, S., Calabria, A., Canale, S., Benedicenti, F., Vallanti, G., Biasco, L., Leo, S., Kabbara, N., Zanetti, G., Rizzo, W.B., Mehta, N.A.L., Cicalese, M.P., Casiraghi, M., Boelens, J.J., Del Carro, U., Dow, D.J., Schmidt, M., Assanelli, A., Neduva, V., Di Sierio, C., Stupka, E., Gardner, J., Van Kalle, C., Bordignon, C., Ciceri, F., Rovelli, A., Roncarolo, M.G., Aiuti, A., Sessa, M., Naldini,

L., 2013. Lentiviral Hematopoietic Stem Cell Gene Therapy Benefits Metachromatic Leukodystrophy. Science 341, 6148. doi:10.1126/science.1233158

Bigras-Poulin, M., Meek, A.H., Martin, S.W., 1985a. Attitudes, management practices, and herd performance—A study of Ontario dairy farm managers. I. Descriptive aspects. Preventive Veterinary Medicine 3, 227–240.

Bigras-Poulin, M., Meek, A.H., Martin, S.W., 1985b. Attitudes, management practices, and herd performance—A study of Ontario dairy farm managers. II. Associations. Preventive Veterinary Medicine 3, 241–250.

Biozzi, G., Mouton, D., Heumann, A.M., Bouthillier, Y., Stiffel, C., Mevel, J.C., 1979. Genetic analysis of antibody responsiveness to sheep erythrocytes in crosses between lines of mice selected for high or low antibody synthesis. Immunology 36, 427–438.

Blair, J.M.A., Webber, M.A., Baylay, A.J., Ogbolu, D.O., Piddock, L.J.V., 2014. Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology AOP 13(1), 42-51. doi:10.1038/nrmicro3380

Blank, J.L., 1992. Phenotypic variation in physiological response to seasonal environments. In: Tomasi, T.E., Horton, T. (Eds.), Mammalian Energetics: Interdisciplinary Views of Metabolism and Reproduction. Comstock Publishing Associates, Ithaca, NY, pp. 186-212.

Blignaut, D.J.C., Petzer, I.M., Thompson, P., 2018. Prevalence of mastitis pathogens in South African pastures and total mixed ration based dairies during 2008 and 2013. Onderstepoort Journal of Veterinary Research 85(1), 1485a. https://doi.org/10.4102/ojvr.v85i1.1482

Boudeaux, K., 2016. Gene therapy and the prevention of mastitis in dairy cattle. Journal of Animal Science 94(Suppl. 5):19. https://doi.org/10.2527/jam2016-0041

Boughton, E., 1979. *Mycoplasma bovis* mastitis. Veterinary Bulletin 49(6), 377-387.

Bradley, A.J., Green, M.J., 2009. Factors affecting cure when treating bovine clinical mastitis with cephalosporin-based intramammary preparations. Journal of Dairy Science 92(5), 1941-1953.

Bradley, A.J., Breen, J.E., Payne, B., White, V., Green, M.J., 2015. An investigation of the efficacy of a polyvalent mastitis vaccine using different vaccination regimens under field conditions in the United Kingdom. Journal of Dairy Science 98(3), 1706-20. doi: 10.3168/jds.2014-8332

Braem, G., De Vliegher, S., Supré, K., Haesebrouck, F., Leroy, F., De Vuyst, L., 2011. (GTG) 5-PCR fingerprinting for the classifica-tion and identification of coagulase-negative Staphylococcus species from bovine milk and teat apices: A comparison of type strains and field isolates. Veterinary Microbiology 147, 67–74.

Bramley, A.J., Dodd, F.H., 1984. Reviews of the progress of dairy science: Mastitis control-progress and prospects. Journal of Dairy Research 51, 481-512.

Brînda, M., 2009. Antibiotic Susceptibility of pathogens isolated from mastitic milk in cattle. Lucrări Tinifice Medicinã Veterinarã XLII, 263-266.

Bruckmaier, R.M., Meyer, H.H.D., 2005. Immunomediator and milk protein gene expression in mammary tissue during endotoxin-induced mastitis. Livestock Production Science 98(1-2), 81-87. https://doi.org/10.1016/j.livprodsci.2005.10.006

Burgos, J.M., Ellington, B.A., Varela, M.F., 2005. Presence of multidrug resistant enteric bacteria in dairy farm topsoil. Journal of Dairy Science 88(4), 1391-1398. doi:10.3168/jds.S0022-0302 (05)72806-X

Burrows, R., Mann, J.A., Greig, A., Chapman, W.G., Goodridge, D., 1971. The growth and persistence of foot-and-mouth disease virus in the bovine mammary gland. Journal of Hygiene 69, 307–321.

Bushnell, R.B., 1984. Mycoplasma mastitis. Veterinary Clinics of North America Large Animal Practice 6(2), 301-312.

Butler, J.A., Sickles, S.A., Johanns, C.J., Rosenbusch, R.F., 2000. Pasteurization of discard mycoplasma mastitic milk used to feed calves: Thermal effects on various mycoplasma. Journal of Dairy Science 83, 2285–2288.

Callaway, T.R., Edrington, T.S., Anderson, R.C., Harvey, R.B., Genovese, K.J., Kennedy, C.N., Venn, D.W., Nisbet, D.J., 2008. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. Animal Health Research Reviews 9(2), 217–225. doi:10.1017/S1466252308001540

Campbell, M.H., Miller, J.K., 1998. Effect of supplemental dietary vitamin E and zinc on reproductive performance of dairy cows and heifers fed excess iron. Journal of Dairy Science 81, 2693–2699.

Carrington, C., du Plessis, A., Naidoo, V. (eds.), 2016, Index of Veterinary Specialities (IVS), October-December 2016, MIMS, Saxonwold.

Chan, B., 2019. Phage therapy killing antibiotic resistant bacteria with viruses. Special to ACSH. https://www.acsh.org/news/2019/02/08/phage-therapy-killing-antibiotic-resistant-bacteria-viruses-13799, last accessed 15 January 2020.

Cobo-Ángel, C, Jaramillo-Jaramillo, A.S., Lasso-Rojas, L.M., Aguilar-Marin, S.B., Sanchez, J., Rodriguez-Lecompte, J.C., Ceballos-Márquez, A., Zadoks, R.N., 2018. *Streptococcus agalactiae* is not always an obligate intramammary pathogen: Molecular epidemiology of GBS from milk, feces and environment in Colombian dairy herds. Plos One 13(12), e0208990. doi:10.1371/journal.pone.020899

Condas, L.A.Z., De Buck, J., Nobrega, D.B., Carson, D.A., Naushad, S., De Vliegher, S., Zadoks, R.N., Middleton, J.R., Dufour, S., Kastelic, J.P., Barkema, H.W., 2017. Prevalence of non-aureus staphylococci species causing intramammary infections in Canadian dairy herds. Journal of Dairy Science 100(7), 5592-5612. doi:10.3168/jds.2016-12478

Corbellini, L., Driemeier, D., Cruz, C., Diaz, M.M., Frerreiro, L., 2001. Bovine Mastitis due to *Prototheca zopfii*: Clinical, Epidemiological and Pathological Aspects in a Brazilian Dairy Herd. Tropical Animal Health and Production 33, 463–470. doi:10.1023/A:1012724412085

Cox, H.U., Hoskins, J.D., Newman, S.S., Foil, C.S., Turnwald, G.H., Roy, A.F., 1988. Temporal study of staphylococcal species on healthy dogs. American Journal of Veterinary Research 49, 747–751.

Cox, H.U., Hoskins, J.D., Newman, S.S., Turnwald, G.H., Foil, C.S., Roy, A.F., Kearney, M.T., 1985. Distribution of staphylococcal species on clinically healthy cats. American Journal of Veterinary Research 46, 1824–1828.

Cox, G., Wright, G.D., 2013. Intrinsic antibiotics resistance: mechanisms, origins, challenges and solutions. International Journal of Medical Microbiology 303(6-7), 287-92. doi:10.1016/j.ijmm.2013.02.009

Crompton, C.W.R., Heuer, C., Parker, K., Mcdougall, S., 2007. Risk Factors for Peripartum Mastitis in Pasture-Grazed Dairy Heifers. Journal of Dairy Science 90(9), 4171-4180.

Cuny, C., Kuemmerle, J., Stanek, C., Willey, B., Strommenger, B., Witte, W., 2006. Emergence of MRSA infections in horses in a veterinary hospital: strain characterisation and comparison with MRSA from humans. Euro Surveillance 11(1), 595.

Da Silva Meira, Q.G., De Medeiros Barbosa, I., Alves Aguiar Athayde, A.J., Siqueira-Júnior, J.P., De Souza, E.L., 2012. Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food-contact surfaces and sensitivity to sanitizers. Food Control 25(2), 469-475.

David, M.Z., Daum, R.S., 2010. Community-Associated Methicillin-Resistant *Staphylococcus aureus*: Epidemiology and Clinical Consequences of an Emerging Epidemic. Clinical Microbiology Reviews 23(3), 616–687.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews 74(3), 417-433.

DeGraves, F.J., Fetrow, J., 1993. Economics of mastitis and mastitis control. Veterinary Clinical Journal of North America: Food Animal Practice 9(3), 421–434. http://dx.doi.org/10.1016/s0749-0720(15)30611-3

Departments of Health and Department of Agriculture, Forestry and Fisheries (DAFF) for the Republic of South Africa, 2018. Antimicrobial Resistance National Strategy Framework 2018 – 2024.

De Pinho, M., Nóbrega, D.B., Facciolo, P.Y., Troncarelli, M.Z., Menozzi, B.D., Langoni, H., 2012. Relationship between teat-end condition, udder cleanliness and bovine subclinical mastitis. Research in Veterinary Science 93(1), 430-434.

De Visscher, A., Supré, K., Haesebrouck, F., Zadoks, R.N., Piessens, V., Van Coillie, E., Piepers, S., De Vliegher, S., 2014. Further evidence for the existence of environmental and host-associated species of coagulase-negative staphylococci in dairy cattle. Veterinary Microbiology 27:172(3-4), 466-474. doi:10.1016/j.vetmic.2014.06.011

De Visscher, A., Piepers, S., Supré, K., Haesebrouck, F., De Vliegher, S., 2015. Short communication: Species group-specific predictors at the cow and quarter level for intramammary infection with coagulase-negative staphylococci in dairy cattle throughout lactation. Journal of Dairy Science 98(8), 5448-53. doi:10.3168/jds.2014-9088

De Visscher, A., Piepers, S., Haesebrouck, F., De Vliegher, S., 2016. Intramammary infection with coagulase-negative staphylococci at parturition: Species-specific prevalence, risk factors, and effect on udder health. Journal of Dairy Science 99(8), 6457-69. doi:10.3168/jds.2015-10458.

Devriese, L.A., Hermans, K., Baele, M., Haesebrouck, F., 2009. Staphylococcus pseudintermedius versus Staphylococcus intermedius. Veterinary Microbiology 133, 206-207.

Devriese, L.A., Hommez, J., 1975. Epidemiology of methicillin resistant *Staphylococcus aureus* in dairy herds. Research in Veterinary Science 19, 23-27.

Devriese, L., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere, A., Haesebrouck, F., 2005. *Staphylococcus pseudintermedius sp.* nov., a coagulase-positive species from animals. International Journal of Systematic and Evolutionary Microbiology 55, 1569–1573.

Dingwell, R.T. Leslie, K.E., Sabour, P., 2006. Influence of the genotype of *Staphylococcus aureus*, determined by pulsed-field gel electrophoresis, on dry-period elimination of subclinical mastitis in Canadian dairy herds. Canadian Journal of Veterinary Research 70, 115-120.

Dodd, F.H., Neave, F.K., 1970. Mastitis control. National Institute for Research in Dairying-Biennial Reviews 3559, 21-60.

Dodd, F. H., 1983. Mastitis - Progress on control. Journal of Dairy Science 66, 1773.

Dohoo, I.R., Martin, S.W., Meek, A.H., 1984. Disease, production and culling in Holstein-Friesian cows. VI. Effects of management on disease rates. Preventive Veterinary Medicine 3, 15-28.

Donkin, E.F. 1981. Choosing a milking parlour. Zimbabwe Agricultural Journal 78(2), 41-48.

Douphrate, D.I., Nonnenmann, C.I.H., Rosecrance, C.P.E., 2009. Ergonomics in Industrialised Dairy Operations. Journal of Agromedicine 14(4), 406-412.

Drackley, J.K., 1999. Biology of dairy cows during the transition period: the final frontier. Journal of Dairy Science 82, 2259–2273.

Dufour, S., Fréchette, A., Barkema, H.W., Mussell, A., Scholl, D.T., 2011. Invited review: Effect of udder health management practices on herd somatic cell count. Journal of Dairy Science 94(2), 563-579. https://doi.org/10.3168/jds.2010-3715

Eberhart, R. J., Buckalew, J.M., 1972. Evaluation of a hygiene and dry period therapy program for mastitis control. Journal of Dairy Science 55, 1683.

Eberhart, R. J., 1975. Control of coliform mastitis. Seminar on Mastitis Control. International Dairy Federation Bulletin Doc 85, 371.

Eberhart, R. J., 1977. Coliform mastitis. Journal of the American Veterinary Medical Association 170, 1160.

Eberhart, R.J., Natzke, R.P., Newbould, F.H.S., Nonnecke, B., Thompson, P.D., 1979. Coliform mastitis - A review. Journal of Dairy Science 62, 1.

Elliott, R.E.W., Tattersfield, J.G., Brookbanks, E.O., 1975. New Zealand National Mastitis survey: 1965-1966. New Zealand Veterinary Journal 24, 80-84.

Emanuele, P., 2010. Antibiotic resistance. American Association of Occupational Health Nurses Journal 58(9).

Enevoldsen, C., Hindhede, J., Kristensen, T., 1996. Dairy herd management types assessed from indicators of health, reproduction, replacement, and milk production. Journal of Dairy Science 79, 1221–1236.

Erasmus, L.J., Robinson, P.H., Ahmadi, A., Hinders, R., Garrett, J.E., 2005. Influence of prepartum and postpartum supplementation of a yeast culture and monensin, or both, or ruminal fermentation and performance of multiparous dairy cows. Animal Feed Science and Technology 122, 219-239.

Erskine, R. J., Eberhart, R.J., Hutchinson, L.J., Spencer, S.B., Campbell, M.A., 1987. Herd management and prevalence of mastitis in dairy herds with high and low somatic cell counts. Journal of the American Veterinary and Medical Association 190, 1411–1416.

Essack, S.Y., 2018. Antibiotic resistance and One Health: a mapping project. The Lancet 6(Special Issue), S27. doi:10.1016/S2214-109X(18)30156-6

European Commission, 2005. Ban on antibiotics as growth promoters in animal feed enters into effect. IP/05/1687 /22/12/2005. European Parliament, Brussels, Belgium.

European Commission, 2009. Green Facts: Effect of biocides on antibiotic resistance. Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), European Parliament, Brussels, Belgium. http://copublications.greenfacts.org/ and at: http://ec.europa.eu/health/ph_risk/popularizing/popularizing_results_en.htm

Faye, B., 1991. Interrelationships between health status and farm management system in French dairy herds. Preventive Veterinary Medicine 12, 133–152.

Faye, B., Lescourret, F., Dorr, N., Tillard, E., MacDermott, B., McDermott, J., 1997. Interrelationships between herd management practices and udder health status using canonical correspondence analysis. Preventive Veterinary Medicine 32, 171–192.

Fessler, A., Scott, C., Kadlec, K., Ehricht, R., Monecke, S., Schwarz, S., 2010. Characterization of methicillin-resistant *Staphylococcus aureus* ST398 from cases of bovine mastitis. Journal of Antimicrobial Chemotherapy 65(4), 619-625.

Field, D., Gaudin, N., Lyons, F., O'Connor, P.M., Cotter, P.D., Hill, C., Ross, R.P., 2015. A bioengineered nisin derivative to control biofilms of *Staphylococcus pseudintermedius*. Plos One. 10(3), e0119684. doi:10.1371/journal.pone.0119684

Fleming, A., 1929. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. Bulletin of the World Health Organization 79(8), 780-90.

Fox, L.K., Gay, J.M., 1993. Update on Bovine Mastitis: Contagious mastitis. Veterinary Clinics of North America: Food Animal Practice 9(3), 475-487.

Francoz, D., Fecteau, G., Desrochers, A., Fortin, M., 2004. Otitis media in dairy calves: A retrospective study of 15 cases (1987 to 2002). Canadian Veterinary Journal 45, 661–666.

Francoz, D., Wellemans, V., Duprè, J.P., Roy, J.P., Labelle, F., Lacasse, P., Dufour, S., 2017. Invited review: A systematic review and qualitative analysis of treatments other than conventional antimicrobials for clinical mastitis in dairy cows. Journal of Dairy Science 100(10), 7751-7770. doi:10.3168/jds.2016-12512

Fuchs, H.-W., 1994. Mastitiden: Virusinfectionen. In: Wendt, K., Bostedt, H., Mielke, H., Fuchs, H.-W. (Eds.), Euter- und Gesa¨ugekrankheiten. Gustav Fisher Verlag Jena (Stuttgart) pp, 422–425.

Fuller, R., 1989. Probiotics in man and animals. Journal of Applied Bacteriology 66, 365-378.

Gagea, M.I., Bateman, K.G., Shanahan, R.A., van Dreumel, T., McEwen, B.J., Carman, S., Archambault, M., Caswell, J.L., 2006. Naturally occurring Mycoplasma bovis-associated pneumonia and polyarthritis in feedlot beef calves. Journal of Veterinary Diagnostic Investigations 18, 29-40.

Geary, U., Lopez-Villalobos, N., Begley, N., McCoy, F., O'Brien, B., O'Grady, L., Shalloo, L., 2012. Estimating the effect of mastitis on the profitability of Irish dairy farms. Journal Dairy Science 95, 3662-3673.

García-Álvarez, L., Holden, M.T.G., Lindsay, H., Webb, C.R., Brown, D.F.J., Curran, M.D., Walpole, E., Brooks, K., Pickard, D.J., Teale, C., Parkhill, J., Bentley, S.D., Edwards, G.F.,

Girvan, E.K., Kearns, A.M., Pichon, B., Hill, R.L.R, Larsen, A.R., Skov, R.L., Peacock, S.J., Maskell, D.J., Holmes, M.A., 2011. Methicillin-resistant *Staphylococcus aureus* with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infectious Diseases 11, 595-603.

Gerber, P., Opio, C., Steinfeld, H., 2007. Poultry production and the environment- a review. Food and Agriculture Organization of the United Nations, FAO Press, from, http://www.fao.org/ag/againfo/home/events/bangkok2007/docs/part2/2_2.pdf

Gill, R., Howard, W.H., Leslie, K.E., Lissemore, K., 1990. Our industry today: Economics of mastitis control. Journal of Dairy Science 73, 3340-3348.

Gillor, O., Nigro, L.M., Riley, M.A., 2005. Genetically Engineered Bacteriocins and their Potential as the Next Generation of Antimicrobials. Current Pharmaceutical Design 11, 1067-1075.

Giraudo, J.A., Calzolari, A., Rampone, H., Rampone, A., Giraudo, A.T., Bogni, C., Larriestra, A., Nagel, R., 1997. Field trials of a vaccine against bovine mastitis. 1. Evaluation in heifers. Journal of Dairy Science 80(5), 845-853.

Goldberg, J.J., Pankey, J.W., Drechsler, P.A., Murdough, A.P., Diantha, H., 1991. An update survey of bulk tank quality in Vermont. Journal of Food Protection 54, 549-553.

Gonzalez, R.N., Sears, P.M., Merrill, R.A., Hayes, G.L., 1992a. Mastitis due to mycoplasma in the state of New York during the period 1972–1990. Cornell Veterinary Journal 82, 29-40.

Gonzalez, R.N., Merril, R.A., Sears, P.M., 1992b., Shedding of *Mycoplasma bovis* from the udder of naturally infected cows and its importance for the diagnosis of bovine intramammary infections. Journal of Dairy Science 75(suppl 1), 259.

Goodger, W. J., Farver, T., Pelletier, J., Johnson, P., de Snayer, G., Galland, J., 1993. The association of milking management practices with bulk tank somatic cell counts. Preventive Veterinary Medicine 15, 235-251.

Gourlay, R.N., Stott, E.J., Espinasse, J., Barle, C., 1974. Isolation of *Mycoplasma agalactiae* var. bovis and infectious bovine rhinotracheitis virus from an outbreak of mastitis in France. Veterinary Records 95, 534–535.

Grindal, R.J., 1988. Update on Mastitis: The role of the milking machine in mastitis. British Veterinary Journal 144, 524.

Gualerzi, C.O., Brandi, L., Fabbretti, A., Pon, C.L., (Edited by)., 2014. Antibiotics: Targets, Mechanisms and Resistance. Wiley publishers, New Jersey, USA. Print ISBN: 9783527333059 |Online ISBN: 9783527659685 |DOI:10.1002/9783527659685

Hajek, V., 1976. *Staphylococcus intermedius*, a new species isolated from animals. International Journal of Systemic Bacteriology 26, 401-408.

Hammami, H., Bormann, J., M'hamdi, N., Montaldo, H.H., Gengler, N., 2013. Evaluation of heat stress effects on production traits and somatic cell score of Holsteins in a temperate environment. Journal of Dairy Science 96(3), 1844-1855.

Hamman, J., 1991. Milking hygiene, milking and mastitis. Dairy, Food and Environmental Sanitation 11(5), 260-204.

Harmon, R.J., 1994. Physiology of mastitis and factors affecting somatic cell counts. Journal of Dairy Science 77(7), 2103–2112. https://doi.org/10.3168/jds.S0022- 0302(94)77153-8

Harmon, R.J., 2001. Somatic cell counts: A primer. 40th Annual meeting, National Mastitis Council, Reno, NV, USA, February 11, 2001, pp. 1–9, viewed 14 January 2015, from http://nmconline.omnibooksonline.com/55008-2-nmc-2001- 1.29290/t-002-1.29685/a-002-1.29714/a-002-1.29715/ap-002-1.961344?qr=1

Henton, M.M., Eagar, H.A., Swan, G.E., van Vuuren, M., 2011. Part VI. Antibiotic management and resistance in livestock production. The South African Medical Journal 101(8).

Heriazon, A., Thompson, K.A., Wilkie, B.N., Mathes-Sears, W., Quinton, M., Mallard, B.A., 2009. Antibody to ovalbumin and delayed-type hypersensitivity to *Candida albicans* and mycobacteria in lactating Holstein cows using Quil A or Freund's complete adjuvant. Veterinary Immunology and Immunopathology 127, 220-227.

Hillerton, J.E., 1999. Redefining mastitis based on somatic cell count. Bulletin of the International Dairy Federation 345, 4-6.

Holmes, M.A., Zadoks, R.N., 2011. Methicillin resistant *Staphylococcus aureus* in human and bovine mastitis. Journal of Mammary Gland Biology and Neoplasia 16(4), 373-82. doi:10.1007/s10911-011-9237-x.

Hutton, C. T., Fox, L.K., Hancock, D.D., 1990. Mastitis control practices: differences between herds with high and low milk SCC. Journal of Dairy Science 73, 1135-1143.

International Dairy Federation, 2017. Annual Report 2016-2017. Brussels, Belgium. International Dairy Federation Press, 2017. https://www.fil-idf.org/wp content/uploads/2017/11/IDF-Annual-Report-2017-low-res-30-nov-2017.pdf

Jasper, D.E., 1981. Bovine Mycoplasmal mastitis. Advances in Veterinary Science and Comparative Medicine 25, 121-159.

Jasper, D.E., 1987. Bovine mastitis due to mycoplasma. Scientific and Technical Review of the Office International des Epizooties (Paris) 6(3), 801-807.

Jevons, M.P., 1961. Celbenin-resistant staphylococci. British Medical Journal 1, 124-125.

Juhász-Kaszanyitzky, E., Jánosi, S., Somogyi, P., Dán, A., van der Graaf-van Bloois, L., van Duijkeren, E., Wagenaar, J.A., 2007. MRSA transmission between cows and humans. Emerging Infectious Diseases 13(4), 630-632.

Kaszanyitzky, E.J., Egyed, Z., Janosi, S., Keseru, J., Gal, Z., Szabo, I., Veres, Z., Somogyi, P., 2004. Staphylococci isolated from animals and food with phenotypically reduced susceptibility to beta-lactamase-resistant beta-lactam antibiotics. ACTA Veterinaria Hungarica (Budapest) 52(1), 7-17.

Kawakami, Y., Kaji, T., Omuro, M., Maruyama, Y., Hiramune, T., Murase, N., Matumoto, M., 1966. Infection of cattle with parainfluenza 3 virus with special reference to udder infection. II. Pathology of the virus to cattle, with particular reference to the mammary gland. Japanese Journal of Microbiology 10, 171–182.

Kiernan, N.E., Heinrichs, A.H., 1994. Identification of farm manager types through cluster analysis of calf and heifer management practices. Preventive Veterinary Medicine 18, 225–236.

King, J. S., 1981. Streptococcus uberis: A review of its role as a causative organism of bovine mastitis. II. Control of infection. British Veterinary Journal 137, 160.

Kingwill, R.G., Neave, F.K., Dodd, F.H., Griffin, T.K., Westgarth, D.R., Wilson, C.D., 1970. The effect of a mastitis control system on levels of subclinical and clinical mastitis in two years. Veterinary Record 87, 94-100.

Kwon, N., Park, K., Jung, W., Youn, H., Lee, Y., Kim, S., Bae, W., Lim, J., Kim, J., Kim, J., Hong, S., Park, Y., 2006. Characteristics of methicillin resistant Staphylococcus aureus isolated from chicken meat and hospitalized dogs in Korea and their epidemiological relatedness. Veterinary Microbiology 117, 304–312.

Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiology Reviews 12, 39-86.

Kluytmans, J.A.J.W., 2010. Methicillin-resistant *Staphylococcus aureus* in food products: cause for concern or case for complacency? Clinical Microbiology and Infection 16(1), 11-15.

Koch, J., 2013. Cutting Antibiotics: Denmark leads Way in Healthier Pig Farming. Spiegel Online International. https://www.spiegel.de/international/europe/danish-pig-farmers-reduce-antibiotics-to-prevent-drug-resistance-a-933344.html (accessed 8 July 2019).

Lactodata, 16(2) November 2013, accessed 07 June 2017, from, www.milksa.co.za/category/all-reports/industry-information

Lactodata, 17(1), May 2014, accessed 07 June 2017, from, www.milksa.co.za/category/all-reports/industry-information

Lamm, C.G., Munson, L., Thurmond, M.C., Barr, B.C., George, L.W., 2004. Mycoplasma otitis in California calves. Journal of Veterinary Diagnostic Investigation 16, 397-402.

Leonard, F.C., Abbott, Y., Rossney, A., Quinn, P.J., O. Mahony, R., Markey, B.K., 2006. Methicillin-resistant *Staphylococcus aureus* isolated from a veterinary surgeon and five dogs in one practice. Veterinary Records 158(5), 155-159.

Leonard, F.C., Markey, B.K., 2008. Meticillin-resistant *Staphylococcus aureus* in animals: a review. Veterinary Journal 175(1), 27-36.

Leslie, K.E., Schukken, Y.H., 1999. Herd programs for eliminating and preventing *Staphylococcus aureus* mastitis. 32nd Annual meeting national mastitis council, INC, Part 2, 1-10.

Leuenberger, A., Sartori, C., Boss, R., Resch, G., Oechslin, F., Steiner, A., Moreillon, P., Graber, H.U., 2019. Genotypes of *Staphylococcus aureus*: On-farm epidemiology and the consequences for prevention of intramammary infections. Journal of Dairy Science. 102(4), 3295-3309. doi:10.3168/jds.2018-15181

Levy, S.B., 2002. Factors impacting on the problem of antibiotic resistance. Journal of Antimicrobial Chemotherapy 49, 25-30.

Lewis, K., 2013. Platforms for antibiotic discovery. Nature Reviews, Drug Discovery 12, 371-387. doi:10.1038/nrd3975

Ligon, B.L., 2004. Penicillin: its discovery and early development. Seminars in paediatric infectious diseases 15(1), 52-7.

Mainda, G., Bessell, P.R., Muma, J.B., McAteer, S.P., ChaseTopping, M.E., Gibbons, J., Stevens, M. P., Gally, D.L., Bronsvoort, B. M. deC., 2015. Prevalence and patterns of antimicrobial resistance among Escherichia coli isolated from Zambian dairy cattle across different production systems. Scientific Reports 5, 12439. doi:10.1038/srep12439

Mallard, B.A., Wilkie, B.N., Kennedy, B.W., Quinton, M., 1992. Use of estimated breeding values in a selection index to breed Yorkshire pigs for high and low immune and innate resistance factors. Animal Biotechnology 3, 257-280.

Mamo, W., Jonsson, P., Flock, J.I., Lindberg, M., Müller, H.P., Wadström, T., Nelson, L., 1994. Vaccination against *Staphylococcus aureus* mastitis: immunological response of mice vaccinated with fibronectin-binding protein (FnBP-A) to challenge with *S. aureus.* Vaccine 12(11), 988-992.

Marais, E., Aithma, N., Perovic, O., Oosthuysen, W., Musenge, E., Duse, A., 2009. Antimicrobial Susceptibility of Methicillin-Resistant *Staphylococcus aureus* isolates from South Africa. South African Medical Journal 99, 170-173.

Maron, D.F., Smith, T.J.S., Nachman, K.E., 2013. Restrictions on antimicrobial use in food animal production: an international regulatory economic survey. Globalization and Health 9, 48.

Martin, S.W., Bateman, K.G., Shewen, P.E., Rosendal, S., Bohac, J.G., Thorburn, M., 1990. A group level analysis of the associations between antibodies to seven putative pathogens and respiratory disease and weight gain in Ontario feedlot calves. Canadian Journal of Veterinary Research 54, 337-342.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D., 2011. *Mycoplasma bovis* Infections in Cattle, ACVIM Consensus statement. Journal of Veterinary Internal Medicine 25, 772–783.

McConnel, C.S., McNeil, A.A., Hadrich, J.C., Lombard, J.E., Heller, J, Franklyn, B.G., 2018. A comparison of a novel time-based summary measure of dairy cow health against cumulative disease frequency. Irish Veterinary Journal 71, 7(2018). doi:10.1186/s13620-018-0119-z

McDougall, S., Agnew, K.E., Cursons, R., Hou, X.X., Compton, C.R.W., 2007a. Parenteral treatment of clinical mastitis with tylosin base or penethamate hydriodide in dairy cattle. Journal of Dairy Science 90, 779-789.

McDougall, S., Arthur, D.G., Bryan, M.A., Vermunt, J.J., Weir, A.M., 2007b. Clinical and bacteriological response to treatment of clinical mastitis with one of three intramammary antibiotics. New Zealand Veterinary Journal 55, 161-170.

Mellenberger, R., Kirk, J., 2001. Mastitis control program for *Staphylococcus aureus* infected dairy cows, Department of Animal studies, Michigan State University and Veterinary medicine

Extension, School of Veterinary Medicine, University of California Davis, from, milkquality.wisc.edu/wp-content/uploads/2011/09/mastitis-controlprogram_staph.-aureus.pdf

Melo, P.C., Sousa, C., Botelho, C., Oliveira R., Nader-Filho, A., 2014. NaOCl effect on *Staphylococcus aureus* biofilm isolated from the milking environment and mastitis infected cows. Pesqisa Veterinaria Brasileira 34(2), 109-113.

Meyer, E., Gastmeier, P., Deja, M., Schwab, F., 2013. Antibiotic consumption and resistance: data from Europe and Germany. International Journal of Medical Microbiology 303(6-7), 388-395.

Middleton, J.R., Luby, C.D., Adams, D.S., 2009. Efficacy of vaccination against staphylococcal mastitis: a review and new data. Veterinary microbiology 134(1-2), 192-198.

Modi, C.M., Patel Hitesh, B., Patel, H.B., Mody, S.K., 2012. A comprehensive review on pharmacotherapeutics of bovine mastitis. Molecular Microbiology Research 2(1), 1-9.

Mørk, T., Tollersrud, T., Kvitle, B., Jørgensen, H.J., Waage, S., 2005. Comparison of *Staphylococcus aureus* genotypes recovered from cases of bovine, ovine, and caprine mastitis. Journal of Clinical Microbiology 43(8), 3979-84. doi:10.1128/JCM.43.8.3979-3984

Motaung, T.E., Petrovski, K.R., Petzer, I.M., Thekisoe, O. & Tsilo, T.J., 2017. Importance of bovine mastitis in Africa. Animal Health Research Reviews 18, 58–69. https://doi.org/10.1017/S1466252317000032

Moxley, J.E., Kennedy, B.E., Downey, B.R., Bowman, J.S.T., 1978. Survey of milking hygiene practices and their relationships to somatic cell counts and milk production. Journal of Dairy Science 61, 1637-1644.

Muylkens, B., Thiry, J., Kirten, P., Schynts F., Thiry, E., 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. Veterinary Research 38, 181-209. doi:10.1051/vetres: 2006059

NAHMS. 2003. Part III: Reference of Dairy Cattle Health and Health Management Practices in the United States, 2002, Ft. Collins, CO: U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, National Animal Health Monitoring System.

National Department of Health, 2018. Surveillance resistance and consumption of antibiotics in South Africa.

Natzke, R. P., 1981. Elements of mastitis control. Journal of Dairy Science 64, 1431.

Neave, F.K., Dodd F.H., Kingwill, R.G., Westgarth, D.R., 1969. Control of Mastitis in the dairy herd by hygiene and management. Journal of Dairy Science 52(5), 696-707.

Nicholas, R.A., Ayling, R.D., Stipkovits, L.P., 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis*: Clinical, cultural, serological and pathological findings. Vaccine 20, 3569-3575.

Nickerson, S.C., Owens, W.E., Boddie, R.L., 1995. Symposium: Mastitis in Dairy Heifers: Initial Studies on Prevalence and Control. Journal of Dairy Science 78, 1607-1618.

Nyman, A.K., Ekman, T., Emanuelson, U., Gustafsson, A.H., Holtenius, K., Waller, K.P., Sandgren, C.H., 2007. Risk factors associated with the incidence of veterinary-treated clinical mastitis in Swedish dairy herds with a high milk yield and a low prevalence of subclinical mastitis. Preventive Veterinary Medicine 78, 142-160.

O'Flaherty, S., Ross, R.P., Meaney, W., Fitzgerald, G.F., Elbreki, M.F., Coffey, A., 2005. Potential of the polyvalent anti-Staphylococcus bacteriophage K for control of antibiotic-resistant staphylococci from hospitals. Applied Environmental Microbiology 71(4), 1836-1842.

OIE. 2007. OIE list of antimicrobials of veterinary importance. The OIE International Committee unanimously adopted the List of Antimicrobials of Veterinary Importance at its 75th General Session in May 2007 (Resolution No. XXVIII), https://www.oie.int/doc/ged/D9840.PDF, (accessed 2 July 2019).

OIE. 2016. Annual report on the use of antimicrobial agents in animals; Better understanding of the global situation, December 2016.

OIE. 2017. Annual report on the use of antimicrobial agents in animals; Better understanding of the global situation, Second report, December 2017.

Olde Riekerink, R.G.M., Barkema, H.W., Kelton, D.F., Scholl, D.T., 2008. Incidence rate of clinical mastitis on Canadian dairy farms. Journal of Dairy Science 91, 1366–1377.

Olive, D.M., Bean, P., 1999. Principles and applications of methods for DNA-based typing of microbial organisms. Journal of Clinical Microbiology 37(6), 1661-1669.

Oliver, S.P., Calvinho, L. F., 1995. Influence of inflammation on mammary gland metabolism and milk composition. Journal of Animal Science 73(Suppl. 2), 18–33.

Pang, M., Sun, L., He, T., Bao, H., Zhang, L., Zhou, Y., Zhang, H., Wei, R., Liu, Y., Wang, R., 2017. Molecular and virulence characterization of highly prevalent *Streptococcus agalactiae* circulated in bovine dairy herds. Veterinary Research 48(1), 65. doi:10.1186/s13567-017-0461-2

Pankey, G.A., Sabath, L.D., 2004. Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram Positive Bacterial Infections. Clinical Infectious Diseases 38(6), 864–870. doi.org/10.1086/381972

Park, J.Y., Fox, L.K., Seo, K.S., McGuire, M.A., Park, Y.H., Rurangirwa, F.R., Sischo, W.M., Bohach, G.A., 2011. Comparison of phenotypic and genotypic methods for the species identification of coagulase-negative staphylococcal isolates from bovine intramammary infections. Veterinary Microbiology 147, 142–148.

Parul, S., Basak, G., Sharma, B., Jain, U., Mishra, R., Vaishali., 2019. Biofilm: An alarming niche in dairy industry. International Journal of Livestock Research 9(4), 2277-1964. doi:10.5455/ijlr.20190207061546

Pavillard, R., Harvey, K., Douglas, D., Hewstone, A., Andrew, J., Collopy, B., Asche, V., Carson, P., Davidson, A., Gilbert, G., Spicer, J., Tosolini, F.,1982. Epidemic of hospital-acquired infection due to methicillin-resistant *Staphylococcus aureus* in major Victorian hospitals. The Medical Journal of Australia 1(11), 451-454.

Perovic, O., Koornhof, H., Black, V., Moodley, I., Duse, A., Galpin, J., 2006. *Staphylococcus aureus* bacteraemia at two academic hospitals in Johannesburg. South African Medical Journal 96(8), 714-717.

Petersen, A., Stegger, M., Heltberg, O., Christensen, J., Zeuthen, A., Knudsen, L.K., Urth, T., Sorum, M., Schouls, L., Larsen, J., Skov, R., and Larsen, A.R., 2013. Epidemiology of methicillin-resistant *Staphylococcus aureus* carrying the novel mecC gene in Denmark corroborates a zoonotic reservoir with transmission to humans. Clinical Microbiological Infections 19, E16-E22.

Petzl, W., Zerbe, H., Günther, J., Yang, W., Seyfert, H.M., Nürnberg, G., Schuberth, H.J., 2008. Escherichia coli, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defence in the udder of the cow. Veterinary Research 571(39), 18.

Petzer, I.M. 2018. Pro-active dairy herd udder health management decisions based on microbiology and cytology of milk samples. PHD Thesis, University of Pretoria.

Petzer, I.M., Karzis, J., Watermeyer, J.C., van der Schans, T.J., Badenhorst, R., 2009. Trends in udder health and emerging mastitogenic pathogens in South African Dairy Herds. Journal of South African Veterinary Association 80(1), 17-22.

Petzer, I.M., Karzis, J., Watermeyer, J.C., Badenhorts, R., van der Schans, T.J., 2012. Trends of mastitogenic bacteria from 2008 to 2012 in South African dairy herds. International Dairy Federation, World Dairy Summit and exhibition, Cape Town, South Africa, IDF Press.

Petzer, I.M., Karzis, J., Donkin, E.F., Webb, E.C., 2016. A pathogen-specific approach towards udder health management in dairy herds: Using culture and somatic cell counts from routine herd investigations. Onderstepoort Journal of Veterinary Research 83(1), a1146. doi:10.4102/ojvr.v83i1.1146

Petzer, I-M., Karzis, J., Donkin, E.F., Webb, E.C., Etter, E.M.C., 2017. Somatic cell count thresholds in composite and quarter milk samples as indicator of bovine intramammary infection status. Onderstepoort Journal of Veterinary Research 84(1), a1269. https://doi.org/10.4102/ojvr.v84i1.1269

Petzer, I.M., Swan, A.C., 2018. The importance of teat liners in preserving teat integrity and udder health. RUVASA, Birchwood Conference centre, 18-20 June 2018.

Petzer, I.M., Karzis, J., van der Schans, T.J., Watermeyer, J.C., Smith, M., 2007. Antibiotic efficacy against staphylococcal udder pathogens in dairy cows in South Africa from 2000 to 2006. Livestock Health and Production Group of the South African Veterinary Association Congress, 20 - 23 June 2007. Pretoria: Vetlink, 33-49.

Piessens, V., Supré, K., Heyndrickx, M., Haesebrouck, F., De Vliegher, S., Van Coillie, E., 2010. Validation of amplified fragment length polymorphism genotyping for species identification of bovine associated coagulase-negative staphylococci. Journal of Microbiology Methods 80, 287-294.

Piessens, V., Van Coillie, E., Verbist, B., Supré, K., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx, M., De Vliegher, S., 2011. Distribution of coagulase-negative Staphylococcus species from milk and environment of dairy cows differs between herds. Journal of Dairy Science 94(6), 2933-44. doi:10.3168/jds.2010-3956

Pieterse, R., Todorov, S.D., 2010. Bacteriocins - exploring alternatives to antibiotics in mastitis treatment. Brazilian Journal of Microbiology 41(3), 542-562. doi:10.1590/S1517-83822010000300003

Pilla, R., Bonura, C., Malvisi, M., Snel, G.G., Piccinini, R., 2013. Methicillin-resistant *Staphylococcus pseudintermedius* as causative agent of dairy cow mastitis. Veterinary Record 173(1), 19. doi:10.1136/vr.101485

Price, L.B., Stegger, M., Hasman, H., Maliha, A., Larsen, J., Andersen, S., Pearson, T., Waters, A.E., Foster, J.T., Schupp, J., Batisti, A., Franco, A., Zmudzki, J., Schwarz, S., Butaye, P., Jouy, E., Pomba, C., Concepción Porrero, M., Ruimy, R., Smith, T.C., Robinson, D.A., Weese, J.S., Arriola, C.S., Yu, F., Laurent, F., Keim, P., Skov, R., Aarestrup, F.M., 2013. *Staphylococcus aureus* CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock mBio 3(1), 305-311.

Rainard, P., Riollet, C., 2006. Innate immunity of the bovine mammary gland. Veterinary Research 37(3), 369-400.

Rainard, P., Foucras, G., Fitzgerald, J.R., Watts, J.L., Koop, G., Middleton, J.R., 2017. Knowledge gaps and research priorities in *Staphylococcus aureus* mastitis control.

Special Issue: Supplement: Current research gaps for advancing control of infectious diseases in production animals. Transboundary and emerging diseases 65(S1), 149-165. doi:10.1111/tbed.12698

Rainard, P., Foucras, G.A., 2018. Critical Appraisal of Probiotics for Mastitis Control. Frontiers in Veterinary Science 5, 251. doi:10.3389/fvets.2018.00251

Rajala-Schultz, P.J., Holland, S., Walker, J.B., DeGraves, F.J., Gebreyes, W.A., Mathews, J., Hogan, J.S., da Costa, L., 2010. Mastitis Research into Practice: Proceedings of the 5th IDF Mastitis Conference, 21-24 March 2010, Christchurch, New Zealand, edited by J.E. Hillerton, Vet Learn, New Zealand.

Reinemann, D., 2005. University of Wisconsin Milking Research Institute Laboratory. Personal communication on December 16.

Reyes, J., Chaffer, M., Rodriguez-Lecompte, J.C., Sánchez, J., Zadocks, R.N., Robinson, N., Cardona, X., Ramirez, N., Keefe, G.P., 2017. Short communication: Molecular epidemiology of *Streptococcus agalactiae* differs between countries. Journal of Dairy Science 100(11), 9294-9297. https://doi.org/10.3168/jds.2017-13363

Sandholm, M., Honkanen-Buzalski, T., Kaartinen, L., Pyörälä, S., 1995. The bovine udder and mastitis, University of Helsinki Faculty of Veterinary medicine, Helsinki.

Ryan, M.P., Meaney, W.J., Ross, R.P., Hill, C., 1998. Evaluation of Lacticin 3147 and a Teat Seal Containing This Bacteriocin for Inhibition of Mastitis Pathogens. Applied Environmental Microbiology 64, 2287-2290.

Sable, S., Pons A.M., Gendron-Gaillard, S., Cottenceau, G., 2000. Antibacterial activity evaluation of microcin J25 against diarrheagenic *Escherichia coli*. Applied Environmental Microbiology. 66, 4595-4597.

Santajit, S., Indrawattana, N., 2016. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. BioMed Research International 2016, 2475067. doi:10.1155/2016/2475067

Sarker, N., Tsudzuki, M., Nishibori, M., Yasue, H., Yamamoto, Y., 2000. Cell-mediated and humoral immunity and phagocytic ability in chicken lines divergently selected for serum immunoglobulin M and G levels. Poultry Science 79, 1705-1709.

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S., Hiramatsu, K., 2007. Reclassification of phenotypically-identified *Staphylococcus intermedius* strains. Journal of Clinical Microbiology 45, 2770-2778.

Schalm, O.W., Carroll, E.J., Jain, N.C., 1971. Bovine Mastitis. Philedelphia, Lea & Febiger, 1971.

Schmidt, T., Kock, M.M., Ehlers, M.M., 2015. Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. Journal of Dairy Science 98, 6256-6269. doi:10.3168/jds.2015-9715

Seguin, J.C., Walker, R.D., Caron, J.P., Kloos, W.E., George, C.G., Hollis, R.J., Jones, R.N., Pfaller, M.A., 1999. Methicillin-resistant *Staphylococcus aureus* outbreak in a veterinary teaching hospital: potential human-to-animal transmission. Journal Clinical Microbiology 37(5), 1459-1463.

Shallcross, L.J., Davies, S.C., 2014. The World Health Assembly resolution on antimicrobial resistance. Journal of Antimicrob Chemotheraphy 69, 2883–2885.

Shryock, T.M., Richwine, A., 2010. The interface between veterinary and human antibiotic use. Annals of the New York Academy of Sciences 1213, 92-105.

Silva, E.R., Silva, N., 2005. Coagulase gene typing of *Staphylococcus aureus* isolated from cows with mastitis in southeast Brazil. Canadian Journal of Veterinary Research 69(4), 260-264.

Smith, B., 2006. The Farming Handbook. University of KwaZulu Natal Press, KwaZulu Natal South Africa & CTA, The Netherlands, ISBN 10:1-86914-090-7, ISBN 13:978-1-86914-090-8.

Smith, K.L., Hillerton, J.E., Harmon, R.J., 2001. NMC guidelines on normal and abnormal raw milk based on SCC and signs of clinical mastitis. National Mastitis Council, Madison, WI,

February 11, 2001, accessed 14 January 2015, from https://www.nmconline.org/docs/abnmilk.htm

Smith, D.L., Smith, T., Rude, B.J., Ward, S.H., 2013. Short communication: Comparison of the effects of heat stress on milk and component yields and somatic cell score in Holstein and Jersey cows. Journal of Dairy Science 96, 3028-3033.

Smith, K.L., Todhunter, D.A., Schoenberger, P.S., 1985. Environmental Mastitis: Cause, Prevalence, Prevention. Journal of Dairy Science 68(6), 1531-1553.

Sneader, W., 2001. History of Sulfonamides. In: eLS [Internet]. John Wiley & Sons, Ltd; 2001 [cited 2014 Nov 28]. Available from: http://onlinelibrary.wiley.com/doi/10.1038/npg.els.0003625/abstract

Sol, J., Sampimon, O.C., Barkema, H.W., Schukken, Y.H., 2000. Physiology and management: Factors Associated with Cure after Therapy of Clinical Mastitis Caused by *Staphylococcus aureus*. Journal of Dairy Science 83, 278–284.

Sordillo, L.M., 2009. Current Concepts on Immunity and Mastitis. WCDS Advances in Dairy Technology. 21, 111-119.

Sordillo, L.M., Aitken, S.L., 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Veterinary Immunology and Immunopathology 128, 104-109.

Sordillo, L.M., Streicher, K.L., 2002. Mammary gland immunity and mastitis susceptibility. Journal of Mammary Gland Biology Neoplasia 7, 135-146.

Spohr, M., Rau, J., Friedrich, A., Klittich, G., Fetsch, A., Guerra, B., Hammerl, J.A., Tenhagen, B.A., 2011. Methicillin resistant *Staphylococcus aureus* (MRSA) in three dairy herds in southwest Germany. Zoonoses Public Health 58, 252–261.

Stalheim, O.H., Page, L.A., 1975. Naturally occurring and experimentally induced mycoplasmal arthritis of cattle. Journal of Clinical Microbiology 2, 165-168.

Stipkovits, L., Rady, M., Glavits, R., 1993. Mycoplasmal arthritis and meningitis in calves. ACTA Veterinaria Hungarica. 41, 73-88.

Stokes, H.W., Gillings, M.R., 2011. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiological Reviews 35(5), 790-819. doi:10.1111/j.1574-6976.2011.00273.x.

Supré, K., De Vliegher, S., Sampimon, O.C., Zadoks, R.N., Vaneechoutte, M., Baele, M., De Graef, E., Piepers, S., Haesebrouck, F., 2009. Technical note: Use of transfer RNA-intergenic spacer PCR combined with capillary electrophoresis to identify coagulase-negative Staphylococcus species originating from bovine milk and teat apices. Journal of Dairy Science 92, 3204–3210.

Supré, K., Haesebrouck, F., Zadoks, R.N., Vaneechoutte, M., Piepers, S., De Vliegher, S., 2011. Some coagulase-negative Staphylococcus species affect udder health more than others. Journal of Dairy Science 94(5), 2329-40. doi:10.3168/jds.2010-3741

Swartz, R., Jooste, P.J., Novello, J.C., 1985. Bacteriophage typing of *Staphylococcus aureus* strains isolated from Bloemfontein Dairy Herds. Journal of South African Veterinary Association 0038, 2809/85/02/0069-0073

Talan, D.A., Staatz, D., Staatz, A., Goldstein, E.J., Singer, K., Overturf, G.D., 1989. *Staphylococcus intermedius* in canine gingiva and canineinflicted human wound infections: laboratory characterization of a newly recognized zoonotic pathogen. Journal of Clinical Microbiology 27, 78-81.

Tenover, T.F., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B. E., Persing, D.H., Swaminathan B., 1995. Interpreting chromosomal DNA restriction patterns produced by Pulsed-Field Gel Electrophoresis: Criteria for bacterial strain typing. Journal of Clinical Microbiology 33(9), 2233-2239.

Ter Laak, E.A., Wentink, G.H., 1992. Increased prevalence of *Mycoplasma bovis* in the Netherlands. The Veterinary Quarterly 14(3), 100-104.

Thomas, A., Dizier, I., Trolin, A., Mainil, J., Linden, A., Ball, H., Bell, C., 2002. Isolation of mycoplasma species from the lower respiratory tract of healthy cattle and cattle with respiratory disease in Belgium. Veterinary Records 151, 472-476.

Thompson-Crispi, K.A., Mallard, B., 2012. Type 1 and Type 2 immune response profiles of commercial dairy cows in 4 regions across Canada. Canadian Journal of Veterinary Research 76(2), 120-128.

Topley, W.W.C., Wilson, G.S., 2010. Topley and Wilson's Microbiology and Microbial Infections, Cumulative Index. 10th Edition, Wiley publishers, New Jersey, USA Print, ISBN:9780470686386, Online ISBN:9780470688618, doi:10.1002/9780470688618

United States Food and Drug Administration (USFDA), 2009, Summary report on antimicrobials sold or distributed for use in food-producing animals, Department of Health and Human Services, FAO Press, from,
www.fda.gov/AnimalVeterinary/NewsEvents/.../ucm277661.htm, accessed 20 January 2020

Van Bambeke, F., Tulken, P.M., 2001. Macrolides: pharmacokinetics and pharmacodynamics. International Journal of Antimicrobial Agents 18(1), S17-23.

Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A., Laximinarayan, R., 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. Lancet Infectious diseases 14(8), 742-750.

Van den Broek, I.V., Van Cleef, B.A., Haenen, A., Broens, E.M., Van der Wolf, P.J., Van den Broek, M.J., Huijsdens, X.W., Kluytmans, J.A., Van de Giessen, A.W., Tiemersma, E.W., 2009. Methicillin-resistant *Staphylococcus aureus* in people living and working in pig farms. Epidemiology Infections 137(5), 700-708.

Vanderhaeghen, W., Piepers, S., Leroy, F., Van Coillie, E., Haesebrouck, F., De Vliegher, S., 2015. Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. Veterinary Journal 203(1), 44-51. doi:10.1016/j.tvjl.2014.11.001

Van Der Lely, C., 2000. Apparatus for milking animals. Switzerland Patent Number: (45) Date of Patent: 6,050,219 Apr. 18, 2000 54.

Vliegher, S.D., Fox, L.K., Piepers, S., McDougall, S., Barkema, H.W., 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. Journal of Dairy Science 95(3), 1025-1040.

Van vuuren, M., Pickard, J., Greyling, J., 2007. South African national veterinary surveillance and monitoring programme for resistance to antimicrobial drugs (SANVAD). Department of Veterinary Tropical diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa.

Vintov, J., Aarestrup, F.M., Zinn, C.E., Olsen, J.E., 2003. Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. Veterinary Microbiology 95, 133-147.

Von Essen, S.G., Auvermann, B.W., 2005. Health effects from breathing air near CAFOs for feeder cattle or hogs. Journal of Agromedicine 10(4), 55-64.

Waksman, S.A., 1947. What is an antibiotic or an antibiotic substance? Mycologia 39(5), 565-569.

Wang, W., Lin, X., Jiang, T., Peng, Z., Xu, J., Yi, L., Li, F., Fanning, S., Baloch, Z., 2018. Prevalence and Characterization of *Staphylococcus aureus* Cultured From Raw Milk Taken From Dairy Cows With Mastitis in Beijing, China. Frontiers in Microbiology 22(9), 1123. doi:10.3389/fmicb.2018.01123

Ward, W.R., Hughes, J.W., Faull, W.B., Cripps, P.J., Sutherland, J.P., Sutherst, J.E., 2002. Observational study of temperature, moisture, pH and bacteria in straw bedding, and faecal consistency, cleanliness and mastitis in cows in four dairy herds. Veterinary Record 151, 199-206.

Wellenberg, G.J., Van der Poel, W.H.M., Van der Vorst, T.J.K., Van Valkengoed, P.H.R., Schukken, Y.H., Wagenaar, F., Van Oirschot, J.T., 2000. Bovine herpesvirus 4 in bovine clinical mastitis. Veterinary Record 147, 222-225.

Wellenberg, G.J., van der Poel, W.H.M., Van Oirschot, J.T., 2002, Viral Infections and bovine mastitis: a review. Veterinary Microbiology 88, 27-45.

White, A., 2010. Mastitis, a practice approach. Journal of Veterinary Surgeon in General Practice, Livestock 15(7), 36–40. doi:10.1111/j.2044-3870

Williams, R., Scholtz, M.M., Neser, F.W.C., 2016. Geographical influence of heat stress on milk production of Holstein dairy cattle on pasture in South Africa under current and future climatic conditions. South African Journal of Animal Science 46(4).

Wilson, C.D., Richards, M.S., 1980. A survey of mastitis in the British dairy herd. Veterinary Record 106, 431-435.

Wingfield, J.C., Kenagy, G.J., 1991. Natural regulation of reproductive cycles. In: Vertebrate Endocrinology: Fundamentals and Biomedical Implications. (eds Pang, P.K.T. & Schreibman, M.P.). Academic Press, New York, pp. 181–241.

Witte, W., 1998. Medical consequences of antibiotic use in agriculture. Science 279, 996-997.

Wooley, R.E., Gibbs, P.S., Schott, E.B., 1999. Inhibition of *Salmonella typhimurium* in chicken intestinal tract by transformed a virulent avian *Escherichia coli*. Avian Disease 43, 245-250.

World Health Organization (WHO), 2002. The World Health Report 2002. Reducing risks promoting life. https://www.who.int/whr/2002/en/, accessed 20 January 2020.

World Health Organization (WHO), 2014. Antimicrobial resistance: global report on surveillance, Geneva, Switzerland, from, http://www.who.int/drugresistance/documents/surveillancereport/en/, accessed 20 January 2020.

Wushouer, H., Tian, Y., Guan, X.D., Han, S., Shi, L.W., 2017. Trends and patterns of antibiotic consumption in China's tertiary hospitals: Based on a 5 year surveillance with sales records, 2011-2015. Plos One 12(12), e0190314. doi:10.1371/journal.pone.0190314

Yoshikawa, H., Xie, B., Oyamada, T., Hiraga, A., Yoshikawa, T., 1997. Detection of bovine leukemia viruses (BLV) in mammary tissues of BLV antibody-positive cows affected by subclinical mastitis. Journal of Veterinary Medical Science 59, 301-302.

Youn, J.H., Hwang, S.Y., Kim, S.H., Koo, H.C., Shin, S., Lim, S.K., Park, Y.H., 2010. *MecA* gene transferrability and antibiogram of zoonotic *Staphylococcus intermedius* from animals, staff and the environment in animal hospitals in Korea. Journal of Microbiology and Biotechnology 20(2), 425-32.

Zhao, X., Lacasse, P., 2007. Mammary tissue damage during bovine mastitis: Causes and control. Journal of Animal Science 86(Suppl. 1), 57-65. doi:10.2527/jas.2007

Appendix A

Intramammary products IVS 2011: Study from 2000 to 2010 (Chapters 2 and 3)

1. Albadry plus, Pfizer AH (Procaine penicillin G & albamycin), DC, still on available in 2018 from Zoetis.
2. Cephudder, Intervet Schering AH (Cephapirin), DC, still on market, MSD AH 2018
3. Cepravin Dry cow, Intervet Schering AH (Cephalonium), DC, MSD still available in 2018.
4. Count down LC, Afrivet (semi-synthetic penicillins, sodium ampicillin & sodium cloxacillin), LC, no longer available.
5. Curaclox, Norbrook (Biotech Vet), (Cloxacillin & ampicillin), DC, DC extra & LC
6. Dispolac, Cooper (Afrivet) (Procaine benzyl penicillin & dihydrostreptomycin sulphate), DC & LC, still available in 2018.
7. Dri-cillin, Bayer AH (Ampicillin & Cloxacillin), DC & LC, no longer available
8. Lactacure LC, Cipla Agrimed (Sodium Ampicillin & Sodium Penicillin), LC, no longer available
9. Lacti-cillin, Bayer AH (Cloxacillin & Ampicillin), LC, no longer available
10. Masticlox DC range, Merial (Cloxacillin & Ampicillin), DC, DC plus, DC plus xtra
11. Masticlox Plus LC, Merial (Cloxacillin & Ampicillin), LC
12. Masticlox QR, Merial (Cloxacillin & blue dye), LC
13. Mastijet Fort, Intervet Schering AH, (Tetracycline, neomycin base, bacitracin & prednisone), LC, still available in 2018.
14. Nafpenzal DC, Intervet Schering AH, (Benzylpenicillin, dihydrostreptomycin & nafcillin), DC, MSD 2018, still available in 2018.
15. Nafpenzal MC, Intervet Schering AH, (Sodiumbenzylpenicillin, dihydrostreptomycin & nafcillin), LC, MSD 2018 still available in 2018.
16. Neo-Mastitar Dry Cow, Intervet Schering AH (Procaine penicillin & neomycin base), DC, no longer available.
17. Orbenin Extra DC Infusion, Pfizer AH (Cloxacillin), LC or DC, still available in 2018.
18. Pendiclox Blue, Pfizer AH (Sodium Ampicillin, sodium cloxacillin & blue tracer dye), LC & DC, no longer available.
19. Penstrep, Bayer AH (Procaine penicillin, dihydrostreptomycin & blue tracer dye), LC & DC, still available in 2018.
20. Rilexine, Virbac (Cephalexin, neomycin sulphate & prednisolone), LC & DC, still available in 2018.

21. Special formula 17900-forte, Pfizer AH (Hydrocortisone acetate, hydrocortisone NA-succinate, procaine penicillin, novobiocin, polymyxin, B sulphate & dihydrostreptomycin), LC, still available in 2018.
22. Spectrazol milking cow, Intervet Schering AH (Cefuroxime), LC, still available in 2018.

Chapter 2: Proactive udder health management in South Africa and monitoring of antibiotic resistance of *Staphylococcus aureus* in dairy herds from 2001 to 2010

Published 7 May 2018: Joanne Karzis, Inge-Marié Petzer, Edward F. Donkin, Vinny Naidoo. 2018. Journal South African Veterinary Association 89, a1490. doi:https://doi.org/10.4102/jsava.v89i0.1490

Significance of the work

Antimicrobial resistance is currently a topic of global interest. This study is the start of much needed retrospective evaluation of much needed surveillance data linked to the pro-active udder health programme, which is lacking in the animal health field. Despite surveillance data being in existence for a few of the main mastitis causing organisms, for this study we focused on *S. aureus* as it is the biggest problem in mastitis of dairy cows. The findings of this study, showed that the accumulated information were generally in accordance with the studies done elsewhere, except for the 20 well-managed herds that were specifically evaluated. These herds all showed a decrease in antibiotic resistance through the implementation of the specified control programme over time. The programme under investigation introduced the use of good management as a novel approach to control bacterial populations and in turn decrease antibiotic resistance in dairy herds. This method has had widespread positive effects on the dairy industry such as, availability of antibiotics for prudent use and on food security through sustaining and assisting in production of safe food products (dairy) for a growing population.

Abstract

Antibiotic resistance of strains of *S. aureus* isolated from bovine milk is of concern internationally. The objective of this study was to investigate trends of resistance of *S. aureus* to antibiotics administered to dairy cows in 19 South African and one Zambian dairy herds (participating in the South African proactive udder health management programme) and to identify possible contributing factors. The resistance of *S. aureus* strains to eight commonly used antibiotics (ampicillin, cloxacillin, penicillin G; cephalexin, cefuroxime, clindamycin,

oxytetracycline and tylosin) in South Africa from 2001 to 2010 was evaluated. *Staphylococcus aureus* isolates (*n* = 2532) were selected from cows with subclinical mastitis from 20 herds routinely sampled as part of the proactive udder health management programme. The isolates were selected from milk samples that had positive bacteriology and somatic cell counts more than 400 000 cells/mL and were tested for antibiotic resistance using a standard Kirby–Bauer test with published clinical breakpoints. The prevalence of antibiotic resistance was evaluated as a percentage of susceptible *S. aureus* isolates out of the total numbers for each antibiotic selected per year. *Staphylococcus aureus* showed a significant increase in percentage of susceptible isolates over time for all antibiotics tested except for ampicillin. The overall prevalence of mastitis did not change during the study period. However, the prevalence of mastitis caused by *S. aureus* (subclinical cases) in the selected herds decreased numerically but not significantly. Reduction in the incidence of antibiotic resistance shown by *S. aureus* was presumed to be a result of the application of the proactive udder health management programme. The fact that the overall prevalence of mastitis was kept stable was possibly because of the influence of the management programme in conjunction with the return of infections caused by non-resistant strains.

Keywords: antimicrobial resistance; *S. aureus*; mastitis; food production

Introduction

South Africa is a developing country in southern Africa. With a population of 55 million people, the average annual milk consumption has been estimated to be approximately 36 L of milk per capita, which is well below the 200 L per capita annually recommended by the World Health Organization (Lassen 2012). Currently 98% of the country's needs Examiner B locally produced, with approximately 10 million litres being imported (Lassen 2012). The South African milk-producing herd was estimated to be approximately 2474 dairy herds in 2012, with an average herd size of 238 and an average production of 20.2 L of milk per cow per day (Milk South Africa 2013), either using thrice or twice daily milking routines. Over the past 10 years, the number of milk producers has decreased, with an increase in average herd size (Milk South Africa 2013).

Mastitis remains the single largest contributor to losses in revenue for dairy producers worldwide (Awale et al. 2012), estimated at approximately $35 billion (Modi et al. 2012). Estimated milk production losses in a specific herd associated with elevated quarter milk

somatic cell counts (SCC) of all lactating cows in the herd were estimated at an annual milk loss of 46 190 L valued at R205 544.84 (Petzer et al. 2016). In South Africa, based on investigation of routine milk samples, the prevalence of mastitis increased from 8.1% in 2002 to 15.4% in 2006 (Petzer et al. 2009). In the herds selected for this study, the overall mastitis prevalence remained stable and did not increase (Petzer et al. 2009).

The most common mastitis pathogens are found in the udder tissues and are spread between cows (contagious or host-adapted pathogens) or from the environment (environmental pathogens), such as bedding materials, manure and soil. This distinction may be important when assessing the challenges present in a specific herd as well as the measures considered to reduce or treat mastitis. Two main mastitis-causing pathogens in South Africa are *Staphylococcus aureus* and *Streptococcus agalactiae.* These organisms are termed major pathogens and are generally regarded as those commonly associated with clinical mastitis in dairy cattle. The causative pathogen should preferably be identified by laboratory testing of milk. There are other bacteria that may be present in the udder and they may have a beneficial effect by preventing the damage caused by major pathogens, because of the production of natural antibacterial substances or competition with other bacteria (Pieterse 2008). These bacteria can erroneously be implicated in instances of increased SCC and thus subclinical mastitis because they usually do not cause clinical mastitis (Schukken et al. 2003). Mastitis pathogens can infect cows both when lactating and during the dry period. Thus, it is important to identify and recognise the source of these infections in order to choose appropriate treatments. Organisms that have been identified are *S. aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*; Gram-negative major pathogens: *Escherichia coli*, *Klebsiella pneumonia*, *Serratia* spp.; minor pathogens: coagulase-negative staphylococci (non-aureus staphylococci) *Micrococcus* spp., *Staphylococcus pseudintermedius*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Streptococcus canis*, *Trueperella pyogenes* and other members of the family Enterobacteriaceae.*Staphylococcus aureus* is the principal cause of mastitis (Petzer et al. 2009, Petzer et al. 2016).

The infected udder is considered the primary reservoir of *S. aureus* and it is believed to be transmitted during milking via contaminated teat liners, milker's hands and communal clothes (Leslie & Schukken 1999). Once *S. aureus* infects the udder, it may cause primary clinical signs such as swelling, heat, redness, and floccules in the milk as well as abscessation and fibrosis of the udder. These bacteria may damage the secretory tissue and cause reappearance of clinical signs or elevated SCCs and may permanently limit an infected

quarter's ability to produce milk and to respond to treatment (Mellenberger & Kirk 2001). *Staphylococcus aureus* is also particularly difficult to treat effectively because it may secrete β-haemolysin, which can lead to potentially fatal gangrenous mastitis (Mellenberger & Kirk 2001). These bacteria can also avoid phagocytosis through biofilm production, which may also lead to poor antibiotic penetration (Ramadhan & Hedges 2005). The production of biofilm may also be correlated with pathogenicity and thus contribute to the virulence of individual strains (Ramadhan & Hedges 2005).

The dairy industry is a major consumer of antibiotics globally and mastitis is the most treated disease of dairy cows. In South Africa, producers have unrestricted access to 12 of 22 registered intramammary products without prescription, while the remaining 10 registered intramammary products are restricted for veterinary use (Carrington et al. 2016). All these intramammary products consist of the ampicillin, cloxacillin, combinations thereof, with a few cephalosporins, lincosamides, tetracyclines available in SA. The antibiotics available without prescription may be used incorrectly (Henton et al. 2011) and may contribute to the emergence and/or persistence of antibiotic-resistant strains in cows, humans or both (Burgos et al. 2005).

The proactive udder health programme of the Milk Laboratory of the Faculty of Veterinary Science, University of Pretoria, was initiated to reduce the development of resistance. The programme is comprehensive and includes routine microbiological and cytological examinations for the whole herd. This allows the early identification of *S. aureus* intramammary infections (IMI) to facilitate eradication of this organism from a herd. For this study, we report on the effectiveness of this programme on 20 herds managed consistently over a 10-year period.

Materials and Methods

A proactive udder health management programme, using the Milk Sample Diagnostic (MSD) computer programme (Abaci Systems, Aretsi SA, Pretoria), was developed and maintained over several years at the University of Pretoria (Petzer et al. 2016). The basic purpose of this management programme was to sample all lactating cows in a herd for both microbiological and cytological evaluations in order to identify *S. aureus*-positive cows. This system identified those animals which were carriers of *S. aureus* despite a low SCC. This information assisted managers and advisors in making management decisions. In this manner, cows with udder infections caused by mainly contagious bacteria could be separated from those not infected

and milked last or if necessary be removed from the herd. The method implemented was thus different from other monitoring systems, where only infected quarters were sampled (Petzer et al. 2016). Producers were inducted into the programme either on their own accord or after being recruited through awareness events on the importance of proactive udder health. Participation in the programme is voluntary and paid for by the producers.

In practice, herds (all lactating cows) were sampled frequently (every 2 to 3 months). Cows that tested positive for *S. aureus* were placed in a separate camp and milked last within 2–3 days of sampling. These cows were kept in the camp with other *S. aureus*-positive cows for the rest of their productive lives. At drying-off, cows were in dry groups with other cows from the herd, but returned to the *S. aureus* group immediately after calving. The positive cows were identified differently in order to facilitate immediate identification should they be in a wrong group. They were always milked last and only after the rest of the lactating cows left the parlour, for their entire productive lives. Udders of all *S. aureus*-positive cows were palpated by an experienced veterinarian just after milking to determine possible chronic udder damage. In addition, the following criteria were used to predict the probability of cure for the individual cow: parity, stage of lactation, level of SCC in the infected quarters, numbers of quarters in an udder infected with *S. aureus* and quarter position according to a suggested formula (Sol et al. 1997). For example, there is a decreased likelihood of cure of cows in second or later lactation, within 1–99 days in milk, with high SCC (> 800 000 cells/mL), with mostly hind quarters affected and 3–4 quarters per udder affected (Sol et al. 1997). Selection of the antibiotic for treatment was based on antibiotic susceptibility testing results and cure based on culture results instead of the SCC. Microbiological cure was monitored, and chronic cases were culled as soon as possible.

Milking hygiene practices included disinfection of milkers' hands or gloves that actually touched the teats (performing stripping), pre- and post-teat dipping and backwashing of clusters with effective and fast-acting disinfectant. All milking systems were tested on an ongoing basis using both pulsographs and teat-end vacuum measurements. The following additional tests were performed weekly by managers as described by the Teat Club International (DeLaval 2017): time from touch to attachment, attachment to milk flow, maximum milk flow and percentage liner slips and residual milk. Teat-end scores were performed on first lactation cows for early detection of incorrect settings used in the maintenance of milking machines. Milking parlour staff and management were trained in implementing the practices of the proactive udder health management programme through constant communication of advice and guidance given by the laboratory manager, based on

the microbiological and cytological results obtained from examinations. These dairy parlours used either the Afimilk or Alpro systems, which are highly technical systems to assist in the monitoring of parlour management.

Each herd was approached on an individual basis. Overall when the udder health status of the herd remained static or deteriorated, a veterinarian visited the herd and the protocol was immediately adjusted according to findings and observations made during the visit. This may have included re-training of milkers or managers in milking procedures, cow handling and operation; cleaning and settings of the milking machine; a new teat dip or disinfectants prescribed; the installation of a Dosatron; improvement of biosecurity or the herd kept as a closed herd thereafter; culling of specific cows; inactivation of udder quarters and introduction or continuation of vaccination with Startvac vaccine (Hipra).

This study presents the results from the first 10 years of the monitoring period. In this period, a total of 363 herds were inducted into the programme. The results from 20 of these herds (19 in South Africa and 1 in Zambia) are presented. These herds were selected because they were continually evaluated (for at least 5 of the 10 years) over this period for impact of these management practices on the development of antibiotic resistance. The herd size varied from 67 to 1253 animals. All were fed on total mixed rations (TMR). The study population included mainly Holstein Friesian, Holstein crossbreeds, crossbreeds and Jersey dairy cows. Cows differed in age, parity, days in milk and milk yield.

Milk samples were taken by professional samplers or milkers trained according to the standard operating procedure (Giesecke et al. 1994). Prior to sampling, the first milk was stripped from all quarters and the teat ends were carefully cleaned and disinfected with methylated alcohol. Approximately 10 mL of milk was collected aseptically into sterile marked sample tubes and kept refrigerated until shipment. In the case of composite milk samples, the same procedure was followed, but approximately equal volumes of milk from each of the four quarters were collected in one sample tube. Samples were transported on ice to reach the Milk Laboratory within 48 h of sampling. Temperatures and conditions such as the cleanliness and appearance of sample tubes were noted on arrival at the laboratory, and samples that were spoiled or of doubtful quality were not processed. Samples were inoculated onto agar plates in the laboratory on the day of their arrival.

Initially a total of 5905 milk samples were collected (Table 2.1) from healthy cows and those suspected to have IMI as part of the routine testing. These samples were mainly from milk

with signs of subclinical IMI (as determined by microbiological examination). Of the samples sent in for analysis, samples with vigorous growth and SCC > 400 000 cells/mL milk were selected for susceptibility testing. Intervals for whole-herd microbiological and cytological herd examinations ranged from monthly to longer intervals (Table 2.3).

Routine bacterial isolation (National Mastitis Council 2017 was performed on all milk samples in accordance with standard laboratory milk culture methodology and preliminary identification was done based on colony morphology (International Dairy Federation 1985). When fewer than two colonies were present, and no organism was identified, the milk sample was noted as 'no growth'. When there were more than two types of organisms present, the sample was noted as contaminated (CU). Where there were two distinct groups of separate growth present, this was noted as 'mixed growth' (MG) or a special code was allocated when major pathogens were involved. Samples classified as 'no growth', CU (contamination) or mixed growth (with or without major pathogens) were not used for antibiotic sensitivity testing. The SCCs were performed by fluoro-optic-electronic methods using a Fossomatic 90 and Fossomatic 5000 (Rhine Rühr, Wendywood, Denmark). Selected isolates of pure cultures of *S. aureus* with vigorous growth from subclinical mastitis cases with an SCC of more than 400 000 cells/mL were utilised for susceptibility testing. Not all *S. aureus* isolates in the herds over the 10-year study period underwent susceptibility testing (the routine practice is to perform one susceptibility test per type of bacteria isolated per investigation).

Antibiotic susceptibility testing was performed on one *S. aureus* isolate for every herd investigation, using the Kirby–Bauer disk diffusion method (Bauer et al. 1966) for eight antibiotics with laboratory quality controls (American Type Culture Collection – ATCC No. 25923 *S. aureus*): ampicillin 10 µg (AMP), cloxacillin 5 µg (OB), penicillin G 10 IU (PEN) (beta-lactams); cephalexin 30 µg (CL), cefuroxime 30 µg (CXM) (cephalosporins); clindamycin 10 µg (DA) (lincosamides); oxytetracycline 30 µg (OT) (tetracyclines) and tylosin 30 µg (TY) (macrolides). Antibiotic susceptibility testing was performed and interpreted by measuring the zone diameter to the nearest whole millimetre for all zones of inhibition, which were categorised as susceptible, intermediate or resistant categories as clinical breakpoints established by the Clinical Laboratory and Standards Institute.

All data were initially captured in the MSD programme (Abaci Systems, Aretsi SA, Pretoria) or in Microsoft Excel. The data in the files of the MSD programme were exported into Excel as CSV files. Excel was used for data sorting and to create pivot tables and figures. All antibiotic susceptibility results that fell into the intermediate category were presumed to be resistant for

the purpose of analysis of data (Wong et al. 2014). The prevalence per herd for susceptibility was listed as a percentage of all samples taken by year. As the interventions started at different times on some of the herds, this introduces a year-by-year bias into the analysis. To correct this, calculations were based on year of intervention. To ascertain if changes per year were significant among the 20 herds, binomial regression (logistic regression) was undertaken per antibiotic evaluated using the IBM SPSS statistics version 22 (IBM Corp., Armonk, NY). The chi-squared test (linear-by-linear association) was additionally conducted to verify the results obtained by binomial regression, using the IBM SPSS statistics version 22 (IBM Corp., Armonk, NY). Trends in resistance from year of programme introduction of each herd are reported.

The assumption of the model was that the longer a herd was included in the programme, the lower the prevalence of resistance of *S. aureus* to a particular drug would become.

Results

The number of samples per herd is presented in Table 2.1. The first year of monitoring for a particular herd is reported as year 1 and subsequently as the years of total intervention. At 509 sampling events, a total of 815 samples positive for *S. aureus* underwent antibiotic susceptibility testing. Cefuroxime and cephalexin had the lowest percentage resistant isolates prior to any intervention on a herd at 24.32% and 15.32%, respectively. After the 10-year monitoring period, this pattern had changed with all herds, demonstrating a significant trend of decreasing incidence of antibiotic resistance ($p > 0.05$) for all products, with the exception of ampicillin, where there was no significant change ($p = 0.104$) (Table 2.1 and 2.2).

Cefuroxime (CXM) was the product that showed the lowest percentage resistance and the highest percentage susceptible samples over the study period.
The prevalence of IMI (SCC ≤ 250 000 + positive culture), irritation (SCC ≤ 250 000 +negative culture) and subclinical mastitis (SCC ≥ 250 000 + positive culture) in the absence of clinical signs is shown in Table 2.3. This was for all the *S. aureus* isolates isolated over the study period (Table 2.3).

An example of how the proactive udder health management programme was implemented is presented in Table 2.4. For each defined monitoring period as shown in the table, the total cases of *S. aureus* were identified, together with first-time infections, repeat infections and

animals cured (bacteriologically cured, which was defined as the absence of bacteria cultured for two consecutive examinations). The overall prevalence of mastitis did not change significantly over this study period (Table 2.3), although a visual decrease (non-significant) in the incidence of *S. aureus* mastitis was shown in a few examples (Table 2.4).

Table 2.1. Total number of antibiotic susceptibility samples of *Staphylococcus aureus* isolates per farm, tested during the 10-year study period.

Herd ID	Herd size	Breed	SCC at first test ($\times 10^3$ cells/mL)		Samples tested per year from implementation of the programme										Total samples tested per herd
			≤250 (%)	≥750 (%)	1	2	3	4	5	6	7	8	9	10	
1	83	H	64.8	2.3	1	2	-	1	2	5	2	5	5	4	27
2	294	H	46	49.9	1	-	4	5	5	4	4	4	3	-	30
3	239	H	52.9	25	7	2	6	5	5	3	1	1	-	-	30
4	699	H	43.4	14.9	1	4	3	4	3	8	7	10	11	1	52
5	296	H	45	10.1	1	-	1		1	1	2	-	-	-	6
6	329	H	42.2	19.8	2	-	4	1	4	1	3	1	-	2	18
7	624	H	80.8	7.3	1	-	1	1	-	3	-	2	-	-	8
8	300	H	79.7	10.6	2	3	1	3	-	1	-	1	2	1	14
9	233	H	50	40.6	1	3	2	2	1	-	3	-	-	-	12
10	1253	H	78	8.4	3	21	11	18	12	13	7	1	-	-	86
11	200	H	7	65	1	4	3	6	3	3	4	1	-	-	25
12	625	HX	71	10.7	2	1	2	-	-	-	2	7	-	-	14
13	576	H	66.6	22.7	7	1	-	1	1	3	-	7	1	-	21
14	200	HX	40.9	19.3	1	1	2	3	1	1	-	-	-	-	9
15	250	X	27.3	20.5	2	-	1	2	1	1	1	-	-	-	8
16	67	X	6.6	67.4	2	2	3	2	1	-	-	-	-	-	10
17	231	H	47.9	33.7	10	15	20	11	9	9	1	-	-	-	75
18	575	J	67.9	15.6	10	3	-	13	8	9	-	-	-	-	43
19	332	J	11.4	72.3	2	1	2	2	1	1	-	-	-	-	9
20	65	J	29.9	16	4	2	3	2	1	-	-	-	-	-	12
Total	7471				61	65	69	82	59	66	37	40	22	8	509

Source: Authors' own work.H, Holstein Friesian; HX, Holstein Friesian crossbreed; X, crossbreed; J, jersey; SCC, somatic cell count; - , no samples tested.

Table 2.2. Numbers of resistant samples of *Staphylococcus aureus* isolates out of the total number of isolates tested per antibiotic, shown for each of the 8 antibiotics used.

Year	AMP	CL	CXM	DA	OB	OT	P	TY
1	50.49 (52/103)	24.32 (9/37)	15.32 (17/111)	53.57 (60/112)	42.99 (46/107)	38.39 (43/112)	64.71 (44/68)	78.38 (58/74)
2	57.01 (61/107)	40.63 (39/96)	25 (28/112)	56.52 (52/92)	46.74 (43/92)	48.65 (54/111)	63.16 (24/38)	79.59 (39/49)
3	60.18 (68/113)	36.11 (26/72)	13.33 (20/150)	59.73 (89/149)	42.18 (62/147)	33.56 (50/149)	60.58 (63/104)	71.7 (38/53)
4	54.25 (83/153)	18 (9/50)	17.98 (32/178)	44.83 (78/174)	34.1 (59/173)	39.43 (69/175)	54.88 (90/164)	74.24 (98/132)
5	56.38 (53/94)	19.61 (10/51)	14.89 (14/94)	46.91 (38/81)	24.18 (22/91)	22.34 (21/94)	59.57 (56/94)	71.67 (43/60)
6	55.84 (43/77)	23.94 (17/71)	6.58 (5/76)	41.67 (30/72)	24.32 (18/74)	24.24 (16/66)	59.21 (45/76)	55.17 (16/29)
7	41.46 (17/41)	15.38 (6/39)	2.5 (1/40)	31.71 (13/41)	19.51 (8/41)	29.41 (10/34)	48.78 (20/41)	53.85 (14/26)
8	45 (18/40)	20 (8/40)	4.88 (2/41)	43.59(17/39)	7.32 (3/41)	27.5 (11/40)	51.22 (21/41)	57.5 (23/40)
9	40.91 (9/22)	27.27 (6/22)	18.18 (4/22)	14.29 (3/21)	36.36 (8/22)	31.82 (7/22)	40 (8/20)	59.09 (13/22)
10	37.5 (3/8)	12.5 (1/8)	12.5 (1/8)	37.5 (3/8)	25 (2/8)	12.5 (1/8)	37.5 (3/8)	50 (4/8)
*p**	0.104	0.004	0.005	>0.000	>0.000	0.001	>0.000	>0.000
*p***	0.104	0.003	0.004	>0.000	>0.000	0.001	0.016	>0.000

Source: Authors' own work

Note: Sample numbers (*n*) indicated in parentheses.

AMP, ampicillin; OB, cloxacillin; CL, cephalexin; DA, clindamycin; P, penicillin G; OT, oxytetracycline; TY, tylosin; CXM, cefuroxime.

*Binomial/logistic regression.

**Chi-squared test (linear-by-linear association).

Table 2.3. Health status and intramammary infections of the 20 herds on which management practices were altered as determined and projected by the MSD program used in practice (for all *Staphylococcus aureus* isolates).

Year	Healthy		IMI		Irritation		Subclinical		Grand Total
2001	41.33	(768)	26.96	(501)	13.19	(245)	18.51	(344)	1858
2002	55.05	(5117)	25.19	(2341)	7.57	(704)	12.19	(1133)	9295
2003	51.34	(14 768)	20.91	(6014)	15.78	(4538)	11.97	(3444)	28 764
2004	46.09	(13 339)	27.35	(7915)	12.33	(3570)	14.23	(4120)	28 944
2005	37.20	(18 500)	29.18	(14 508)	13.62	(6772)	20.00	(9945)	49 725
2006	32.13	(15 445)	36.89	(17 734)	10.71	(5150)	20.26	(9741)	48 070
2007	30.39	(9410)	38.83	(12 022)	9.60	(2973)	21.18	(6559)	30 964
2008	37.28	(12 589)	24.57	(8299)	11.10	(3748)	27.05	(9136)	33 772
2009	48.69	(7844)	13.13	(2115)	16.59	(2673)	21.59	(3479)	16 111
2010	46.86	(14 104)	20.12	(6054)	10.84	(3263)	22.18	(6675)	30 096

Source: Authors own work

IMI, intramammary infection: somatic cell counts (SCC) ≤ 250 000 + positive culture; irritation: SCC ≤ 250 000 + negative culture; subclinical: SCC ≥ 250 000 + positive culture in the absence of clinical signs. Results are presented as percentage with the number of samples in parentheses.

This data was from a retrospective dataset from 2000 to 2010.

Table 2.4. Monitoring udder health results (*Staphylococcus aureus*) from a typical herd of the 20 herds over time (herd size, n = 231).

Dates	STA total (n)	STA new (n)	STA repeat (n)	STA cured (n)
May-05	30	29	1	1
Jun-05	39	33	6	21
Jul-05	42	32	10	27
Intervention				
Feb-06	4	4	0	3
Mar-06	7	7	0	1
Nov-08	3	3	0	0
Dec-08	9	7	2	3
Jan-09	7	7	0	6
Aug-10	8	8	0	0
Sep-10	4	3	1	7
Oct-10	2	2	0	3
Nov-10	6	5	1	0

Source: Authors' own work

STA, *S. aureus*; STA total (n), total cases of STA isolated per examination; STA new, first time infections of STA isolated from a cow; STA repeat, STA isolated more than twice from the same cow (probable chronically infected cows); STA cured, animal positive for STA on the previous test and negative for two or more consecutive examinations (bacteriological cure); intervention, application of proactive udder health management programme.

Discussion

The commercialisation of antibiotics in the 1940s has revolutionised medicine in many respects with many lives having been saved. However, the overuse of these once highly effective antibiotics has been accompanied by the rapid selection for resistant strains of bacteria (Davies & Davies 2010). A recent WHO health report has warned that resistance to antibiotics in general is a 'global' threat (World Health Organization 2014), and one that impacts both human health and the agricultural industry. The exposure of humans to antibiotic-resistant pathogens through agricultural products and contact with animals has increased from the beginning of the 21st century (Kluytmans 2010). This is noteworthy when compared to previous historic exposures that were limited to hospital nosocomial infections (Witte 1998). Of the various veterinary uses of antibiotics, our concern has been the high levels of resistance seen in mastitis-causing organisms in South Africa. The resistance of *S. aureus* to beta-lactam antibiotics in a limited study carried out in the KwaZulu-Natal province has been found to be 48% during this same monitoring period (Schmidt 2011). However, this cannot be compared with our results because of the limited data upon which the KwaZulu-Natal study was based.

In the past, most selection of antibiotics for mastitis therapy was reactive, with the predominant practice being the management and treatment of only cows with clinical mastitis. The idea when initiating the proactive herd management programme was to promote a change in herd management through other control strategies, with the aim of decreasing the need for antibiotic treatment. The programme allowed for the early specific identification of *S. aureus* as the causative organism of mostly subclinical mastitis, through a combination of the monitoring of both SCCs and bacterial culture (Table 2.1). The reason for considering both criteria was based on previous findings from our laboratory that have shown that 15.4% of *S. aureus* IMI might be missed when using SCC alone (Petzer et al. 2016). In the proactive system, animals with a positive diagnosis were subjected to specific management practices such as changes in the order of their milking to reduce transmission to healthy animals, and initiation of treatment based on antibiotic susceptibility testing, with chronic cases being culled as soon as possible, to rid the herd of resistant bacteria that pose a risk of new *S. aureus* IMI.

This programme has focused on procedures that would assist in decreasing the spread of this organism between animals as well as between animals and people. The process involved a combination of integrated herd and parlour hygiene, milker and manager education (and re-training), milking machine monitoring, early responsible treatment as well as constant supervision (Barkema et al. 2006). The importance of education in the value chain cannot be

overlooked, as shown by Dufour et al. (2012), who found that the dairy producers who believed that they were already doing enough about mastitis (i.e. those who were not open to new management strategies) had a higher chance of acquiring new *S. aureus* infections in their herds. What was also important in the use of antibiotics was to convey the message that antibiotic treatment is only one aid in the treatment and prevention of clinical mastitis at the herd level.

Unexpectedly, we did not find a significant decrease in the overall prevalence of mastitis (mostly subclinical) (Table 2.3). This was possibly because of the influence of the management programme in conjunction with the return of infections caused by non-resistant strains. However, a numerical decrease (non-significant) in mastitis caused by *S. aureus* was shown in a few of the examples from these herds (Table 2.4). Another South African study showed the overall trend of mastitis increasing from 8.1% in 2002 to 15.4% in 2006 from all routine samples during that time period (Petzer et al. 2009). However, the 20 herds in this study, with continuous evaluation and correct application of this management programme in practice (at 1–4-month intervals) for the 10-year study period, showed an overall stable prevalence of mastitis (Table 2.3). Only a significant reduction in the prevalence of antibiotic resistance in *S. aureus* was found (Table 2.2). This highlights the possibility of being able to farm successfully using less antibiotics for milk production. This management programme works well only when the information obtained is put into practice correctly. While the significant decrease in the resistance is an important finding (Table 2.2), it is not suggested that bacterial resistance can be reversed, but rather that the improved udder health management had naturally selected for less resistant/pathogenic strains of *S. aureus*. Therefore, we believe that the *S. aureus* strains that were identified in milk samples from these herds were being effectively removed from the infectious cycle (Table 2.4). This was done by successful and early treatment of both subclinical and clinical IMI, inactivation of quarters or culling of cows. The risk of new infections by these bacteria was also limited by isolating infected cows, by milking them last and by improving milking hygiene. This was based on the epidemiology of *S. aureus* mastitis strains, which appear to come from both other cows and humans. The rationale for this was that the chronic *S. aureus*-infected animals were repeatedly infected animals that failed to respond to treatment, thus likely representing the reservoirs of resistant pathogens. It is also plausible that good hygiene limited the spread of *S. aureus* from the farm workers to the animals. In more than eight previous *S. aureus* outbreaks in the country, a link has been shown between bacteria isolated from throat swabs of dairy parlour staff and from cows' udders (Petzer et al. 2009).

The change in bacterial population resulting from good biosecurity measures is not an unknown phenomenon. The best examples come from intensive care units of hospitals that have implemented good hygiene practices (Sydnor & Perl 2009). In one medical study, a 9% year-on-year decrease in methicillin-resistant *S. aureus* (MRSA) cases was reported (Kallen et al. 2010). These medical studies have illustrated how the resistance profiles of bacteria can change under intensive care unit biosecurity programmes. As for the reason for the change in resistance (Table 2.2), this is most likely because of a replacement of the more resistant pathogens with environmental or 'wild type' strains of bacteria that are not yet antibiotic resistant. The term 'wild type' refers to the phenotype of the typical form of a species as it occurs in nature. Originally, the 'wild type' was conceptualised as a product of the standard 'normal' allele at a locus, in contrast to that produced by a non-standard, 'mutant' allele, typically without resistance (Merriam-Webster's Collegiate Dictionary 1999). The environmental pathogens are more genetically diverse and able to colonise environments from which the pathogenic organisms have been removed. While we have not yet characterised the change in phenotype of the organisms over time, the resistance profiles found after the 10-year period were very similar to the results of the South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD) report (Van Vuuren et al. 2007), which indicated surprisingly low resistance of *S. aureus* among South African dairy cows to the classes of antibiotic tested. In the SANVAD results, the greatest antibiotic resistance was recorded for gentamicin, ampicillin and enrofloxacin. In spite of this finding, 12.4% of isolates were multi-resistant, with most of them being resistant to ampicillin (Crestani et al. 2016, Van Vuuren et al. 2007). Ampicillin was the only product used in this study which did not show a significant decrease in antibiotic resistance over the study period (p = 0.104) (Table 2.2).

Despite the introduction of management practices, the overall prevalence of mastitis was not significantly reduced (Table 2.3). Mastitis is a complex disease involving interaction between the stress experienced by the cow, the level of milk production, parity, immune competence, udder conformation and parlour management (milking machine factors and parlour hygiene). This suggests that merely controlling the external variables such as the milking machine pressure and general hygiene is not in itself sufficient to reduce the overall prevalence of mastitis significantly, although mastitis caused by *S. aureus* decreased numerically (non-significant) during the study period (Table 2.4) (Petzer et al. 2009). While techniques of good hygiene and management have been used for the control of mastitis in dairy herds for many years (Neave et al. 1969), this has now also been shown to have an impact on antibiotic resistance, through prudent treatment selection criteria and protocols based on susceptibility

testing, which can assist dairy producers to manage effectively and eliminate *S. aureus* udder infections. These aspects should be integrated with management techniques such as general farm hygiene, milker education and supervision, routine assessment of all cows using both microbiology and cytology and strict culling programmes (Petzer et al. 2016). Attention should also be given to cow factors, and selecting for those factors that are likely predictors of resistance against mastitis. This would require that the management system should include the collection of more information related to these predictors in the management programmes. Such predictors should be determined in future studies where other associated factors in mastitis such as volume of milk produced, udder confirmation and general health could be considered.

Conclusion

The findings of this study indicate that proactive dairy herd management for mastitis control can have a major beneficial influence on the population of bacteria. This type of information will assist in the development of government and industry policy on access and use of antibiotics and inform users on some general aspects and trends of effective antibiotics. Through this proactive udder health management practice, the results show that currently used antibiotics can still be effective. The attitudes of producers and their staff also play an important role. While it is believed that these results indicate that change in management practices is a valuable tool, these types of interventions are only of value if all role players are properly integrated into the process.

Acknowledgements

The authors thank the producers for allowing them to use data obtained on their farms, and the staff of the Milk Laboratory, Department of Production Animal Studies, Faculty of Veterinary Science, Onderstepoort, for the laboratory analysis, J.C. Watermeyer and R. Badenhorst.

References

Awale, M.M., Dudhatra, G.B., Avinash, K., Chauhan, B.N., Kamani, D.R., Modi, C.M., Patel, H.B., Mody, S.K., 2012. Bovine mastitis: A threat to economy. Scientific Reports 1, 295.

Barkema, H.W., Schukken, Y.H., Zadoks, R.N., 2006. The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis, Journal of Dairy Science 89, 1877–1895. doi:10.3168/jds.S0022-0302 (06)72256-1

Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M., 1966. Kirby Bauer method antimicrobial susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology 45(4), 493–496. https://doi.org/10/1093/ajcp/45.4 ts.493

Burgos, J.M., Ellington, B.A., Varela, M.F., 2005. Presence of multidrug resistant enteric bacteria in dairy farm topsoil. Journal of Dairy Science 88(4), 1391–1398. doi:10.3168/jds.S0022-0302 (05)72806-X

Carrington, C., Du Plessis, A., Naidoo, V. (eds.), 2016. Index of Veterinary Specialities (IVS), October-December 2016, MIMS, Saxonwold.

Crestani, C., Mancini, M., Bonamico, S., Segalin, C., Busa, A., Rosa, G., Dall'Ava, B., Nannoni, E., Ostanello, F. & Barberio, A., 2016. Sensibilità in vitro nei confronti dei principali antibiotici da parte degli agenti di mastite isolati nel Triveneto negli anni 2010–2014. Large Animal Review 22, 99–106.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews 74(3), 417–433. Doi:10.1128/MMBR.00016-10

DeLaval, Milk production.com: Teat Club International, accessed 13 July 2017, from http://www.milkproduction.com/Library/Authors/Teat-Club-International/

Dufour, S., Dohoo, I.R., Barkema, H.W., Descoteaux, L., Devries, T.J., Reyher, K.K., Roy, J.P., Scholl, D.T., 2012. Manageable risk factors associated with the lactational incidence, elimination, and prevalence of *Staphylococcus aureus* intramammary infections in dairy cows. Journal of Dairy Science 95(3), 1283–1300. doi:10.3168/jds.2011-4711

Giesecke, W.H., Du Preez, J.H., Petzer, I.M., 1994. Practical mastitis control in dairy herds. Butterworths, Durban, South Africa. ISBN: 0409109231.

Henton, M.M., Eager, H.A., Swan, G.E., Van Vuuren, M., 2011. Antibiotic management and resistance in livestock production. South African Medical Journal 101(8), 583–586.

International Dairy Federation, 1985. Laboratory methods for use in mastitis work. IDF Document 132, International Dairy Federation Press, Brussels.

Kallen, A.J., Mu, Y., Bulens, S., Reingold, A., Petit, S., Gershman, K., Ray, S.M., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., Schaffner, W., Patel, P.R., Fridkin, S.K., 2010. Health care-associated invasive MRSA infections, 2005–2008. Journal of the American Medical Association 304, 641–648. doi:10.1001/jama.2010.1115

Kluytmans, J.A., 2010. Methicillin-resistant *S. aureus* in food products: Cause for concern or case for competency? Clinical Microbiology and Infectious Diseases 16(1), 11–15.

Lassen, B., 2012. Dairy production in South Africa–impressions – Country Report 2012/1. Agri Benchmark Dairy, accessed 07 June 2017, from http://www.agribenchmark.org/fileadmin/Dateiablage/B-Dairy/Country Report/12 country information SA.pdf

Leslie, K.E., Schukken, Y.H., 1999. Herd programs for eliminating and preventing *S. aureus* mastitis', in Proceedings of 32nd Annual meeting National Mastitis Council, 16 to 21 May 2016, Nashville, Tennessee, INC Part 2, 1–10.

Mellenberger, R., Kirk, J., 2001, Mastitis control program for *Staphylococcus aureus* infected dairy cows, Department of Animal Studies, Michigan State University and Veterinary Medicine Extension, School of Veterinary Medicine, University of California Davis, accessed 28 February 2018, from http://www.milkquality.wisc.edu/wp-content/uploads/2011/09/mastitis-controlprogram staph.-aureus.pdf

Merriam-Webster's Collegiate Dictionary, 1999, 10th edn., Merriam-Webster Incorporated, Springfield, MA.

Milk South Africa, Lacto Data 2013, accessed June 07, 2017, from https://milksa.co.za/reports?page=59

Modi, C.M., Patel, Hitesh, B., Patel, H.B., Mody, S.K., 2012. A comprehensive review on pharmaco-therapeutics of bovine mastitis. Molecular Microbiology 2(1), 1–9.

National Mastitis Council. Staphylococci. In Laboratory Handbook on Bovine Mastitis, 3rd ed.; National Mastitis Council: Verona, WI, USA, 2017, 43–52.

Neave, F.K., Dodd F.H., Kingwill, R.G., Westgarth, D.R., 1969. Control of mastitis in the dairy herd by hygiene and management. Journal of Dairy Science 52(5), 696–707.doi:10.3168/jds.S0022-0302(69)86632-4

Petzer, I.M., Karzis, J., Donkin, E.F., Webb, E.C., Etter, E.M.C., 2016. Pathogen specific approach towards udder health management in dairy herds using micro-cytology from routine herd investigations. Onderstepoort Journal of Veterinary Research 83(1), 1146. doi:10.4102/ojvr.v83i1.1146

Petzer, I.M., Karzis, J., Watermeyer, J.C., Van der Schans, T.J., Badenhorst, R., 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. Journal of South African Veterinary Association 80(1), 17–22. doi:10.4102/jsava.v80i1.163

Pieterse, R., 2008. Control of bacterial pathogens associated with mastitis in dairy cows with natural antimicrobial peptides produced by lactic acid bacteria. MSc thesis, Department of Microbiology, University of Stellenbosch.

Ramadhan, A.A., Hedges, E., 2005. Biofilm formation and esp gene carriage in enterococci. Journal of Clinical Pathology 58, 685–686. doi:10.1136/jcp.2004.024109

Schmidt, T., 2011. In vitro antimicrobial susceptibility of *Staphylococcus aureus* strains from dairy herds in KwaZulu-Natal. Journal of South African Veterinary Association 82(2), 76–79. doi:10.4102/jsava.v82i2.38

Schukken, Y.H., Wilson, D.J., Welcome, F., Garrison-Tofosky, L., Gonzalez, R.N., 2003. Monitoring udder health and milk quality using somatic cell counts. Veterinary Research 34, 579–596. doi:10.1051/vetres:2003028

Sol, J., Sampimon, O.C., Snoep, J.J., Schukken, Y.H., 1997. Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by *Staphylococcus aureus.* Journal of Dairy Science 80, 2803–2808. http://www.journalofdairyscience.org/article/S0022-0302(97)76243-X/pdf

Sydnor, E.R.M., Perl, T.M., 2011. Hospital epidemiology and infection control in acute-care settings. Clinical Microbiology Reviews 24(1), 141–173. doi:10.1128/CMR.00027-10

Van Vuuren, M., Picard, J., Greyling, J., 2007. South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs, University of Pretoria, Pretoria, ISBN 978-1-86854-673-2.

Witte, W., 1998. Medical consequences of antimicrobial use in agriculture. Science 279, 996–997. doi:10.1126/science.279.5353.996

Wong, P.H.P., Krosigk, M., Roscoe, D.L., Lau, T.T.Y., Yousefi, M., Bowie W.R., 2014. Antimicrobial co-resistance patterns of Gram-negative bacilli isolated from bloodstream infections: A longitudinal epidemiological study from 2002–2011. BMC Infectious Diseases 14, 393. doi:10.1186/1471-2334-14-393

World Health Organization (WHO), 2014. Antimicrobial resistance: Global report on surveillance. Geneva, Switzerland, accessed 07 June 2017, from http://www.who.int/drugresistance/documents/surveillancereport/en/

Chapter 3: Climatic and regional antibiotic resistance patterns of *Staphylococcus aureus* in South African dairy herds.

Published 10 July 2019: Joanne Karzis, Inge-Marié Petzer, Edward F. Donkin, Vinny Naidoo, Eric M.C. Etter. 2019. Onderstepoort Journal of Veterinary Research 86(1), a1674. https://doi.org/10.4102/ojvr.v86i1.1674

Significance of the work

Some of the most important topics of global relevance over the last decade are climate change and antibiotic resistance. For this study we evaluated antibiotic resistance in accordance with the effects of the specified climatic conditions in the different provinces of South Africa which is a multi-climatic country over time. The findings showed significant effects of weather conditions related to the different climatic conditions at different times of the year on antibiotic resistance in different seasons and regions. Such information will assist producers and veterinarians in decision making for improved prudent use of antibiotics in different seasons and regions.

Abstract

South Africa is a large country of approximately 1.22 million km^2, made up of nine provinces with three climatic zones. Farming in the country is mostly defined by regional differences. Of the different organisms isolated from milk samples of dairy cows, *S. aureus* poses a challenge to maintain udder health and wholesome dairy products for human consumption. Antibiotic resistant bacteria are therefore a potential health hazard. The objective of this study was to investigate the seasonal and regional relationships of antibiotic resistance of *S. aureus*, of which little is known. This study was undertaken to evaluate a data set of 3410 *S. aureus* isolates, taken from milk samples with somatic cell count >400 000 cells/mL from 830 commercial dairy herds. These isolates were tested for antimicrobial susceptibility using the Kirby Bauer method for ampicillin, cloxacillin, penicillin G, clindamycin, oxy-tetracycline, cephalexin, cefuroxime and tylosin. The samples were from 830 dairy herds, out of the estimated 2000 commercial dairy herds in South Africa. All the antibiotics tested, except for

cephalosporins, showed a predicted prevalence of resistance of above 50% in most provinces, which is a concern. The lowest prevalence of resistance to most of the categories of antibiotics tested was present in KwaZulu-Natal during spring. The cephalosporins had the lowest levels of prevalence of bacterial resistance in Gauteng during winter. Resistance patterns of *S. aureus* to the eight antibiotics varied in the different seasons and provinces, possibly because of different weather conditions, and action and spectrum of antibiotics.

Keywords: antibiotic resistance; *S. aureus*; mastitis; seasons; regions; dairy cattle

Introduction

Mastitis remains the most important economic disease in dairy cattle in first world countries despite the progress made in improving general udder health in recent years. The discovery and use of antimicrobial agents in the 20th century has been one of medical science's greatest achievements. However, bacteria are becoming increasingly resistant to these agents (Roberts 2002). Bacterial antimicrobial resistance in humans is interlinked with antimicrobial resistance in other populations, especially farm animals, which are exposed to enormous quantities of antibiotics (despite attempts at reduction) and which act as another reservoir of resistance genes (Woolhouse et al. 2015).

According to Wingfield and Kenagy (1991) and Blank (1992), seasonal changes are cyclic, largely predictable and represent the strongest and most abundant source of external variation influencing human and natural systems. Although generalisations can be made about the climate in the various provinces, there are considerable variations within each province. KwaZulu-Natal has a subtropical climate with mostly summer rainfall, while the Western Cape has a Mediterranean climate with mostly winter rainfall. The Eastern Cape has generally dry and cold winters and hot summers, but with rain mostly in late summer (Smith 2006). The Free State has mostly summer rainfall, and late summer rainfall in some parts, with cold dry winters. Limpopo province has a hot dry winter climate with summer rainfall. Mpumalanga is a summer rainfall area, divided into the Highveld with cold winters and the Lowveld which has a subtropical climate similar to that of KwaZulu-Natal. Gauteng and North West province have cold dry winters and hot summers with rainfall (Smith 2006). The incidence and duration of frost occurs in winter in the higher altitudes of the Western Cape, Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Mpumalanga and Limpopo, rather than in the rest of the country (Smith 2006). The Western Cape, Eastern Cape, Free State and North West provinces all

experience lower average temperatures than those in Gauteng and the Mpumalanga Highveld, but Limpopo province has the highest average temperatures (Smith 2006). A commonly used climate classification map is that of Wladimir Köppen (Kottek et al. 2006) which was used to develop a climate classification map for South Africa (Figure 3.1). This shows the extreme variability of the weather and climate across different parts of South Africa.

In South Africa, intramammary remedies are available by prescription from a veterinarian according to Act 101 of 1965, but also without prescription under Act 36 (Stock Remedies Act); and this enables producers to buy such products freely, which may lead to incorrect use of these products and an increase in antibiotic resistance. This unique situation explains the necessity of such a study as this, concerning antibiotic resistance, and perhaps a review of the relevant legislation.

In South Africa, little information is available on the variation of the prevalence of antibiotic resistance of *Staphylococcus aureus* (in different seasons and regions). Such knowledge will have an important impact on the treatment of animals with antibiotics, especially in South Africa with the unique climatic variations of the environment amongst the different provinces of the country.

The aims of this study were to identify the seasonal weather effects in nine provinces, in relation to the prevalence of *S. aureus* resistance to eight antibiotics that had been tested in 830 South African dairy herds over an 11-year study period.

The objectives included the calculation of the prediction of the prevalence of resistance for the different antibiotics in different seasons and provinces; a comparison of the proportions of the prevalence of bacterial resistance between all antibiotics used; the creation of time series to show trends of resistance over time; as well as to identify any relationship between seasons and or regions and the prevalence of bacterial antibiotic resistance.

Materials and Methods

From 2000 to 2010, a total of 3410 susceptibility tests were performed on *S. aureus* isolated from milk samples of commercial dairy herds in South Africa. *Staphylococcus aureus* was chosen for this study as it is one of the most important mastitis-causing bacteria. The samples represented 830 dairy herds, out of the total of approximately 2000 commercial dairy herds in

the nine provinces of South Africa, namely Gauteng (*n* = 301), KwaZulu-Natal (*n* = 369), Free State (*n* = 67), Eastern Cape (*n* = 464), Western Cape (*n* = 646), Northern Cape (NC) (*n* = 4), North West (*n* = 52), Limpopo (North) (*n* = 56) and Mpumalanga (*n* = 68) (Figure 3.2).

Most of these dairy producers send composite milk samples to the Milk Laboratory, Faculty of Veterinary Science, University of Pretoria, for testing (microbiology and cytology) on a routine basis, as part of a proactive udder health management programme. In the case of outbreaks or clinical cases, follow up testing may be done with quarter milk sample testing. The minority of producers that do not test on a routine basis may be those that test when a crisis occurs in the udder health of their herds. The milk samples were collected either by the farmers themselves according to a standard operating procedure (NMC 2004) or by professional milk samplers.

All samples were analysed at the Milk Laboratory, Faculty of Veterinary Science, University of Pretoria. Microbiological and cytological examinations were performed on all milk samples (NMC 2004). Isolates tested for antimicrobial susceptibility were all selected from milk samples with a somatic cell count (SCC) (Fossomatic 5000, Rhine Ruhr) of more than 400 000 cells/mL (NMC 2004). The Kirby Bauer method (Bauer et al. 1966) with published breakpoints was used to determine antimicrobial susceptibility. The results were based on the diameter of the inhibition zones and were classified as sensitive, intermediate or resistant in accordance with the Clinical and Laboratory Standard Institute (CLSI 2008, CLSI 2012).

Eight antibiotics used in intramammary treatment (dry and lactating remedies) that are available in South Africa were tested. These were beta-lactams (ampicillin 10 *µ*g, cloxacillin 5 *µ*g, penicillin G 10 IU), cephalosporins (cephalexin 30 *µ*g, cefuroxime 30 *µ*g), lincosamides (clindamycin 10 *µ*g), tetracyclines (oxy-tetracycline 30 *µ*g) and macrolides (tylosin 30 *µ*g). The South African national average daily milk yield was 20.2 kg in 2013, and average yield varied slightly over the years of the study period; and it would have varied between herds, within herds between cows, and in different stages of lactation (Lactodata 2013).The lactating cow numbers of the herds used varied from approximately 30 (smallest herd) to 1700 cows (largest herd), with an average of about 100–400 lactating cows tested during the period of this investigation.

The producers in this study were part of the proactive udder health programme (Karzis et al. 2018). Antibiograms were performed with every routine herd examination and the results were used by the veterinarians and producers when deciding on antibiotic treatment which was

administered by trained staff. As part of the proactive udder health programme, *S. aureus* positive cows were identified, placed in a separate group for life, milked last and culled in due course (Petzer et al. 2009).

Statistical analysis

Data from NC province were removed from analysis as they were not sufficient compared to the information available from other provinces. The results were grouped into two categories as: resistant or susceptible. Results that were originally listed as being intermediate (CLSI guidelines) were grouped together with the resistant results in this analysis, as this could only accommodate for two categories.

Comparisons of proportions of resistance of antibiotics using the Bonferroni p-value adjustment method (Benjamini & Hochberg 1995) were done to compare differences in proportions of resistance of *S. aureus* to the different antibiotics between all the antibiotics used in all provinces and for all seasons over time.

Time series analysis was used to identify both seasonality and the trend of antibiotic resistance of *S. aureus* over time for the eight antibiotics used. The 'zoo', 'xts' and 'forecast' packages of the R software© (version 3.3.3 for Mac) were used to perform these time series analyses. Data were averaged per month. Missing data were filled using the seasonal Kalman filter. A local polynomial regression smoothing (LOESS 'LOcal regrESSion') was applied to the data in order to extract first the effect of seasonality. The seasonal component was removed from the data and the same method was applied to obtain the trend (Cleveland et al. 1990). The final result of the stepwise analysis of the data into seasonal, trend and remainder components was obtained after several iterations of this process. To test the goodness of fit of the stepwise analysis, the autocorrelation function of the 'remainders' (residues) was analysed to check if they were stationary. An augmented Dickey–Fuller test (ADF test) was used to confirm stationary residues compared to the null hypothesis which was that the time series had a unit root.

Further apparent relationships between season and province on the prevalence of antibiotic resistance of all *S. aureus* isolates were tested with a general linear mixed model (GLMM) ('glmer' within 'lme4' package with R software© version 3.3.3) using a logit link-function. This model allowed the random effect from the different herds and from the repetition of data collection over time, to be taken into account. This type of GLMM takes into account this

random effect when comparing the different provinces and the different seasons as well as the interactions between seasons and provinces. The existence of interaction between provinces and seasons was checked by using logistic regression. In case of the existence of these interactions, all the potential interactions were compared with the specific interaction showing the lowest level of antibiotic resistance prevalence (according to univariate pre-analysis). Odds ratios (OR), their confidence interval and the associated p-value (using the threshold $p = 0.05$ for statistical significance) were calculated from the results of the GLMM. When no statistically significant interaction was found, the GLMM was re-run to remove the interaction and to compare seasons and provinces, respectively, using for both of them the specific seasons and or provinces showing the lowest antibiotic resistance prevalence as reference (according to univariate pre-analysis). Comparisons were also based on OR calculated from the GLMM using the following formula:

OR = exp (Estimate),
$OR-LL_{95\%}$ = exp (Estimate−1,96*se),
OR−exp (Estimate+1,96*se), [Eqn 1]

where 'Estimate' is the estimate of the fixed effect obtained from the GLMM and 'se' the standard error associated with the estimate of the fixed effects. $LL_{95\%}$ and $UL_{95\%}$ are the lower limit of the 95%confidence interval and the upper limit of the 95% confidence interval, respectively.

To calculate the prevalence of resistance for each season, province or interactions and their confidence intervals with an accepted error of 5% the results of the GLMM could not be used directly. Indeed, the formula applied on the results of the 'glmer' function to obtain the respective prevalence of resistance for all seasons, provinces or for all the potential interaction does not take into consideration the random effect. Therefore, the prediction of level of resistance was used, using the GLMM as a model applied to the complete data set based on the assumption that the data set was truly a good representative sample of the population of interest. The 'predict' function of the 'lme4' package adapted for fitted mixed-effect models was used to calculate the prevalence of resistance per province, per season or per province within season.

The data analysis accounted for all seasons: spring (n = 482) (01 September to 30 November); summer (n = 404) (01 December to 28 February); autumn (n = 530) (01 March to 31 May);

winter (n = 607) (01 June to 31 August). This is a broad classification as used as by the weather services, although definitions of seasons and rainfall patterns can be variable.

Results

In this study, the overall comparison of proportions of resistance of *S. aureus* between the antibiotics investigated was significantly different for all pairs indicated, except for penicillin G and ampicillin, clindamycin and ampicillin and for oxy-tetracycline and cloxacillin. Of the eight antibiotics tested, tylosin and penicillin G showed the highest prevalence of resistance (67.1% and 50.3%, respectively), cefuroxime and cephalexin the lowest (14.39% and 23.05%, respectively) (Table 3.1).

From the time series analysis, the stepwise analysis of the data for all the antibiotics resulted in stationary remainders confirmed by the analysis of the correlogram and the ADF test. The trends, seasonal relationships of the data (repeated pattern per year) and remainders were calculated in the time series carried out for all eight antibiotics used.However, a meaningful trend from the time-series stepwise analysis was shown only for ampicillin (Figure 3.3)

Concerning the GLMM, we found that the repetition of the data collected over time did not have an effect, whereas there was a clear effect of farm, for all the antibiotics tested.

The GLMM applied to the data for *S. aureus* resistance to ampicillin, penicillin G, clindamycin and tylosin showed that there was no interaction between province (Table 3.2) and season (Table 3.3); thus, these were tested separately. In addition, no significant differences were shown between the prevalence of antibiotic resistance according to the season for tylosin.

For the prevalence of antibiotic resistance for *S. aureus* to cefuroxime, cephalexin, oxy-tetracycline and cloxacillin, the GLMM analysis showed significant interactions between season and province (Table 3.4). The prevalence of *S. aureus* resistance to cloxacillin in winter was significantly higher than in spring, and the prevalence of antibiotic resistance in North West and Free State provinces was also significantly higher than the prevalence of antibiotic resistance in KwaZulu-Natal.

The results presented in Tables 3.2, 3.3 and 3.4 show the predictions according to the model with presentation of the significant associations of seasons or provinces or the interactions of seasons and provinces for each antibiotic investigated.

In KwaZulu-Natal during spring, the *S. aureus* isolates had the lowest prevalence of antibiotic resistance for clindamycin (lincosamide) and for the beta-lactam group (ampicillin, cloxacillin and penicillin G) when compared to all other provinces and seasons. Findings generated in KwaZulu-Natal during spring (lowest level of antibiotic resistance) were therefore used as baseline against which the other provinces and seasons were compared (Tables 3.2 and 3.3). Conversely, cefuroxime and cephalexin showed a different trend to the rest of the antibiotics, by having the lowest prevalence of *S. aureus* antibiotic resistance in Gauteng and in winter (Table 3.4). Autumn in KwaZulu-Natal was the season that showed the lowest prevalence of *S. aureus* antibiotic resistance for oxy-tetracycline (Table 3.4). In addition, the prevalence of resistance of *S. aureus* had a direct relationship to season and provinces as well as the interaction of season and provinces.

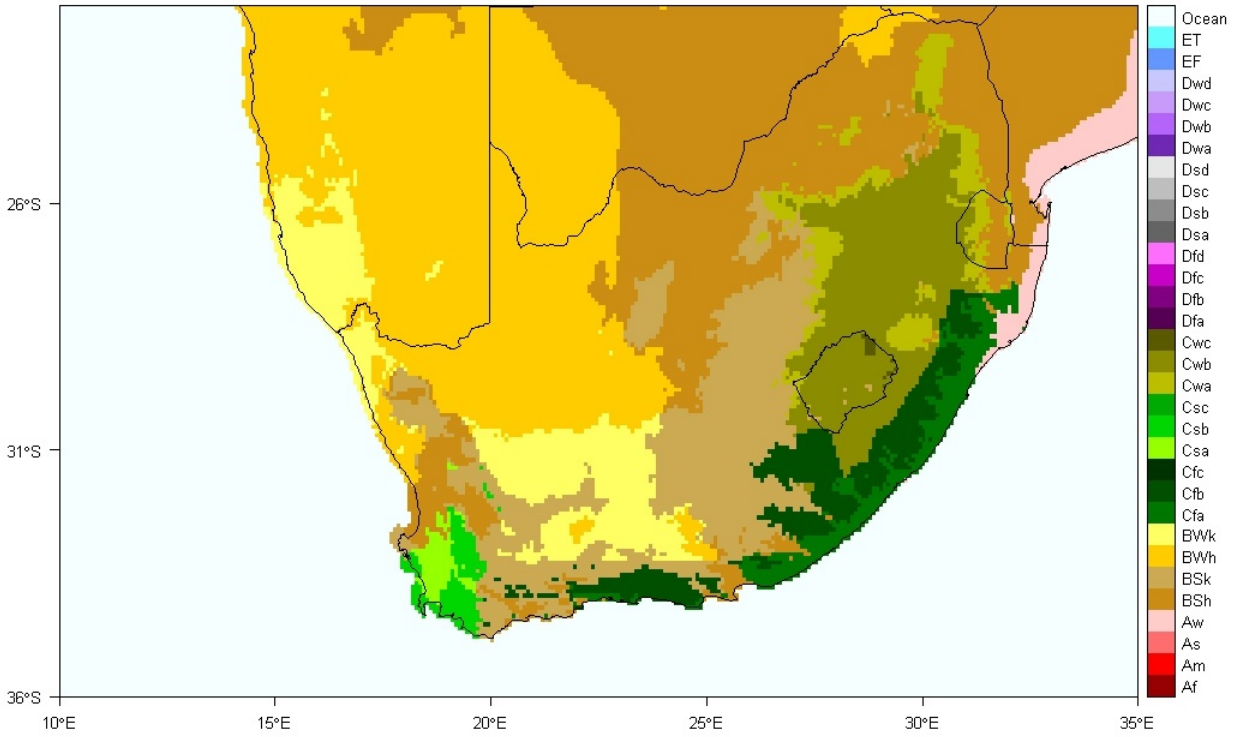

Figure 3.1 Map of South Africa with the Köppen-Geiger climate classification.

ET, Tundra Climate; EF, Ice cap climate; Dwd/Dsd/Dfd, sub-arctic or boreal climate with severe winters; Dwc/Dfc/Dsc, sub-arctic or boreal climates; Dwb/Dsb/Dfb, warm summer continental or hemi-boreal climates; Dwa/Dsa/Dfa, hot summer continental climates; Cwc, temperate dry winter cold summer; Cwb, dry winter highland climate; Cwa, subtropical dry winter; Csc, Mediterranean warm/cool summer climates; Csb, Mediterranean cold summer climates; Csa, Mediterranean hot summer climates; Cfc, sub-polar oceanic climate; Cfb, oceanic climate; Cfa, humid subtropical climates; BWk, arid desert cold climate; BWh, arid desert hot climate; BSk, arid steppe cold climate; BSh, arid steppe hot climate; Aw, tropical savanna wet climate; As, tropical savanna dry climate; Am, tropical monsoon climate; Af, tropical rainforest climate.

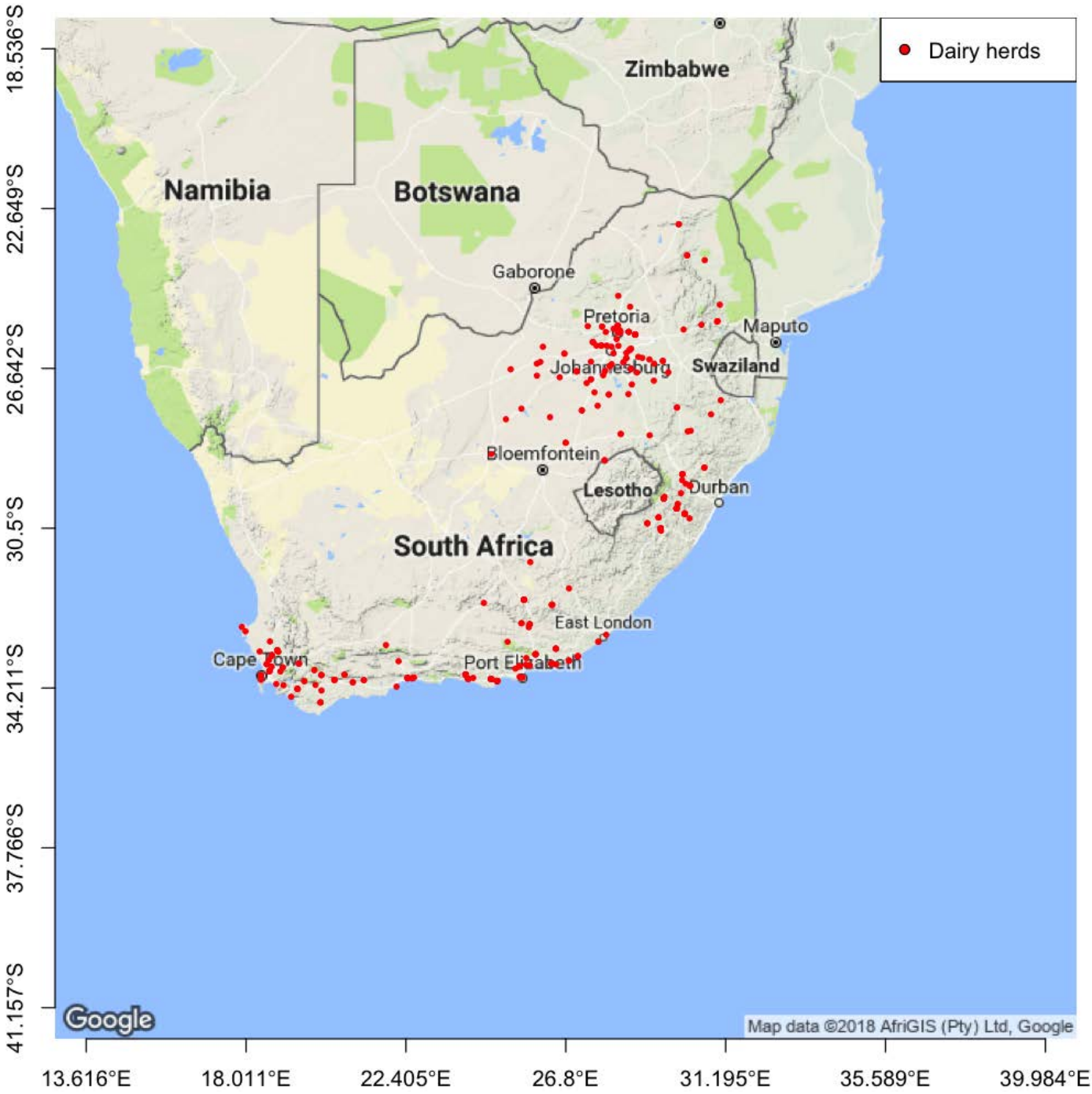

Figure 3.2 Distribution of milk sampling locations in South Africa.

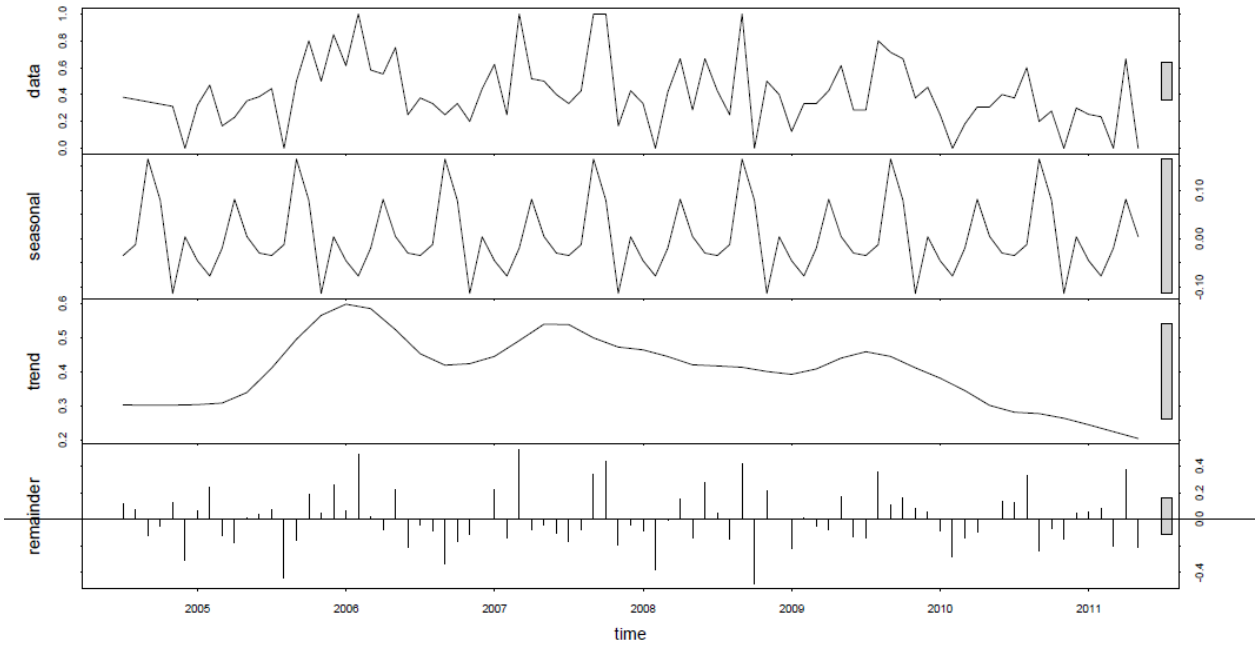

Figure 3.3 Time series of resistance of *Staphylococcus aureus* to ampicillin, showing the data, trend, seasonal effects and the remainder.

Table 3.1 P-values obtained from the pairwise comparison of proportions of prevalence between the eight antibiotics tested for antibiotic resistance of *Staphylococcus aureus* (over the 11-year period and in all seasons), using the Bonferroni adjustment method.

Antibiotics	**Ampicillin**	**Cefuroxime**	**Cloxacillin**	**Penicillin G**	**Cephalexin**	**Clindamycin**	**Oxy-tetracycline**	**Tylosin**
Cefuroxime	**	-	-	-	-	-	-	-
Cloxacillin	**	**	-	-	-	-	-	-
Penicillin G	1	**	**	-	-	-	-	-
Cephalexin	**	**	**	**	-	-	-	-
Clindamycin	0.23	**	**	**	**	-	-	-
Oxy-tetracycline	**	**	1	**	**	**	-	-
Tylosin	**	**	**	**	**	**	**	-
Resistance (%)	47.28	14.39	30.04	50.30	23.05	42.94	31.99	67.10

Note: Resistance, overall antibiotic resistance.

**, means p-value = $<10^{-5}$.

Table 3.2 Prediction of expectation of *Staphylococcus aureus* antibiotic resistance to ampicillin, penicillin G, clindamycin and tylosin for the provinces showing significant differences according to the general linear mixed model.

Product	Province	Resistance (%)
Ampicillin	KZN†	34
	WC‡	56
Penicillin G	KZN†	37
	WC‡	57
Clindamycin	KZN†	23
	NW‡	58
Tylosin	KZN†	56
	L‡	83

KZN, KwaZulu-Natal; L, Limpopo, NW, North West; WC, Western Cape.

†, Indicates the province with the lowest prevalence of antibiotic resistance within which a significant difference existed.

‡, Indicates the provinces which had significantly higher antibiotic resistance than KZN.

Table 3.3 Prediction of expectation of *Staphylococcus aureus* antibiotic resistance to ampicillin, penicillin G and clindamycin for different seasons where significant differences were shown according to the analysis using the general linear mixed model.

Product	Season	Resistance (%)
Ampicillin	Spring†	39
	Summer‡	48
Penicillin G	Spring†	41
	Summer‡	53
Clindamycin	Spring†	36
	Autumn‡	43

†, Indicates the season which showed the lowest level of antibiotic resistance within which a significant difference existed.

‡, Indicates the seasons with significantly higher level of antibiotic resistance than spring.

Table 3.4 Prediction of expectation of *Staphylococcus aureus* of antibiotic resistance to cefuroxime, cephalexin, oxy-tetracycline and cloxacillin for different seasons and provinces where significant differences were shown according to the analysis using the general linear mixed model.

Product	Season	Province	Resistance (%)
Cefuroxime	Winter†	GP†	3
	Autumn‡	NW	33
Cephalexin	Winter†	GP†	8
	Summer‡	FS	73
Oxy-tetracycline	Autumn†	KZN†	12
		GP	61
		L	61
Cloxacillin	Spring†	KZN†	17
	Winter‡	FS	57
		NW	57

FS, Free State; GP, Gauteng; KZN, KwaZulu-Natal; L, Limpopo; NW; North West.

†, Indicates the season and province together which showed the lowest level of antibiotic resistance within which a significant difference existed.

‡Indicates the seasons and provinces with significantly higher level of antibiotic resistance.

Discussion

The antibiotics used in this study differed in their action and also in their spectra. The beta-lactams are bactericidal, while clindamycin, oxy-tetracycline and tylosin are bacteriostatic. Ampicillin, cloxacillin, cefuroxime and oxy-tetracycline are broad spectrum antibiotics, while penicillin G, cephalexin, clindamycin and tylosin are active against Gram-positive micro-organisms. There have been variations in the prevalence of *S. aureus* antibiotic resistance overall (Table 3.1) and in different provinces and different seasons (Tables 3.2, 3.3 and 3.4). Reasons for these variations are unclear. Antibiotic resistance could also occur from random genetic mutations and subsequent natural selection of bacteria in order for them to survive.

The decreasing trend of antibiotic resistance found in a previous study conducted in South Africa (Karzis et al. 2018) was confirmed for ampicillin, using the time series analysis in the current study (Figure 3.3). The lowest level of antibiotic resistance of *S. aureus* to beta-lactams and clindamycin was in KwaZulu-Natal during spring, which implied a significant positive effect or association of this season on the prevalence of resistance. This could be possibly because of the increased daily milk production potential of cows grazing on pastures as well as the calving patterns in KwaZulu-Natal in spring. A previous study, in which seasonal effects were not investigated, showed that the overall prevalence of mastitis did not change during the same 11-year study period (Karzis et al. 2018).

A possible reason for lower prevalence of antibiotic resistance of *S. aureus* to cephalosporins during winter in Gauteng may be that the dry cold season has generally a lower probable prevalence of intramammary infections that would require less treatment. This would be supported by the occurring higher average incidence of frost duration in Gauteng (Smith 2006), which would have suppressed insect vectors associated in mastitis pathogen transmission (Zadocks et al. 2011). Mastitis prevalence in different seasons and provinces of South Africa must be investigated further in future to evaluate any possible association with bacterial antibiotic resistance. Mastitis has been known to increase in wet conditions because of muddy paddocks and bedding, which increases hygiene and management challenges during these periods. In this study, the highest number of samples used was from the main dairy producing provinces, namely KwaZulu-Natal, Eastern Cape and Western Cape (Figure 3.2). Most provinces showed a prediction of the occurrence of bacterial resistance for *S. aureus* to these antibiotics of more than 50%, which is of concern (Tables 3.2, 3.3 and 3.4).The lowest prevalence of resistance of *S. aureus* to oxy-tetracycline (12%) was shown in KwaZulu-Natal in autumn; and the lowest levels of resistance to tylosin (56%) were shown in KwaZulu-Natal, with no apparent difference between seasons. Tylosin is one of the few intramuscular products registered for use in the treatment of mastitis in South Africa, which has been found not to be effective.

The highest prevalence of *S. aureus* resistance against penicillin G and ampicillin was indicated during the hot summer months in South Africa (Table 3.3). The only antibiotic that showed an increase in the prevalence of resistance in *S. aureus* in winter was cloxacillin in North West and the Free State (Table 3.4). However, these observed effects were only on a small group of antibiotics (ampicillin and penicillin G), compared to the overall number of eight antibiotics used in this study. Thus, these observed effects probably indicated no general effect. It is difficult to account for the reasons causing these differences.

The data also showed that most of the identified resistance of *S. aureus* was to the beta-lactam group. These are the products most often used currently as intramammary remedies in South Africa (as there is only a small selection of remedies available) (Eagar, Swan & Van Vuuren 2012).

The seasonal and regional effects on antibiotic resistance in South Africa cannot be compared directly to studies in other countries, as climatic conditions, rainfall patterns and related management systems differ. Based on the findings of this study on seasonal and regional differences, veterinarians in practice should rather use local surveillance data in preference to national or international data.

In addition to the different weather and ecological conditions (Figure 3.2), there are also differences in feeding systems [such as with total mixed rations (TMRs)] where cows are kept both in free-stall barns and camps on the one hand or on pastures on the other. The number of milkings per day and

in the average herd size in the different provinces also vary (468 in Eastern Cape, 367 in KwaZulu-Natal and 203 in Western Cape, with herd size in the rest of the country ranging between 96 and 175) (Lactodata 2013).

KwaZulu-Natal, the Eastern Cape and Western Cape have mostly pasture-based herds that are milked twice per day. In the rest of South Africa, the high producing large herds are mostly herds fed TMRs which are milked three times per day. This may have had an effect on antibiotic efficiency and the development of resistance because the intramammary products widely available in South Africa are mostly time-dependant. Three times a day milking could also have had an effect on the prevalence of mastitis pathogens, as the cows are handled more frequently (eight hourly), and have the potential to be exposed to more pathogens in the parlour if the milking hygiene is not excellent. This would explain the results of this study, which showed that in KwaZulu-Natal (where herds were generally milked twice per day and were mainly pasture fed herds), there was the lowest level of resistance for almost all antibiotics tested. The different usage of intramammary antibiotics (lactating and dry cow therapies) in the different provinces could also possibly have contributed to a difference in bacterial antibiotic resistance in different provinces. Unfortunately, these data were not made available by suppliers. A study by Fox et al. (1995) also found that location, herd and season significantly influenced prevalence of intramammary infections, which is similarly implied in this study which found that prevalence of antibiotic resistance can be influenced by provinces and seasons.

Challenging weather conditions and climatic differences may cause bacteria to form protective mechanisms such as biofilm which might cause an increase in the prevalence of antibiotic resistance (Da Silva Meira et al. 2012, Melo et al. 2014). Many normal microbiota bacteria produce a capsular polysaccharide matrix (glycocalyx) to form a biofilm. A biofilm is a system that can be adapted to changing environmental conditions and constitutes a physical barrier, which protects the encased bacteria from detergents and sanitizers (Potera 1999). The hypothesis is that the variation in prevalence of antibiotic resistance of bacteria between seasons that show extreme weather conditions may be because of the variation in the population of bacteria that can produce biofilm in the different seasons. The findings of this study might be related to such a hypothesis.

The ability of *S. aureus* strains to produce biofilm and conditions enhancing this production have not been studied under South African conditions in the different seasons and provinces.

According to Atulya et al. (2014), an acidic pH has a positive correlation with biofilm formation. The current intramammary antibiotics available for use for mastitis in South Africa include highly acidic antibiotics such as penicillins, which could favour the formation of biofilm and the establishment of infections in udder tissue. However, lipid soluble (non-ionised) antibiotics such as clindamycin are able to penetrate the biofilm (Kundukad et al. 2017). The ionised form of an antibiotic has low lipid

solubility (but high water solubility) and high electrical resistance, and thus cannot penetrate cell membranes easily (e.g. ampicillin, cloxacillin, penicillin G and oxy-tetracycline) (Shargel et al. 2012). Evidence indicates that photoperiod-driven physiologic changes are typical in mammalian species, including some in humans (Dowell 2001). These are examples of how the environment affects survival of organisms in general.

Future studies need to be conducted to determine antibiotic resistance genes present in *S. aureus* isolated from dairy cattle in South Africa, as new *S. aureus* strains have been identified (Monistero et al. 2018). In addition, the determination of biofilm expression of these isolates should be checked for seasonal and regional patterns. Other factors such as calving pattern and parlour design that may also contribute to the development of mastitis and antibiotic resistance in different seasons and regions also need to be investigated.

Conclusion

There were seasonal and regional apparent effects on the prevalence of antibiotic resistance in *S. aureus* in South Africa.

Reasons for these associations may be attributed to factors such as the different weather, ecological and rainfall patterns and differences in management systems, such as milking intervals during the different seasons and in different provinces, as well as the feeding systems used in different provinces. Resistance patterns were also found to differ between bacteriostatic and bactericidal antibiotics depending on spectrum (narrow vs. broad).

Management and hygiene challenges are increased under the warmer and wetter more challenging conditions in the various South African provinces and at different times of the year.

It is of concern that all the antibiotics tested, except for cefuroxime, showed a predicted prevalence of resistance of above 50% in most provinces. The lowest predicted prevalence of resistance was in KwaZulu-Natal in spring for all antibiotics tested except for the cephalosporins which had a lower prevalence in Gauteng during winter.

Acknowledgements

The authors would like to thank the producers for allowing them to use data obtained on their farms to obtain the knowledge, and the staff of the Milk Laboratory, Department of Production Animal Studies, Faculty of Veterinary Science, Onderstepoort, for the laboratory analysis, J.C. Watermeyer and R Badenhorst.

References

Atulya, M., Jesil, A.M., Venkata, J.R., Mallikarjuna, C.R., 2014. Influence of milk components in establishing biofilm mediated bacterial mastitis infections in cattle: A fractional factorial approach. Research in Veterinary Science 96, 25–27. doi:10.1016/j.rvsc.2013.12.001

Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M., 1966. Kirby Bauer method antimicrobial susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology 45(4), 493–496. doi:10.1093/ajcp/45.4_ts.493

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 57, 289–300, accessed 08 January 2019, from http://www.jstor.org/stable/2346101

Blank, J.L., 1992. Phenotypic variation in physiological response to seasonal environments. in T.E. Tomasi & T. Horton (eds.), Mammalian energetics: Interdisciplinary views of metabolism and reproduction, pp. 186–212, Comstock Publishing Associates, Ithaca, NY.

Cleveland, R.B., Cleveland, W.S., McRae, J.E., Terpenning, I., 1990. STL: A seasonal-trend decomposition procedure based on loess. Journal of Official Statistics 6, 3–73.

Clinical and Laboratory Standards (CLSI) formerly NCCLS General Susceptibility testing (SCM03), 2012, Clinical and Laboratory Standards Institute, 32(3), accessed 01 August 2017, from www.techstreet.com/CLSI

CLSI, 2008, Performance standards for antimicrobial susceptibility testing: Twentieth information supplement, CLSI document M100-S20, Clinical and Laboratory Standards Institute, Wayne, PA.

Da Silva Meira, Q.G., Barbosa, I.M., Aguiar Athayde, A.J.A., Siqueira-Junior, J.P., Souza, E.L., 2012, ‘Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food-

contact surfaces and sensitivity to sanitizers'. Food Control Journal 25(2), 469–475. doi:10.1016/j.foodcont.2011.11.030

Dowell, S.F., 2001. Seasonal variation in host susceptibility and cycles of certain infectious diseases. Emerging Infectious Diseases 7(3), 369–374. doi:10.3201/eid0703.017301

Eagar, H., Swan, G., Van Vuuren, M., 2012. A survey of antimicrobial usage in animals in South Africa with specific reference to food animals. Journal of the South African Veterinary Association 83(1), Art. #16, 1–8.

Fox, L.K., Chester, S.T., Hallberg, J.W., Nickerson, S.C., Weaver, L.D., 1995. Survey of intramammary infections in dairy heifers at breeding age and first parturition. Journal of Dairy Science 78, 1619–1628. doi:10.3168/jds.S0022-0302 (95)76786-8

Karzis, J., Petzer, I.M., Donkin, E.F. & Naidoo, V., 2018. Proactive udder health management in South Africa and monitoring of antibiotic resistance of *Staphylococcus aureus* in dairy herds from 2001 to 2010. Journal of South African Veterinary Association 89, a1490. doi:10.4102/jsava v89i0-1490

Kottek, M., Grieser, J., Beck, C., Rudolf, B., Rubel, F., 2006. World map of the Köppen-Geiger climate classification updated'. Meteorological Journal 15(3), 259–263. doi:10.1127/0941-2948/2006/0130

Kundukad, B., Schussman, M., Yang, K., Seviour, T., Yang, L., Rice, S.A., Kjelleberg, S., Doyle, P.S., 2017. Mechanistic action of weak acid antibiotics on biofilms. Nature 7, 4783. doi:10.1038/s41598-017-05178-3

Lactodata, Volume 16(2), November 2013, accessed 07 June 2017, from www.milksa.co.za/category/all-reports/industry-information

Melo, P.C., Sousa, C., Botelho, C., Oliveira, R., Nader-Filho, A., 2014. NaOCl effect on *Staphylococcus aureus* biofilm isolated from the milking environment and mastitis infected cows. Brazilian Journal of Veterinary Research 34(2), 109–113.doi:10.1590/S0100-736X2014000200002

Monistero, V., Graber, H.U., Pollera, C., Cremonesi, P., Castiglioni, B., Bottini, E., Ceballos-Marquez, A., Lasso-Rojas, L., Kroemker, V., Wente, N., Petzer, I.M., Santisteban, C., Runyan, J., Veiga Santos, M., Gomes Alves, B., Piccinini, R., Bronzo, V., Abbassi, M.S., Said, M.B., Moroni, P., 2018. *Staphylococcus aureus* isolates from bovine mastitis in eight countries: Genotypes, detection

of genes encoding different toxins and other virulence genes. Preprints. doi:10.20944/preprints201804.0325.v1

National Mastitis Council (NMC), 2004. Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality, 4th edn., NMS, accessed 01 August 2017, from www.nmconline.org

National Mastitis Council (NMC), 2017. Staphylococci. In Laboratory Handbook on Bovine Mastitis, 3rd ed.; National Mastitis Council: Verona, WI, USA, 2017, 43–52.

Petzer, I.M., Karzis, J., Watermeyer, J.C., van der Schans, T.J. & Badenhorst, R., 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. Journal of South African Veterinary Association 80(1), 17–22. doi:10.4102/jsava.v80i1.163

Potera, C., 1999. Forging a link between biofilms and disease. Science 283(5409), 1837–1839. doi:10.1126/science.283.5409.1837

Roberts, M.C., 2002. Resistance to tetracycline, macrolide-lincosamide-streptogramin, trimethoprim, and sulfonamide antibiotic classes. Molecular Biotechnology 20(3), 261–283. doi:10.1385/MB:20:3:261

Shargel, L., Wu-Pong, S., Yu, A.B.C., 2012. Applied biopharmaceutics and pharmacokinetics, 6th edn., McGraw-Hill Education, New York.

Smith, B., 2006. The farming handbook, University of KwaZulu Natal Press, KwaZulu Natal South Africa & CTA, The Netherlands, ISBN 10:1-86914-090-7, ISBN 13:978-1-86914-090-8.

Wingfield, J.C., Kenagy, G.J., 1991. Natural regulation of reproductive cycles. in Pang, P.K.T., Schreibman, M.P., (eds.), Vertebrate endocrinology: Fundamentals and biomedical implications, pp. 181–241, Academic Press, New York.

Woolhouse, M., Ward, M., van Bunnik, B., Farrar, J., 2015. Antimicrobial resistance in humans, livestock and the wider environment. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences Royal Society (Great Britain) 370(1670) 1–7. doi:10.1098/rstb.2014.0083

Zadocks, R.N., Middleton, J.R., McDougall, S., Katholm, J., Schukken, Y.H., 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. Journal of Mammary Gland Biology and Neoplasia 16(4), 357–372. doi:10.1007/s10911-011-9236-y

Chapter 4: Challenging the conventional identification of coagulase positive staphylococci through the use of molecular techniques

Significance of the work

Bacterial evolution is a phenomenon which is constantly challenging the conventional identification of many microorganisms, which may lead to the discovery of new micro-organisms or new strains of micro-organisms or to the re-classification of existing micro-organisms. At the start of this research the focus was on antibiotic resistance profiles of *S. aureus* . However, during the routine testing of *S. aureus* (microbiology, cytology and antibiotic susceptibility testing) a different strain of *Staphylococcus* spp. was identified. Initially this was noticed in a single dairy herd in South Africa with many *S. aureus* (as identified at the time), with a somatic cell count (SCC) below 100 000 cells/ml milk. This unusual finding led to the further investigation of this emerging pathogen which differed from *S.* aureus identified according to the usual procedures in use at that time as well as further phenotypic differentiation of *Staphylococcus* spp. using maltose agar. This initial evaluation showed the strain to be maltose negative compared to the usual *S. aureus.* Final genome analysis confirmed the strain to be *S. aureus* albeit with an abnormal maltase gene. This was an important finding and consequently the maltose negative isolates were reported to clients to explain that these staphylococci were different from the maltose positive staphylococci and therefore they required different management and treatment procedures.

The MLST (multi locus sequence typing) results identified the maltose negative *S. aureus* as *S. aureus* ST 2992, an identical strain of single origin, isolated repeatedly over time. The later molecular identification and sequencing of the maltose negative *S. aureus* ST 2992 led to the discovery of the stop codon on the *malA* gene of this strain which most likely terminates the α-glucosidase protein which is responsible for the maltose reduction. This important finding explains the phenotypic difference between the maltose negative and maltose positive *S. aureus* which react differently in practice. The maltose negative *S. aureus* is less pathogenic and shows greater antibiotic resistance and thus needs to be treated differently. Although these results are from a relatively small sample, this finding also highlights the importance of differentiation between maltose positive and maltose negative *S. aureus* strains in routine veterinary diagnostics. However, the relatively quick and cost effective conventional microbiology methods, although not entirely accurate, are still acceptable to use for routine diagnostics. This study also shows that the maltose negative phenotype can probably be attributed to differences on the *malA* and/or *malR* genes.

Submitted for publication, December 2019: Joanne Karzis, Inge-Marié Petzer, Edward F. Donkin, Vinny Naidoo, Christiaan Labuschagne. (Journal of Dairy Science)

Abstract

The most clinically relevant staphylococci in veterinary medicine are those that are coagulase positive, namely *S. aureus* and *S. pseudintermedius*. Over years of detailed monitoring of dairy herds, a new strain of coagulase positive and maltose negative *S. aureus* appears to be emerging as a pathogen in South Africa. This study challenges the adequacy of the conventional microbiological method by following up with MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionisation Time-of-Flight, Mass Spectrometry) and the 16S rRNA sequencing method (test of superior quality which serves as a point of reference against which other tests of its type may be compared). Initially, a limited number of bacterial colonies of the maltose negative staphylococcus used were confirmed as being *S. aureus* and not another species of Staphylococcus, tentatively identified by the conventional microbiological methods as *Staphylococcus pseudintermedius* (*S. pseudintermedius)*. The strains of coagulase positive and maltose negative *S. aureus* tested positive for both *malA* and *malR* genes (using a maltose positive *S. aureus* ATCC culture as a control. Thus the α-glucosidase gene was shown to be present, but it was not being expressed phenotypically, which may possibly be attributed to the abnormal stop codon present. This newly identified maltose negative *S. aureus* was less pathogenic than the conventionally identified maltose positive *S. aureus,* and can be treated more like coagulase negative staphylococci where only clinical cases are treated, rather than the usual treatment for *S. aureus* (maltose positive).

Keywords: *Staphylococcus aureus*, maltose negative, conventional identification, MALDI-TOF MS, 16s rRNA, Multi-Locus Sequence Typing, *malA*, *malR*

Introduction

Staphylococcus aureus is one of the leading sources of intra-mammary infections (IMI) in dairy cows (Dufour et al. 2012, Zecconi & Scali 2013). It has been reported that 11% of the IMI are caused by *S. aureus* in South Africa (Petzer et al. 2009) and 10–40% in other countries (Kateete et al. 2013, Basanisi et al. 2017, Liu et al. 2017). The most clinically relevant staphylococci in veterinary medicine are those that are coagulase positive, namely *S. aureus* and members of the *Staphylococcus intermedius* group (SIG), in particular *S. pseudintermedius* (previously part of the *S. aureus* classification). The colonization of dairy herds and the subsequent contamination of raw milk by *S. aureus*, especially those isolates which express multi-drug resistance, biofilm formation and

the ability to produce toxins, remains an important concern for both the dairy producer and public health (Wang et al. 2018).

The Milk Laboratory at the Faculty of Veterinary Science of the University of Pretoria provides an extensive dairy cow udder monitoring programme in South Africa. Since 2005, the Laboratory has been isolating an increasing number of coagulase positive and maltose negative staphylococci which were tentatively identified as *S. pseudintermedius*. *Staphylococcus intermedius* was described as a species in 1976 based on guanine and cytosine content and phenotypic tests (Hajek 1976).

The *S. intermedius group* forms part of the normal microflora of the skin and mucosa of dogs and cats (Cox et al. 1985, Cox et al. 1988, Talan et al. 1989) and has also been found in a wide range of other animals including horses, goats, minks, foxes, raccoons and pigeons. During the past few years, there has been confusion about the classification of *S. intermedius*. In 2005, an organism named *S. pseudintermedius* was described based on 16S rRNA gene sequence analysis of isolates from a cat, a dog, a horse and a parrot (Devriese et al. 2005). More recently, isolates that were all maltose negative, and formerly identified as *S. intermedius* by phenotypic characteristics were reclassified (Sasaki et al. 2007) into three clusters namely: *S. intermedius, S. pseudintermedius* and *Staphylococcus delphini* (*S. delphini*), based on the nucleotide sequence analysis of the sodA and hsp60 genes (Sasaki et al. 2007). The *S. intermedius* strain from dairy cattle has yet to be characterised. In this study we attempted to characterise this emerging strain of staphylococcus by evaluating the isolates through conventional microbiology (maltose test), MALDI-TOF MS and the genetic 16s r RNA sequencing and further multi-locus sequence typing (MLST).

Conventional microbiological methods identify *Staphylococcus* spp. based on colony morphology, haemolysis patterns, Gram staining, catalase and coagulase production (Jorgensen et al. 2015). The coagulase test determines whether bacteria produce clots in plasma based on the coagulase and von Willebrand factor binding protein (vWbp) (Yu et al. 2017). The clumping test is based on agglutination in plasma, that is mediated by Coa, vWbp and clumping factor A (ClfA) from the bacterial and cell envelope (Yu et al. 2017). *Staphylococcus aureus* produces coagulase while coagulase negative staphylococci (NAS) as the name indicates lack coagulase activity. However, several staphylococcal species are now known to be variable regarding coagulase and Coa and vWbp genes (De Buck et al. 2017, Becker et al. 2014). These genes protect *S. aureus* from phagocytosis (McAdow et al. 2011).

In South Africa, as with routine veterinary diagnostics worldwide, the focus is on a fast turnaround time and relatively low cost. Therefore staphylococci are identified by conventional methods that use the coagulase test (Staphylase test, Oxoid), as the core test. However, in more recent years, further diagnosis of coagulase positive staphylococci on maltose agar has been known to differentiate *S.*

aureus from the *S. intermedius* group (Devriese et al. 2005). The purpose of the maltose fermentation test is to detect whether a microbe can ferment the carbohydrate maltose and utilize it as a carbon source. The fermentation of maltose to produce acid end products, will decrease the pH of the maltose agar, which will in turn cause a colour change due to the pH indicator in the agar, thus confirming *S. aureus* (Jorgensen et al. 2015).

The rRNA genes (5S, 16S, and 23S) and intergenic regions are specific for prokaryotes and are commonly used for taxonomic purposes (CLSI MM18-A 2010). These genes are present in all organisms and are considered to be only weakly affected by horizontal gene transfer and they contain mosaics of sequence stretches ranging from highly conserved to variable (CLSI MM18-A 2010). These characteristics make the 16S rRNA gene the most widely used region for bacterial taxonomy and identification (CLSI MM18-A 2010).

The balance of strain predominance and heterogeneity in *S. aureus* is such that a considerable effort has been invested in the identification of strain specific outcomes of infection. This has led to a demonstration of strain-specific associations with somatic cell count (Dingwell et al. 2006, van den Borne et al. 2010, Zadocks et al. 2000), milk yield (Middleton & Fox 2002), biofilm production (Fox et al. 2005), clinical signs (Haveri et al. 2005, Zadocks et al. 2000), persistence (Haveri et al. 2007) and treatment response (Dingwell et al. 2006, Graber et al. 2009, van den Borne et al. 2010). Not all studies support an association between clinical outcomes and strain (Larsen et al. 2000, Middleton & Fox 2002, Middleton et al. 2002).

The conventional identification method used in this study initially in the routine microbiological testing was based on the study by Hajek (1976) and later verified by (Sasaki et al. 2007). However, because of new molecular information and classification of a maltose negative *S. aureus* (Johler et al. 2019), the purpose of this study was to be certain that this organism was a classical *S. aureus* (maltose positive) compared to *S. pseudintermedius* (maltose negative) or a similar organism and also to revalidate the phenotype identification system that is currently in use in South Africa. A previous study in the Netherlands has identified a coagulase negative, maltose negative *S. aureus* strain (Johler et al. 2019) which can make diagnosis based on the maltose phenotype potentially inaccurate.

The main aim of this study was to challenge and to improve the current conventional microbiological diagnosis of coagulase positive staphylococci as described above.

Materials and Methods

Bovine milk samples received for routine udder health investigations at the Milk Laboratory, Faculty of Veterinary Science, University of Pretoria were cultured by using conventional microbiological methods. *Staphylococcus* spp. (maltose positive and maltose negative) isolates identified from such milk samples were collected from 2009 to 2018. These samples were taken by professional samplers or milkers trained according to a standard operating procedure (Giesecke et al. 1994). The samples were transported on ice to reach the Milk Laboratory within 48 hours after sampling. The temperatures and other conditions such as sample tube cleanliness and appearance, were noted on arrival at the laboratory and the samples that were spoiled (for example, sour milk samples) or of doubtful quality were not processed. The samples were plated out at the laboratory on the day of their arrival (Petzer et al. 2017). From these, a total of 31 maltose negative (the only ones collected) and 31 maltose positive *Staphylococcus* isolates (selected equal number of maltose positive randomly from isolates collected during the same time period) were collected between 2009 and 2018.

In this study 31 isolates were identified phenotypically using conventional microbiology methods during routine diagnosis as potential *S. pseudintermedius* (Figures 4.1 and 4.2) and were sent for further testing to confirm the diagnosis on MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) and genotypic identification, using 16S RNA ribosomal sequencing. Three of these isolates were further analysed by using multi locus sequence typing (MLST).

The conventional microbiological identification was done at the Milk Laboratory according to the method that was recommended by the National Mastitis Council (2017). Milk from samples were plated out on a blood-tryptose agar plate (Oxoid, Quantum Biotechnologies (Pty) Ltd, South Africa) and incubated aerobically at 37°C ± 1°C for 24 ±2 hours. Phenotypic differentiation of staphylococci was initially identified based on colony morphology, pigmentation, haemolysis, catalase, staphylase, maltose and potassium hydroxide (Petzer et al. 2017). Samples with a growth of two and more colonies from the same bacterial species were defined as a pure culture, when only one species grew on an inoculum (National Mastitis Council Guidelines 2017). A sample was considered contaminated when three or more dissimilar colony types were observed. The catalase reaction was used to differentiate between Gram-positive staphylococci and streptococci. Staphylase, a coagulase test (Oxoid, supplied by Quantum Biotechnologies [Pty] Ltd, Ferndale, South Africa), was used to distinguish between coagulase-positive and coagulase-negative staphylococci. Maltose agar plates (Merck NT Laboratory Supplies, Halfway House, South Africa) (Petzer et al. 2017) were used. This conventional microbiological identification scheme (Jorgensen at al. 2015) was used for further identification of staphylococci. Thirty-one isolates from dairy cattle were phenotypically identified as *S. pseudintermedius* (coagulase positive, maltose negative staphylococci) and another 31 isolates

identified as maltose positive *S. aureus* collected during the same time period, were used (Figure 4.1).

These 31 maltose negative staphylococci and the 31 maltose positive *S. aureus*, were submitted for MALDI-TOF MS, (Bruker) identification (NHLS-NICD, Sandringham). All MALDI-TOF MS identifications (with scores of >2) were carried out by using the direct transfer method (Figure 4.2). Biological material (single colony) was smeared as a thin film directly onto a spot on a MALDI target plate. The material was overlaid with 1µl of α-cyano-4-hydroxy-cinnamic acid (HCCA) solution within 1 hour and allowed to dry at room temperature. The screening was automated without any user interference. Flex Control software (Bruker Daltonics) recorded spectra set for bacterial identification. MALDI Biolayer 3.0 software (Bruker Daltonics) with an integrated pattern-matching algorithm was used to compare generated peak lists against the reference library (van Dyk et al. 2016).

The 31 maltose negative staphylococci were submitted to Inqaba Biotec ™ for identification using the genetic 16S rRNA Sanger sequencing analysis (Figure 4.2). Genomic DNA was extracted from the cultures using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005) following the manufacturer's instructions. The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (New England Biolabs, Catalogue No. M0486) with the primers presented in Table 4.1. The PCR products were run on a gel and gel purified with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The purified fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on an ABI 3500xl Genetic Analyzer using a 50cm array and POP-7 (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyse the .ab1 files and identification results were obtained by a BLAST search (BLASTN 2.2.31+) (Altschul et al. 1997). Included in all the above testing methods were *S. aureus* ATCC 25923 and *S. pseudintermedius* ATCC 49444, which served as the reference strains for quality control purposes.

Table 4.1 Primers used in the current study.

Name of Primer	Target	Sequence (5' to 3')	Reference
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG	Weisburg et al. 1991
16S-1492R		CGGTTACCTTGTTACGACTT	
arc-Up	Arc	TTGATTCACCAGCGCGTATTGTC	Enright et al. 2000
arc-Dn		AGGTATCTGCTTCAATCAGCG	
aroE-Up	aroE	ATCGGAAATCCTATTTCACATTC	
aroE-Dn		GGTGTTGTATTAATAACGATATC	
glpF-Up	glpF	CTAGGAACTGCAATCTTAATCC	
glpF-Dn		TGGTAAAATCGCATGTCCAATTC	
gmk-Up	Gmk	ATCGTTTTATCGGGACCATC	
gmk-Dn		TCATTAACTACAACGTAATCGTA	
pta-Up	Pta	GTTAAAATCGTATTACCTGAAGG	
pta-Dn		GACCCTTTTGTTGAAAAGCTTAA	
tpi-Up	Tpi	TCGTTCATTCTGAACGTCGTGAA	
tpi-Dn		TTTGCACCTTCTAACAATTGTAC	
yqiL-Up	yqiL	CAGCATACAGGACACCTATTGGC	
yqiL-Dn		CGTTGAGGAATCGATACTGGAAC	
JO-MALAR-F	MalA+MalR	CTATAGGTCTACAAATGGCC	This study
JO-MALAR-R		CAGGAGGTGATTAAATGGTTAC	
Jo-Mal-Int		GATGTAATGACAGCAAC (Internal sequencing primer)	

Molecular Phylogenetic analysis was performed using the Maximum Likelihood method (Figure 4.3). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-1593, 46) is shown (Figure 4.3). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then by selecting the topology with the superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1, 3634)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 78, 40% sites). The tree was drawn to scale (Figure 4.3), with branch lengths measured in the number of substitutions per site. The analysis involved 54 nucleotide sequences. Codon positions that were included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There was a total of 641 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

The Gram-positive bacterium *Bacillus subtilis* was used as the outgroup (a more distantly related group of organisms that serves as a reference group when determining the evolutionary relationships of the organisms under study).

The absence of maltose activity was evaluated by determining the presence of the *malA* and *malR* genes (Egeter & Brückner 1995), with a maltose positive *S. aureus* ATCC 25923 used as the control

(Figure 4.2). Three of the maltose negative staphylococci isolates (from 2009, 2013 and 2017), in the same large group of *S. aureus*, were shown to be identical based on results of the 16s rRNA sequencing analysis (Figure 4.3) and were submitted to Inqaba Biotec ™ for further analysis by utilizing MLST. The internal fragments of seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi,* and *yqiL*) were amplified as previously described (Enright et al. 2000) and sequenced by using Brilliant Dye V3.1 (Nimagen, Netherlands) and an ABI 3500 XL Genetic analyser with a 50cm array and POP-7 (Applied Biosystems). The sequence of each locus was compared to allele sequences in the MLST database (http://saureus.mlst.net) in order to define the allelic profile for each isolate and to assign a sequence type (ST), which identified this strain as *S. aureus* ST 2992.

Results

The maltose negative *Staphylococcus* spp. were initially identified as *S. pseudintermedius* phenotypically.

The MALDI-TOF MS analysis (Bruker) identified 23 of these isolates as *S. aureus* (maltose negative), one each as *S. capitis and S. xylosus*, four as *S. chromogenes* and two isolates with no possible identification (Figure 4.2).

These same 31 coagulase positive, maltose negative staphylococci were sent for 16s RNA sequencing which similarly identified 23 of these isolates as *S. aureus*, one each as *S. capitis*, *S. saprophyticus, S. xylosus and Macrococcus caseolyticus and* four as *S. chromogenes* (Figure 3). The two isolates with no possible identification using the MALD-TOF MS, were identified as *S. saprophyticus* and *Macrococcus caseolyticus* by 16s rRNA sequencing. The Gram-positive bacterium *Bacillus subtilis* was used as the outgroup (as described in Materials and Methods), although there was also a built in outgroup in the samples (Figure 4.3), which was identified as *Macrococcus caseolyticus* (Figure 4.3).

Further 16S identification and MLST confirmed all 23 maltose negative *S. aureus* isolates as an identical strain of single origin, repeated over time (Figure 4.4).The *S. aureus* ST 2992 strain was assigned, based on the results of the MLST data (Figure 4.2).

The *malA* and *malR* genes were present in the maltose negative *S. aureus* ST 2992. However, a stop codon was discovered at base pair 844 of the *malA* gene caused by a cytosine to thymine transition which may render a truncated α-glucosidase protein.
Nucleotide sequences of genes encoding the α-glucosidase (*malA*) that show the stop codon (Figure 4.4) and its upstream region in the investigated strain ST 2992, have been deposited in GenBank (accession number to be added once the submission has been processed.

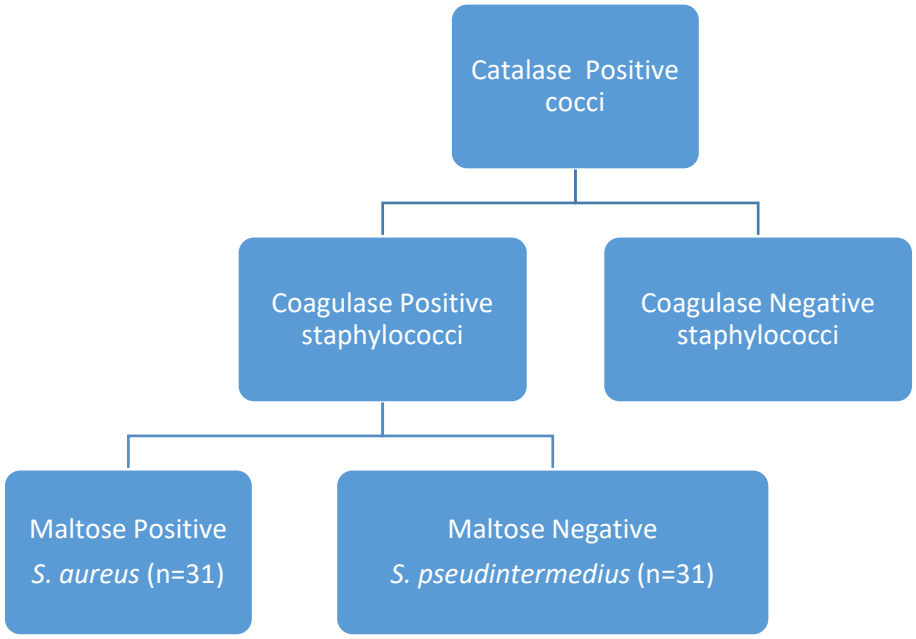

Figure 4.1 Conventional microbiological procedures for identification of staphylococci.

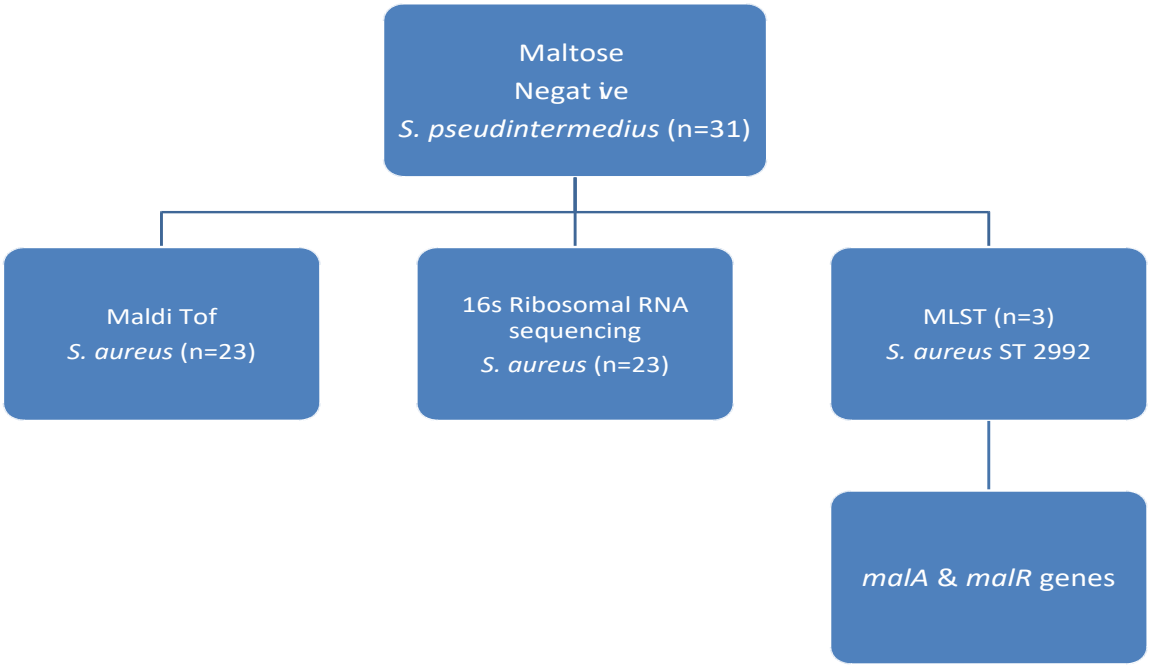

Figure 4.2 Procedures for confirmation of *S. pseudintermedius* identification by further testing.

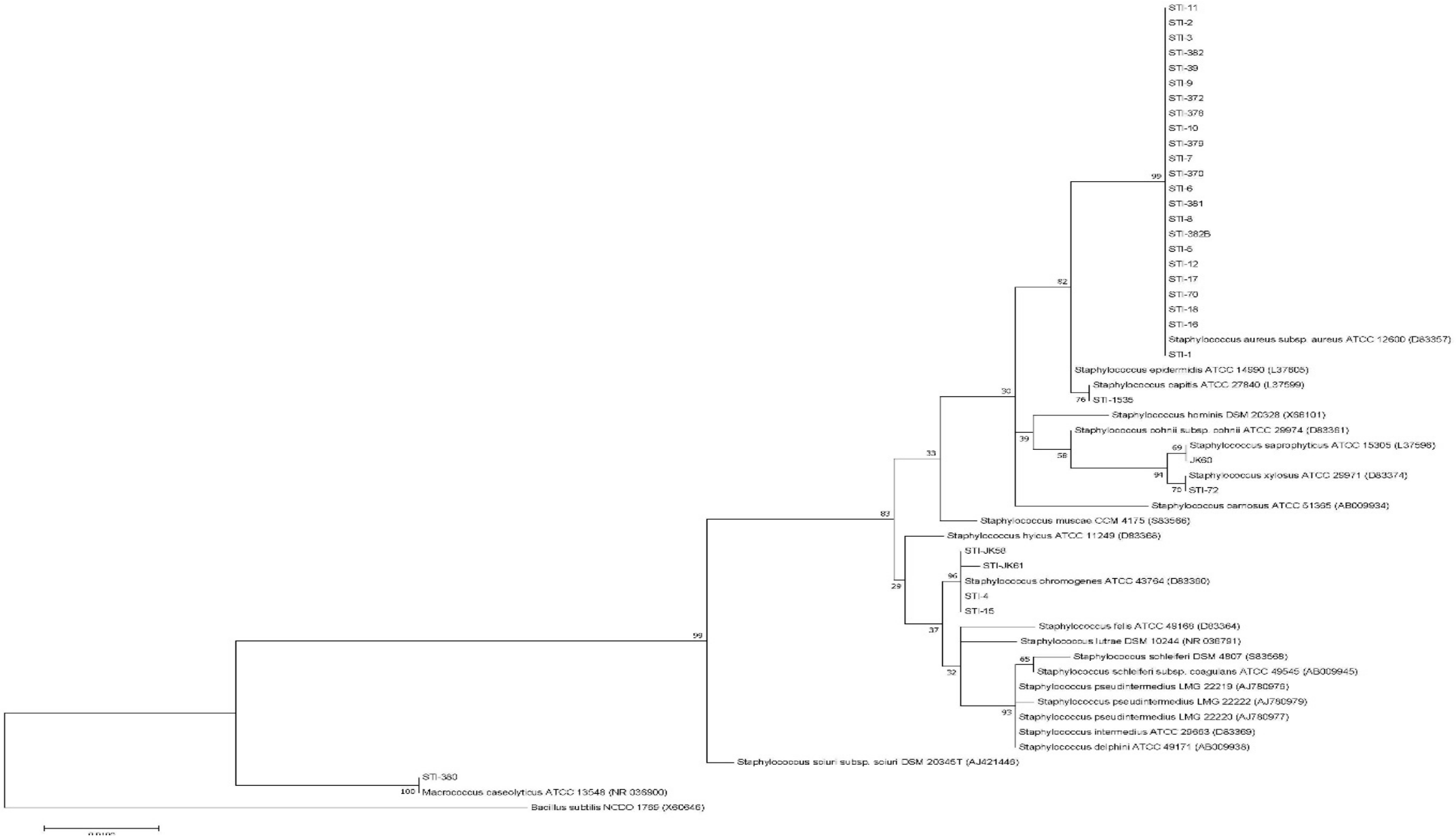

Figure 4.3 Molecular Phylogenetic analysis by Maximum Likelihood method.

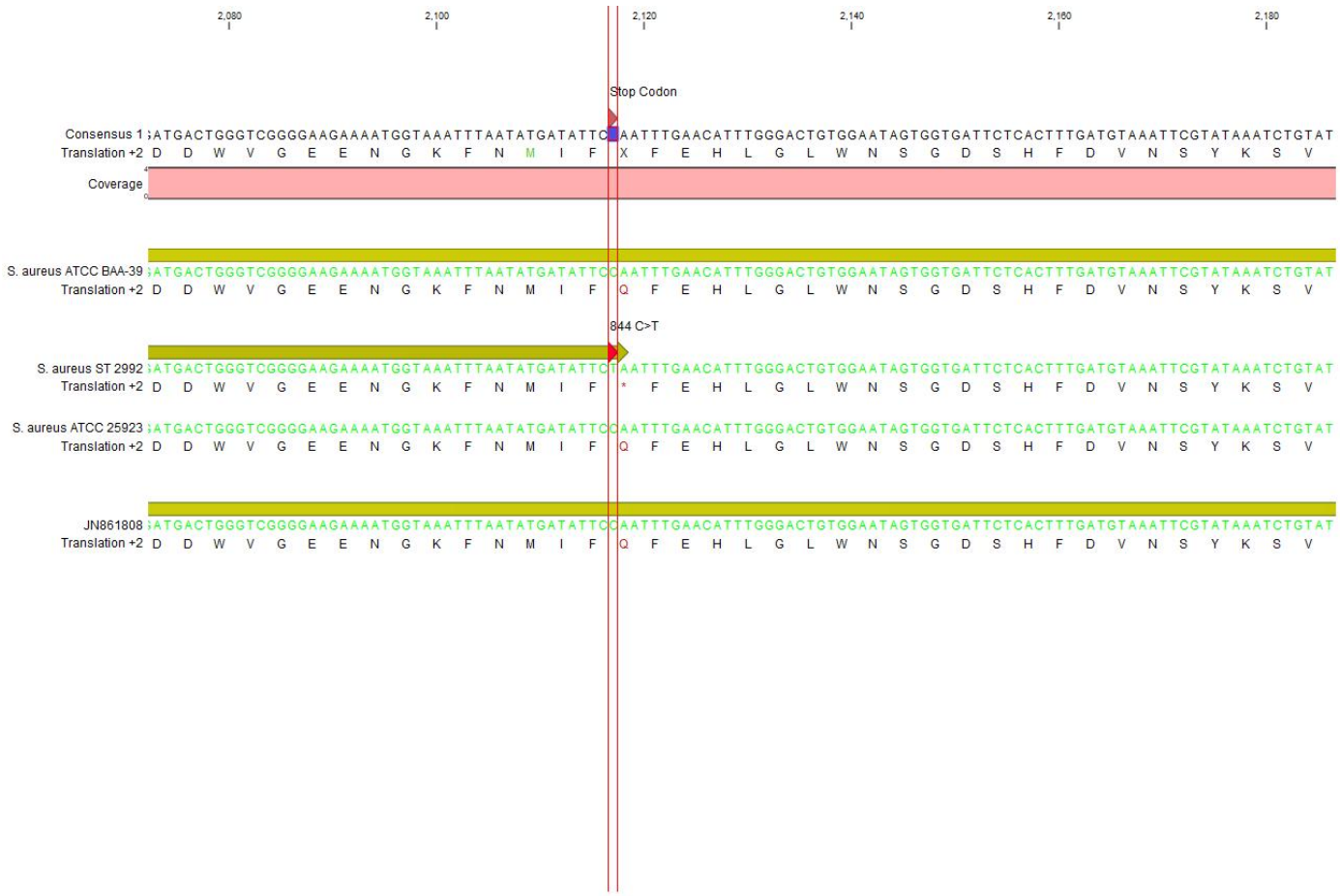

Figure 4.4 Comparison of the alignment of *malA* gene of the maltose negative *S. aureus* ST 2992 with that of the *S. aureus* ATCC BAA-39 (NCBI database), conventionally identified maltose positive *S. aureus* ATCC 25923 and coagulase negative, maltose negative *S. aureus* JN 861808 (Johler et al. 2012), highlighting the mutation that resulted in the stop codon.

Discussion

The initial phenotypic identification (by conventional methods) was not supported by the MALDI-TOF MS identification with the suspected *S. pseudintermedius* isolates being identified as *S. aureus* (Figures 4.1 and 4.2). These different results when using the conventional identification and the MALDI-TOF MS, could have perhaps initially been explained to some extent by previous analytical procedures that had been used. In the past in South Africa, questionable MALDI-TOF MS identification of the *S. intermedius* group of organisms had been the result of the lack of specific strains of *S. pseudintermedius* in the MALDI-TOF MS identification library. Nevertheless, IDEXX Germany has subsequently added about 30 *S. pseudintermedius* strains to this library, 10 of which were sent from South Africa and identified by the same phenotypic method used in this study (maltose test) (personal communication, 2018). Thus, this addition of *S. pseudintermedius* strains should have resolved the possible identification error due to lack of strains in the MALDI-TOF MS library. However, this possible discrepancy was not in agreement with a study done in the

Netherlands that found the MALDI-TOF MS method of identification to be effective to differentiate between *S. aureus* and *S. pseudintermedius* (Verstappen et al. 2017). However, the MALDI-TOF MS (Bruker) used in this study was accurate in the differentiation between *S. aureus* ATCC 25923 and *S. pseudintermedius* ATCC 49444 control cultures.

The further 16s rRNA sequencing results correlated completely with the results of the MALDI-TOF MS for the identification of the 23 identical *S. aureus* isolates, and *Macrococcus caseolyticus* was an inbuilt outgroup (Figure 4.3). Macrococcus is a genus of Gram-positive cocci that was identified separately in 1998 and belongs to the family Staphylococcaceae. This organism is a large Gram-positive coccus that is related to the *Staphylococcus* spp. and is often associated with meat and milk of sheep, goats and cattle. Methicillin-resistant *Macrococcus caseolyticus* strains from bovine and canine origins have been found to carry a novel *mecD* gene that conferred resistance to all classes of β-lactams, including anti-methicillin resistant *S. aureus* cephalosporins. (Mašlaňová et al. 2018, Schwendener et al. 2017).

The identification of the *S. aureus* ST 2992 strain was assigned based on the further multi-locus sequence typing (MLST). This MLST data combined with the 16s identification confirmed that three isolates from 2009, 2013 and 2017 (large identical group of *S. aureus* n= 23) respectively, were a maltose negative strain of *S. aureus* (ST 2992) and not *S.pseudintermedius*. This was shown to be an identical strain of single origin isolated repeatedly over time (Figure 4.3). This isolate has been shown to belong to the common bovine lineage CC97 (Schmidt et al. 2017) and has not yet been identified from humans in the country to date. Although, if this strain were to be identidied in humans as has been the case for other isolates from the CC97 lineage, this would suggest zoonotic transfer (Schmidt et al. 2017). It appears at this stage that this maltose negative strain of *S. aureus* (ST 2992) is unique to South Africa, and no related epidemiology from other countries has been found yet.

The presence of *femA* gene (Kobayashi et al. 1994), also further confirmed that these isolates were a type of *S. aureus* with an odd biochemical profile and not *S. pseudintermedius*. The *malA* and *malR* genes were present in the maltose negative *S. aureus* ST 2992 and in the maltose positive *S. aureus* ATCC 25923, control used (Figure 4.2). These isolates have the α-glucosidase protein, but it is not expressed. The *malA* gene encodes an α-1, 4-glucosidase or maltase, which also liberates glucose from sucrose and it seems that the *malR* gene participates in the regulation of the gene(s) for maltose transport and would thus be needed for the full expression of these genes (Egeter & Brückner 1995). The inactivation of *malA* in a sucrose-6-phosphate hydrolase-deficient *S. xylosus* strain has been shown to result in the complete loss of the residual sucrose hydrolase activity (Egeter & Brückner 1995).

This study discovered a stop codon at base pair 844 of the *malA* gene where there was a cytosine to thymine transition. This stop codon resulted in the early termination of the α-glucosidase protein (Figure 4.4). This truncated protein is most likely the cause of the maltose negative phenotype of *S. aureus* ST 2992. In Figure 4, the sequence of the *malA* gene of the maltose negative *S. aureus* ST 2992 is compared to the following isolates: the *S. aureus* ATCC BAA-39 (NCBI database), the "classical" maltose positive *S. aureus* ATCC 25923 and the coagulase negative maltose negative *S. aureus* JN 861808 (Johler et al. 2012). This comparison illustrated the cytosine to thymine transition at base pair 844 of the maltose negative *S. aureus* ST 2992, which is not present in the *malA* sequences of the other isolates compared (Figure 4.4). However, the *malR* gene of the maltose negative *S. aureus* ST 2992 is identical to that of the ATCC strain used. The isolate from the Netherlands which was coagulase negative and maltose negative however, did not show as few mutations on the *malA* gene sequence compared to those of maltose positive control strains used (Johler et al. 2012). The Dutch strain was reported as a coagulase deficient *S. aureus* isolated from clinical cases of bovine mastitis (Johler et al. 2012). Thus, the maltose negative phenotype can most likely be caused by various differences occurring in either the *malA* or *malR* genes or in both, and therefore further research is needed to explore these differences.

The practical implications of such maltose negative staphylococcus isolates that are identified as *S. pseudintermedius* by conventional microbiology and phenotypic tests are not as serious as might be initially perceived. The reason for this is that these maltose negative *S. aureus* strains react differently from the reactions of the conventionally identified maltose positive *S. aureus* in practice. This maltose positive *S. aureus* is still a problem in udder health in South Africa (Petzer et al. 2009) and worldwide (Pyörälä & Pyörälä 1997, Sol et al. 1997, Tenhagen et al. 2009). Maltose negative *S. aureus* differs in the prevalence per herd, with only very small numbers occurring per herd, and it does not seem to be contagious (personal communication, 2018). In comparison, maltose positive *S. aureus* occurs with a high prevalence per herd and is highly contagious (Petzer et al. 2016). For maltose negative *S. aureus* identified there have also not yet been any repeat cases, so that it does not seem to become chronic, and the udder parenchyma damage is limited (personal communication, 2018). Both of these aspects are common with maltose positive *S. aureus*. Therefore, different management and treatment strategies should apply in practice. In the case of maltose positive *S. aureus,* parlour hygiene is imperative, positive cows should be separated for life and milked last, and they should be followed up with quarter milk samples (microbiology and cytology) (Petzer et al. 2016). This is done, in order to be able to decide on the prognosis, and to treat or to cull as necessary. However for maltose negative *S. aureus* only clinical cases are treated, as can be done with the coagulase negative staphylococci (Pyörälä & Taponen 2009). The genetic difference found in the sequence of the *malA* gene (Figure 4.4) of the maltose negative *S. aureus* ST 2992 could possibly also be the reason for this strain to react differently from the way the maltose positive strains react in practice.

Another study on the same isolates in (Chapter 5) has also found that the antibiotic resistance trends of maltose negative and maltose positive *S. aureus* differ. In general, more antibiotic resistance is shown by the maltose positive *S. aureus*, including multi-drug resistance (resistance to products from three or more antibiotic classes) in comparison to the maltose negative *S. aureus* isolates. This indicates that although both are identified as *S. aureus*, there are a few very important differences between maltose negative and maltose positive *S. aureus* in practice, with the maltose negative *S. aureus* being overall less pathogenic and less contagious than the maltose positive *S. aureus.* These differences could perhaps also be attributed to the stop codon on the *malA* gene of *S. aureus* ST 2992 (Figure 4.4).

Future work on whole genome sequencing of the maltose negative *S. aureus* ST 2992 isolate is necessary in order to investigate any other possible differences at other regions of the genome, which may be contributing factors to the different phenotype and/or the differences in pathogenicity and antibiotic resistance of this *S. aureus* ST 2992 strain. This maltase gene mutation can serve as a target for future research to discover whether this is indeed responsible for the changes in virulence.

Conclusion

The identification by the MALDI-TOF MS and 16S sequencing of maltose negative *S. aureus* with this relatively small sample were found in both cases to support for the identification of maltose negative *Staphylococcus* spp. as a *S. aureus* strain. Multi locus sequence typing (MLST) has further identified this emerging pathogen as the maltose negative *S. aureus* ST 2992 strain, which in this limited sample was shown to be a strain of single origin isolated repeatedly. It is not possible to speculate on the origin of this strain, which could be from a local genetic change, or introduced from elsewhere. Although conventional microbiological methods are not entirely correct in the strain identification of coagulase positive and maltose negative staphylococci phenotypically, it is important to differentiate between maltose negative and maltose positive *S. aureus* isolates during routine diagnosis of mastitis causing organisms. This is because maltose negative and maltose positive *S. aureus* organisms react differently and thus need to be treated differently in practice. While the maltose negative *S. aureus* ST 2992 identified tested positive for both *malA* & *malR* genes the α-glucosidase gene present was not being expressed due to the abnormally placed stop codon.

Acknowledgements

The authors thank the producers for allowing them to use data and samples obtained on their farms to obtain the knowledge. Thank you also to the staff of the Milk Laboratory, Department of Production

Animal Studies, Faculty of Veterinary Science, Onderstepoort, for the laboratory analysis and in particular Mrs J.C. Watermeyer, Miss R. Badenhorst, Mrs E.F. Konaite, Mr K.S. Malekane, Miss N.I. Memma and Miss O.P. Nchako. The authors also thank Prof Olga Perovic and Mrs Marshagne Smith of National Health Laboratory Services (NHLS)-NICD, Sandringham for the MALDI-TOF MS analysis.

References

Altschul, S.F., Madden, T.L., Alejandro A., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Basanisi, M. G., La Bella, G., Nobili, G., Franconieri, I., and La Salandra, G., 2017. Genotyping of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk and dairy products in South Italy. Food Microbiology 62, 141–146. doi:10.1016/j.fm.2016.10.020

Becker, K., Heilmann, C., Peters, G. 2014. Coagulase-negative staphylococci. Clinical Microbiological Reviews 27(4), 870-926. doi:10.1128/CMR.00109-13

CLSI, MM18-A, 2010. Interpretive criteria for identification of bacteria and fungi by DNA target sequencing. Approved guideline. 28(12).

Cox, H.U., Hoskins, J.D., Newman, S.S., Foil, C.S., Turnwald, G.H., Roy, A.F., 1988. Temporal study of staphylococcal species on healthy dogs. American Journal of Veterinary Research. 49, 747–751.

Cox, H.U., Hoskins, J.D., Newman, S.S., Turnwald, G.H., Foil, C.S., Roy, A.F., Kearney, M.T., 1985. Distribution of staphylococcal species on clinically healthy cats. American Journal of Veterinary Research 46, 1824–1828.

De Buck, J., Nashad, S., Nóbrega, D.B., Montero, A., Carson, D., Luby, C., Barkema, H.W., 2017. Whole genome sequences, a treasure trove of information on non-aureus staphylococci. Proceedings of the 2nd Seminar on coagulase-negative staphylococci, 18-19 May 2017, Ghent, Belgium, 19-28.

Devriese, L., Vancanneyt, M., Baele, M., Vaneechoutte, M., De Graef, E., Snauwaert, C., Cleenwerck, I., Dawyndt, P., Swings, J., Decostere, A., Haesebrouck, F., 2005. *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. International Journal of Systematic and Evolutionary Microbiology 55, 1569–1573.

Dingwell, R.T., Leslie, K.E., Sabour, P., Lepp, D., Pacan, J., 2006. Influence of the genotype of *Staphylococcus aureus*, determined by pulsed-field gel electrophoresis, on dry-period elimination of subclinical mastitis in Canadian dairy herds. Canadian Journal of Veterinary Research 70(2), 115-120.

Dufour, S., Dohoo, I. R., Barkema, H. W., Descôteaux, L., Devries, T. J., Reyher, K. K., Roy, J.P,, Scholl, D.T., 2012. Manageable risk factors associated with the lactational incidence, elimination, and prevalence of *Staphylococcus aureus* intramammary infections in dairy cows. Journal of Dairy Science 95, 1283-1300. doi:10.3168/jds.2011-4711

Egeter, O., Brückner, R., 1995. Characterization of a Genetic Locus Essential for Maltose-Maltotriose Utilization in *Staphylococcus xylosus*. Journal of Bacteriology p, 2408–2415.

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., Spratt, B.G., 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. Journal of Clinical Microbiology 38, 1008–1015.

Fox, L.K., Zadoks, R.N., Gaskins, C.T., 2005. Biofilm production by *Staphylococcus aureus* associated with intramammary infection. Veterinary Microbiology 107(3-4), 295-299.

Giesecke, W.H., Du Preez, J.H., Petzer, I.M., 1994. Practical mastitis control in dairy herds, Butterworths, Durban.

Graber, H.U., Naskova, J., Studer, E., Kaufmann. T., Kirchhofer, M., Brechbühl, M., Schaeren, W., Steiner, A., Fournier, C., 2009. Mastitis-related subtypes of bovine *Staphylococcus aureus* are characterized by different clinical properties. Journal of Dairy Science 92(4), 1442-1451. doi:10.3168/jds.2008-1430

Hajek, V., 1976. *Staphylococcus intermedius*, a new species isolated from animals. International Journal of Systematic Bacteriology 26, 401–408.

Haveri, M., Taponen, S., Vuopio-Varkila, J., Salmenlinna, S., Pyörälä, S. 2005. Bacterial genotype affects the manifestation and persistence of bovine *Staphylococcus aureus* intramammary infection. Journal of Clinical Microbiology. 43(2), 959-961.

Haveri, M., Roslöf, A., Rantala, L., Pyörälä, S. 2007. Virulence genes of bovine *Staphylococcus aureus* from persistent and non-persistent intramammary infections with different clinical characteristics. Journal of Applied Microbiology 103(4), 993-1000.

Johler, S., Moser, M., Engl, C., Tasara, T., Corti, S., Chen, J., Stephan, R., 2012. A coagulase- and α-glucosidase negative variant of *Staphylococcus aureus*: A challenge for routine microbiological diagnostics. Journal of Clinical Microbiology p, 1827-1828. doi:10.1128/JCM.06345-11

Jolley, K.A., Bray, J.E., Maiden, M.C.J., 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Research 24(3), 124. doi:10.12688/wellcomeopenres.14826.1. eCollection 2018, 3:124 (version 1; referees: 2 approved). The development of this site has been funded by the Wellcome Trust.

Edited by, Jorgensen, J.H., Pfaller, M.A., Landry, M.L., Richter, S.S., Warnock, D.W., Carrol, K.C., Funke, G. 2015. Manual of Clinical Microbiology 11th Edition. Volume 1, ASM Press, Washington DC. Print ISBN:9781555817374, e-ISBN:9781555817381, doi:10.1128/9781555817381

Kateete, D. P., Kabugo, U., Baluku, H., Nyakarahuka, L., Kyobe, S., Okee, M., Najjuka, C.F., Joloba, M.L., 2013. Prevalence and antimicrobial susceptibility patterns of bacteria from milkmen and cows with clinical mastitis in and around Kampala, Uganda. Plos One 8, e63413. doi:10.1371/journal.pone.0063413

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16, 111-120.

Kobayashi, N., Wu, H., Kojima, K., Taniguchi, K., Urasawa, S., Uehara, N., Omizu, Y., Kishi, Y., Yagihashi, A., Kurokawa, I., 1994. Detection of mecA, femA, and femB genes in clinical strains of staphylococci using polymerase chain reaction. Epidemiology and Infection 113(2), 259-66. doi:10.1017/s0950268800051682

Kumar S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33, 1870-1874.

Larsen, H.D., Sloth, K.H., Elsberg, C., Enevoldsen, C., Pedersen, L.H., Eriksen, N.H., Aarestrup, F.M., Jensen, N.E., 2000. The dynamics of *Staphylococcus aureus* intramammary infection in nine Danish dairy herds. Veterinary Microbiology 71(1-2), 89-101.

Liu, H., Li, S., Meng, L., Dong, L., Zhao, S., Lan, X., Wang, J., Zheng, N., 2017. Prevalence, antimicrobial susceptibility, and molecular characterization of *Staphylococcus aureus* isolated from dairy herds in northern China. Journal of Dairy Science 100, 8796–8803. doi:10.3168/jds.2017-13370

Mašlaňová, I., Wertheimer, Z., Sedláček, I., Švec, P., Indráková, A., Kovařovic, V., Schumann, P., Spröer, C., Králová, S., Šedo, O., Krištofová, L., Vrbovská, V., Füzik, T., Petráš, P., Zdráhalm, Z., Ružičková, V., Doškař, J., Pantuček, R., 2018. Description and Comparative Genomics of *Macrococcus caseolyticus* subsp. *hominis* subsp. nov., *Macrococcus goetzii* sp. nov., *Macrococcus epidermidis* sp. nov., and *Macrococcus bohemicus* sp. nov., Novel Macrococci From Human Clinical Material With Virulence Potential and Suspected Uptake of Foreign DNA by Natural Transformation. Frontiers in Microbiology 13(9), 1178. doi:10.3389/fmicb.2018.01178

Mc Adow, M., Kim, H.K., Dedent, A.C., HendrickX, A.P.A., Schneewind, O. and Missiakas, D.M., 2011. Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood. Plos Pathogens 7, e1002307

Middleton, J.R., Fox, L.K. 2002. Influence of *Staphylococcus aureus* strain on mammary quarter milk production. Veterinary Record 150(13), 411-413.

Middleton, J.R., Fox, L.K., Gay, J.M., Tyler, J.W., Besser, T.E., 2002. Influence of *Staphylococcus aureus* strain-type on mammary quarter milk somatic cell count and N-acetyl-beta-D-glucosaminidase activity in cattle from eight dairies. Journal of Dairy Science 85(5), 1133-1140.

National Mastitis Council. 2017. Staphylococci. In Laboratory Handbook on Bovine Mastitis, 3rd ed.; National Mastitis Council: Verona, Winsconsin, USA, p, 43–52.

Petzer, I.M., Karzis, J., Watermeyer, J.C., Van der Schans, T.J. & Van Reenen, R. 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. Journal of South African Veterinary Association 80(1), 17-22. doi:10.4102/jsava.v80i1.163

Petzer, I.M., Karzis, J., Donkin, E.F., Webb, E.C. 2016. A pathogen-specific approach towards udder health management in dairy herds: Using culture and somatic cell counts from routine herd investigations**.** Onderstepoort Journal of Veterinary Research 83(1), 12. doi:10.4102/ojvr.v83i1.1146

Petzer, I.M., Karzis, J., Donkin E.F., Webb, E.C. and Etter, E.M.C. 2017. Pathogen specific bovine intramammary infections: The validity of a somatic cell count threshold as an indicator of

intramammary infection in quarter and composite milk samples. Journal of South African Veterinary Association 88(1), April 2017. doi:10.4102/jsava.v88i0.1465

Pyörälä, S., Pyörälä, E., 1997. Accuracy of methods using somatic cell count and N-acetyl-beta-D-glucosaminidase activity in milk to assess the bacteriological cure of bovine clinical mastitis. Journal of Dairy Science 80(11), 2820-2825.

Pyörälä, S., Taponen, S., 2009. Coagulase-negative staphylococci – emerging mastitis pathogens. Veterinary Microbiology 134(1-2), 3.doi:10.1016/j.vetmic.2008.09.015

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S., Hiramatsu, K., 2007. Reclassification of phenotypically-identified Staphylococcus intermedius strains. Journal of Clinical Microbiology 45, 2770–2778.

Schmidt, T., Kock, M.M., Ehlers, M.M., 2017. Molecular characterization of *Staphylococcus aureus* Isolated from bovine mastitis and close human contacts in South African dairy herds: Genetic diversity and inter-species host transmission. Frontiers in Microbiology 8(511), 1-15. doi:10.3389/fmicb.2017.00511

Schwendener, S., Cotting, K., Perreten, V., 2017. Novel methicillin resistance gene mecD in clinical *Macrococcus caseolyticus* strains from bovine and canine sources. Scientific Reports 7, 43797. doi:10.1038/srep43797

Sol, J., Sampimon, O.C., Snoep, J.J., Schukken, Y.H., 1997. Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by *Staphylococcus aureus*. Journal of Dairy Science 80(11), 2803-2808.

Talan, D.A., Staatz, D., Staatz, A., Goldstein, E.J., Singer, K., Overturf, G.D., 1989. *Staphylococcus intermedius* in canine gingiva and canine inflicted human wound infections: laboratory characterization of a newly recognized zoonotic pathogen. Journal of Clinical Microbiology 27, 78–81.

Tenhagen, B. A., Hansen, I., Reinecke, A., Heuwieser, W., 2009. Prevalence of pathogens in milk samples of dairy cows with clinical mastitis and in heifers at first parturition. Journal of Dairy Research 76(2), 179-187.

van den Borne, BH., Nielen, M., van Schaik, G., Melchior, M.B., Lam, T.J., Zadoks, R.N., 2010. Host adaptation of bovine *Staphylococcus aureus* seems associated with bacteriological cure after

lactational antimicrobial treatment. Journal of Dairy Science 93(6), 2550-2558. doi:10.3168/jds.2009-2971

Vanderhaeghen, W., Piepers, S., Leroy, F., Van Coillie, E., Haesebrouck, F. and De Vliegher, S., 2015. Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. W.B. Saunders. Available at: https://www-sciencedirect-com.uplib.idm.oclc.org/science/article/pii/S109002331400447X (Accessed: 1 July 2019).

Van Dyk, B.N., De Bruin, W., Du Plessis, E.M., Korsten, L., 2016. Microbiological Food Safety Status of Commercially Produced Tomatoes from Production to Marketing. Journal of Food Production 79(3), 392-406. doi:10.4315/0362-028X.JFP-15-300

Verstappen, K.M., Huijbregts, L., Spaninks, M., Wagenaar, J.A., Fluit, A.C., Duim, B., 2017. Development of a real-time PCR for detection of *Staphylococcus pseudintermedius* using a novel automated comparison of whole-genome sequences. Plos One 12(8), e0183925. doi:10.1371/journal.pone.0183925

Wang, W., Lin, X., Jiang, T., Peng, Z., Xu, J., Yi, L., Li, F., Fanning, S., Baloch, Z., 2018. Prevalence and characterization of *Staphylococcus aureus* cultured from raw milk taken from dairy cows with mastitis in Beijing, China. Frontiers in Microbiology 9, 1123. doi:10.3389/fmicb.2018.01123

Weisburg, W. G., Barns, S. M., Pelletier, D. A., Lane, D. J., 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173, 697–703.

Yu, W., Kim, H.K., Rauch, S., Schneewind, O., Missiakas, D.M., 2017. Pathogenic conversion of coagulase-negative staphylococci. Microbes and Infection 19, 101-109.

Zadoks, R., van Leeuwen, W., Barkema, H., Sampimon, O., Verbrugh, H., Schukken, Y.H., van Belkum, A. 2000. Application of pulsed-field gel electrophoresis and binary typing as tools in veterinary clinical microbiology and molecular epidemiologic analysis of bovine and human *Staphylococcus aureus* isolates. Journal of Clinical Microbiology 38(5), 1931-1939.

Zecconi, A., Scali, F. 2013. *Staphylococcus aureus* virulence factors in evasion from innate immune defenses in human and animal diseases. Immunology Letters 150, 12-22. doi:10.1016/j.imlet.2013.01.004

Chapter 5: Surveillance of antibiotic resistance of maltose negative *Staphylococcus aureus* in South African dairy herds

Significance of the work

This study evaluated antibiotic resistance data (disc diffusion method) of this emerging pathogen (maltose negative *S. aureus,* identified in the previous chapter) as described for *S. aureus* studied and reported previously (Chapters 1 and 2). In addition to the retrospective information, minimum inhibitory concentration (MIC) testing was undertaken on a limited number of maltose positive and maltose negative *S. aureus* isolates taken over a total time period of nine years. The findings of this study highlighted differences in antibiotic resistance between maltose negative and maltose positive *S. aureus* isolates. The maltose negative *S. aureus* isolates showed overall increased antibiotic resistance when compared to the maltose positive strain, as well as multi-drug resistance These findings also showed resistance of the maltose negative strain isolated from milk samples to antibiotics that are only used in human medicine, which implies a possible anthroponosis (transfer from humans to animals) and requires further studies under the “one health” approach. This study also highlighted the differences in antibiotic resistance profiles between the maltose positive and maltose negative *S. aureus* which is very useful information for producers and veterinarians in the field when treating cows infected with such organisms in practice.

Submitted for publication, December 2019: Joanne Karzis, Inge-Marié Petzer, Edward F. Donkin, Vinny Naidoo & Eric M.C. Etter (BMC Veterinary Research Journal)

Abstract

The discovery of antibiotics in the 1930s was one of the greatest achievements in medical history. However, bacterial resistance to antibiotics was already first observed in the 1940s. Since then bacterial resistance to antibiotics has been reported in both human and veterinary medicine, including in dairy cows. Many years of monitoring milk samples have led to the identification of a new strain of *Staphylococcus aureus* (*S. aureus)*, which is maltose negative and appears to be an emerging pathogen in South Africa. In this study the differences in susceptibility to antibiotics of this strain were evaluated over time, over different seasons, in different provinces, and according to somatic cell count (SCC) categories.

A data set of 271 maltose negative *S. aureus* isolates, cultured from milk samples from 117 herds out of the estimated 2000 commercial dairy herds in South Africa between 2010 and 2017, was

studied using the Kirby Bauer method. Initially in this method, the reactions of bacteria to specific antibiotics were classified as resistant, or susceptible, or intermediate (previously used system). This test was carried out using the nine antibiotics that were available as intramammary remedies in South Africa at the time. The analysis was done to compare the previously used system (intermediate category grouped with resistant) and the more recent system, (intermediate category grouped with susceptible) Clinical Laboratory Standards Institute (CLSI) breakpoints. The results between the previously used system and the more recent system, differed for tylosin, cefalonium, oxy-tetracycline and cloxacillin. Neither the analysis using the previous system nor the more recent system showed an effect of province for the maltose negative *S. aureus*. This was in contrast to the results for maltose positive *S. aureus* where differences between provinces were shown in a previous study (Karzis et al. 2019).

For the susceptibility testing of 57 maltose negative *S. aureus* and 57 maltose positive *S. aureus* the minimum inhibitory concentration (MIC) results for the maltose negative *S. aureus* confirmed the results of the Kirby Bauer. The results for the maltose negative strains of *S. aureus* differed in general, in their antibiotic resistance patterns over time, in comparison to maltose-positive *S. aureus* results. MIC testing also indicated more multi-drug resistant isolates that were seen with the maltose negative *S. aureus* than in the maltose positive strains.

Keywords: antibiotic resistance; maltose negative mastitis; minimum inhibitory concentrations; regions; seasons; Somatic Cell Count; *Staphylococcus aureus*

Introduction

The genus *Staphylococcus* consists of a variety of opportunistic pathogens of variable relevance in veterinary medicine. The most clinically relevant staphylococci in veterinary medicine are the coagulase positive *Staphylococcus aureus* and members of the *S. intermedius* group (SIG) (Sasaki et al., 2007), particularly *Staphylococcus pseudintermedius (S. pseudintermedius)*. A noted property of staphylococci is their ability to become resistant to antimicrobials. Methicillin resistance is of particular relevance, because it is conferred by a presence of the mecA gene, which encodes for the production of an altered penicillin binding protein (PBP) (PBP2a or PBP2') that has a low affinity for all beta-lactam antimicrobials (penicillins, cephalosporins, carbapenems) (Kwon et al. 2006). Methicillin-resistant *S. aureus* (MRSA) is recognised as a significant problem in human medicine and it is among the most important infections in hospitalized individuals (Klevens et al. 2007). Recently, MRSA has emerged as a significant community-associated pathogen that has caused disease in people in the general population (not only in hospitals), including those that would previously have been considered as low risk for infection (Frazee et al. 2005).

In veterinary medicine *S. aureus* (maltose positive) is the biggest problem in the dairy industry in South Africa and globally (Petzer et al. 2009, Petzer et al. 2012). The infected udder is considered to be the primary reservoir of *S. aureus* and the organism is believed to be transmitted during milking. Despite this, a proportion of heifers which are already infected with *S. aureus,* enter the milking herd (Nickerson et al. 1995). This suggests routes of transmission in addition to the milking equipment and the milking parlour. A good understanding of *S. aureus* reservoirs and transmission is essential for the effective control of the organism in a herd. The probability of treatment resulting in cure of *S. aureus* (maltose positive) infection is calculated by taking the following factors into account: parity; the stage of lactation; the SCC level; the specific teat position on the udder; the number of quarters infected; and the duration of treatment required (Sol et al. 1997).

The Milk Laboratory at the Faculty of Veterinary Science at the University of Pretoria has provided an extensive dairy cow udder monitoring programme in South Africa. Since 2005, an increasing number of coagulase positive, maltose negative staphylococci have been isolated, that were confirmed as maltose negative *S. aureus* by molecular methods. These organisms were first identified from a dairy cow in a single South African dairy herd with a somatic cell count of less than 100 000 cell/ml of milk. As early as three years later these organisms were isolated from numerous other dairy herds in South Africa, albeit with effective susceptibility to antibiotics that were tested routinely and with a low SCC. However in more recent years 2016/2017/2018, coagulase positive and maltose negative staphylococci were isolated showing resistance (MRSA, cefoxitin disc) where some of the milk samples started to show higher SCC (> 400 000cells/ml milk) than initially shown (personal experience). In addition to the previous study which attempted to characterise this emerging pathogen, a further evaluation was carried out of the resistance trends evident in historic Kirby Bauer susceptibility data and more recent MIC data.

Materials and Methods

Retrospective data analysis of maltose negative *S. aureus* (Kirby Bauer test):

From 2010 to 2017, susceptibility tests were performed on maltose negative staphylococci that were isolated from milk samples of commercial dairy herds in South Africa (n= 271), using the Kirby Bauer method (Bauer et al. 1966). The total of 271 samples originated from 117 dairy herds out of approximately 2000 commercial dairy herds (Lactodata 2009). These herds were located in all nine provinces of South Africa, but with greatly varying numbers in the different provinces, namely: Gauteng (n= 8), KwaZulu Natal (n= 170), Free State (n= 4), Eastern Cape (n= 56), Western Cape (n= 27), Northern Cape (n= 1), North West (n= 1), Limpopo (North) (n= 1) and Mpumalanga (n= 3). Most of these dairy producers sent milk samples to the Milk Laboratory, Faculty of Veterinary Science, University of Pretoria , for testing (microbiology and cytology) on a routine basis, as part of

the pro-active udder health management programme, while a few were part of post-mastitis diagnosis only. The milk samples were collected in an aseptic manner either by the farmers themselves according to a standard operating procedure (NMC 2004) or by professional milk samplers. The samples were then cultured according to the method recommended by the National Mastitis Council (2004). Milk from samples were plated out on a blood-tryptose agar plate (Oxoid, Quantum Biotechnologies (Pty) Ltd, South Africa) and were incubated aerobically at 37°C ± 1°C for 24 ± 2 hours. Phenotypic differentiation of staphylococci was initially based on colony morphology, pigmentation, haemolysis, catalase, staphylase, maltose and potassium hydroxide tests that were used (Petzer et al. 2017). A positive maltose agar reaction (colour changed to yellow) confirmed *S. aureus* and a negative maltose reaction (colour remained purple) confirmed an organism from the *Staphylococcus intermedius* group (Hajek 1976, Sasaki et al. 2007).

All milk samples were analysed at the Milk Laboratory by microbiological and cytological examinations. (NMC 2017. Isolates to be tested for routine antimicrobial susceptibility were all selected from milk samples with a somatic cell count (SCC) (Fossomatic 5000 and Fossomatic FC, Rhine Ruhr) of more than 400 000 cells/ml (NMC 2017), when applicable, in order to include cases of subclinical mastitis. This was the general rule for routine antibiotic susceptibility testing. However, when a maltose negative *S. aureus* was isolated from a herd, antibiotic sensitivity testing was done on that organism regardless of SCC. The disc diffusion, Kirby Bauer method (Bauer et al. 1966) with published breakpoints was used to determine the antimicrobial susceptibility of the routine diagnostic samples that were used for the retrospective data analysis. The initial results were based on the diameter of the inhibition zones and were classified as sensitive, intermediate or resistant (intermediate grouped with resistant) in accordance with the clinical breakpoints that were established by the Clinical Laboratory and Standards Institute according to CLSI Vol. 32 No.3 (CLSI 2012) and (CLSI 2008a) (previous system). These data were later re-classified according to the CLSI M31-A3 2015 and CLSI M100-S25 2015 (intermediate grouped with susceptible), and the results were compared.

Nine antibiotics used in intramammary treatment (dry and lactating remedies) that were available for use in South Africa were tested. These included the penicillins (ampicillin 10 µg, cloxacillin 5 µg, penicillin G 10 IU), cephalosporins (cephalexin 30 µg, cefuroxime 30 µg), lincosamides (clindamycin 10 µg), tetracyclines (oxy-tetracycline 30 µg) and macrolides (tylosin 30 µg and cefoxitin 30 µg). The lactating cow numbers of the herds in this study varied from approximately 30 (smallest herd) to 1 700 cows, (largest herd) (Lactodata 2013). The South African national average daily milk yield was 20.2kg in 2013, and average yield varied slightly over the years of the study period. It would also have varied between herds, within herds between cows, and in different stages of lactation, but this detailed information is not available (Lactodata 2013).

All herds from which these isolates were collected participated in the pro-active udder health programme at the Milk Laboratory (Karzis et al. 2018). Antibiograms were done with every routine herd examination and the results were used by the veterinarians and producers when deciding on antibiotic treatment, which was then administered by trained staff. As part of the pro-active udder health programme. *S. intermedius* isolates from cows were distinguished from maltose positive *S. aureus* isolates.

Minimum Inhibitory Concentration (MIC) Testing:

For this part of the study we made use of 57 of these coagulase positive, maltose negative *S. aureus isolates* (2012-2013 n=15; 2018-2019 n=42) and 57 maltose positive *S. aureus* isolates (2012-2014 n=11; 2017-2018 n= 46), isolated from 38 dairy herds mainly from KwaZulu Natal and Eastern Cape provinces, but also with a few samples from Western Cape, Gauteng and Mpumalanga. Antimicrobial agents that were selected were based upon the availability of commercial intramammary infusion products or as representatives of their respective antimicrobial classes such as: ampicillin, oxacillin, erythromycin, penicillin and tetracycline. The MICs were determined by using the automated broth microdilution method (Staneck et al. 1985) (PM 32 panels and Microscan 40 Walkaway system, Beckman Coulter, California, USA) and the results evaluated according to the Clinical Laboratory Standards Institute (CLSI M31-A3, 2015 and CLSI M100-S25, 2015) and EUCAST. *Staphylococcus aureus* ATCC 25923 and *S. pseudintermedius* ATCC 49444 served as the reference strains for quality control purposes. The MIC data analysis used the LabPro software of the Microscan 40 Walkaway system, (Beckman Coulter, California, USA) in order to determine the MIC 90 values and the MIC 50 was calculated manually using the results from the automated MIC system (Microscan 40 Walkawaysystem, Beckman Coulter, USA).

Statistical Analysis

The results were grouped into two categories namely, resistant or susceptible. Results that were originally listed as being intermediate were grouped together with the resistant results in this analysis, according to the previous conventional system. However, after the introduction of the new CLSI & EUCAST recommendations that intermediate results should be grouped with those classified as susceptible, this analysis was repeated using this recently introduced system, and the two sets of results were compared.

The SCC categories that were used were low (< 150 $x10^3$ cells per ml milk), med (150 $x10^3$ to 400 $x10^3$ cells per ml milk) and high (> 400 $x10^3$ cells per ml milk).

The Chi^2 test was used to check for the existence of any effect of year, province, season or SCC category on the response variable, the resistance to the different antibiotics. This Chi^2 analysis also allowed classification of the categories of each variable in order to introduce the one with the lowest

level of resistance as the reference category in the following GLMM (general linear mixed model) analysis. Apparent relationships between season, province and SCC category on the prevalence of antibiotic resistance of all *S. aureus* isolates were tested in a multivariate model, a ('glmer' function within 'lme4' package of the R software © version 3.3.3) using a logit link-function. This model allowed the analysis to take account of the random effect from the different herds. This type of general linear mixed model (GLMM) can be used to take account of this random effect when comparing the different provinces, the different seasons and the SCC category as well as the interactions. Under the "goal of parsimony", a stepwise approach based on the Akaike Information Criterion, was used to select the best model.

Results

The first part of this study was the retrospective data analysis (Kirby Bauer) which was done only on maltose negative *S. aureus*, as a previous study had been done with a similar analysis of maltose positive *S. aureus* that combined the resistant and intermediate results and reported susceptible results separately (Karzis et al. 2018). The eight antibiotics that were used in this retrospective study were the commonly used antibiotics that are available as intramammary remedies in South Africa. The original classification (CLSI 2008, CLSI 2012) and the more recent classification (intermediate grouped with susceptible) (CLSI M31-A3 2015, CLSI M100-S25 2015) of antibiotic resistance showed similar trends. These trends of resistance to ampicillin, cephalexin, cefalonium, cloxacillin, oxy-tetracycline and penicillin peaked (at highest) in 2011, and for tylosin in 2013, and then decreased over time. However, in 2014 there was a slight increase in antibiotic resistance seen for cloxacillin and in 2016 for ampicillin, cephalexin, cefalonium, penicillin and tylosin (Figures 5.1, 5.2, 5.3, 5.4, 5.5 & 5.6). The analysis of antibiotic resistance for cloxacillin showed significant differences between SCC categories only according to the GLMM analysis ($p<0.05$). Cefoxitin, cloxacillin and cefuroxime showed no significant differences for years and provinces (Table 5.1). The analysis of antibiotic resistance of maltose negative *S. aureus* for oxy-tetracycline, cephalexin, ampicillin, tylosin, cefalonium and penicillin G showed significant differences according to the GLMM ($p<0.05$) over time (years). Similar trends (but not significant) were shown for clindamycin which was used from 2009 to 2012 and increased in percentage antibiotic resistance and (also not significant) for cefoxitin which was used from 2014 to 2017 and showed a decreased antibiotic resistance over time.

These data were re-evaluated according to the more recent classification system (CLSI M31-A3 2015, CLSI M100-S25 2015) that grouped results from susceptible and intermediate data together, so that the resistance data were reported on separately. Some differences were apparent as a detailed examination of Tables 5.1 and 5.2 will show:

Tylosin, cloxacillin, oxy-tetracycline and cefalonium showed differences in the effects of year, season, province and SCC categories on antibiotic resistance as seen between the original system

of classification (Table 5.1) compared to the more recent system of classification (Table 5.2) CLSI guidelines. According to the new guidelines, tylosin, oxy-tetracycline and cefalonium showed no significant effects at all on antibiotic resistance from the factors measured (Table 5.2), whereas they had showed some effects of year, season and SCC categories when using the previous classification (Table 5.1). Cloxacillin showed an effect of SCC category only when using the previous classification system for analysis (Table 5.1), but there appeared to be possibly a slight effect of season as well as when using the more recent classification (Table 5.2). There was no effect of province on antibiotic resistance when using both the previous system (Table 5.1) and the more recent system (Table 5.2) CLSI guidelines. Ampicillin, penicillin G, cefoxitin and cefuroxime showed similar effects of year, province, season and SCC categories for both the previous system (Table 5.1) and the more recent system (Table 5.2) CLSI guidelines. For ampicillin, comparing the results from the previous system of analysis, seasonal effects showed differences only for autumn compared to spring (Table 5.1).However, by using the more recent system, the results for ampicillin appeared to show an effect of both summer and autumn (Table 5.2) with high resistance at those times, this could be compared to spring where there was low resistance.

This comparison between the analysis of the effects of year, season, province and SCC categories of antibiotic resistance of maltose negative *S. aureus* shows that the new breakpoints (CLSI) as well as the grouping of intermediate readings with susceptible readings instead of with resistant readings, as was done in the past, does indeed make a difference to the results (Tables 5.1 & 5.2). The logic behind the change of this grouping, was that in the past, intermediate readings were grouped with resistant readings to create more strictly defined categories.

Antibiotic products shown in Tables 5.3 and 5.4 are all the products from the PM 32 panel (Beckman Coulter) that showed resistance to any of the isolates tested. The 57 maltose positive and the 57 maltose negative *S. aureus* isolates were all resistant to ampicillin. Out of the total of 114 isolates overall, only 37 were resistant to more than one product namely, 30 maltose negative *S. aureus* and seven maltose positive *S. aureus* (Table 5.4). There was a total of 25 multidrug resistant (MDR) isolates (isolates resistant to an antibiotic from three or more antibiotic classes), 3 maltose positive and 22 maltose negative *S. aureus* (Table 5.4).

Table 5.1 Summary of differences in antibiotic resistance (GLMM) between years, seasons, provinces and SCC categories according to CLSI Vol. 32 No.3 (CLSI 2012) and (CLSI 2008a), with intermediate responses grouped with resistant responses (the original system).

Antibiotic	Year	Season	Province	SCC Category
Tylosin	P < 0.01*	P=0.08 Autumn(Highest R) & Spring (Lowest R) *	NS	NS*
Penicillin G	P = 0.04*	P<0.01 Autumn (highest R)& Spring (Lowest R)*	NS	NS*
Ampicillin	P = 0.02*	P=0.02 Autumn (highest R)& Spring (Lowest R)*	NS	NS*
Clindamycin	N/A	N/A	N/A	N/A
Cefuroxime[a]	NS	NS	NS	NS*
Cefalonium (2011-2017)	P = 0.08*	NS	NS	P= 0.01 Med SCC (Highest R)& High SCC (Lowest R)*
Cefoxitin (2014-2017)	NS	NS	NS	NS*
Oxy-tetracycline	P = 0.003*	NS	NS	NS*
Cephalexin[b]	P = 0.007*	P=0.07 Summer(Highest R)&Autumn(Lowest R)*	NS	NS
Cloxacillin	NS	NS	NS	P<0.05 Low SCC (Highest R) & High SCC (Lowest R)*

See text for detailed explanation.

*The variable/s included in the best statistical model (GLMM), even if NS.

NS = Not Significant, R = Resistance, N/A = Not applicable.

Table 5.2 Summary of differences in antibiotic resistance (GLMM) between years, seasons, provinces and SCC categories according to CLSI M31-A3 2015 and CLSI M100-S25 2015, with intermediate responses grouped with susceptible responses (the more recent system).

Antibiotic	Year	Season	Province	SCC Category
Tylosin	NS	NS	NS	NS*
Penicillin G	P = 0.06*	P=0.0011 Autumn & P = 0.0019 Summer (Highest R) & Spring (Lowest R)*	NS	NS*
Ampicillin	P = 0.031*	P=0.000374 Autumn & P = 0.042 Summer (Highest R) & Spring (Lowest R)*	NS	NS*
Clindamycin	N/A	N/A	N/A	N/A
Cefuroxime[a]	NS	NS	NS	NS*
Cefalonium (2011-2017)	NS	NS	NS	NS
Cefoxitin (2014-2017)	NS	NS*	NS	NS*
Oxy-tetracycline	NS	NS	NS	NS*
Cephalexin[b]	P = 0.008*	P=0.0 12 Summer (Highest R) & Winter (Lowest R)*	NS	NS*
Cloxacillin	NS	P=0.0 58 Summer (Highest R) & Autumn (Lowest R)*	NS	P = 0.067 Low SCC (Highest R) & High SCC (Lowest R)*

See text for detailed explanation.

*The variable/s included in order to achieve the best statistical model (GLMM), even if NS.

NS = Not Significant, R = Resistance, N/A = Not applicable.

Table 5.3 Distribution of minimum inhibitory concentrations (MIC) cumulative percentage inhibited by antibiotic level for maltose positive (shown as STA +) (n=57) and maltose negative *Staphylococcus aureus* (shown as STA -) (n=57).

Product	% Resistance	STA (+) (n = 57) Distribution of MICs % (µg/ml)												% Resistance	STA (-) (n = 57) Distribution of MICs % (µg/ml)										MIC 90		MIC 50	
		0.03	0.06	0.12	0.25	0.5	1	2	4	8	32	64	256		0.03	0.06	0.12	0.25	0.5	1	2	4	8	32	64	STA+	STI-	STA+	STI-
Amox/K Clav	3.5	a	a	a	a	89[a]*	99[a]*	99[a]*	99[a]*	99*				21.1	a	a	a	a	77[a]*	89[a]*	91[a]*	95*	100*			1	2	0.5	0.5
Ampicillin		a	a	a	a	70*	78*	85*	90*	90*					a	a	a		56*	63*	68*	93*	96*			4	4	0.5	0.5
Azithromycin	3.5	a	a	a	a	a	98	98		b	b	b	b	1.8	a	a	a	a		98*	98*		b	b	b	1	1		
Cefepime	1.8	a	a	a	a	a	a	a	100[a]	100[a]	b	b	b	22.8	a	a	a	a	a	a	a	98[a]	98*	b	b	4	4		
Cefotaxime	1.8	a	a	a	a	a	93[a]*	100[a]*	a	a	b	b	b	22.8	a	a	a	a	a	88[a]*	96[a]*	a		b	b	1	2		
Cefoxitin	1.8	a	a	a	a	a	a	a	100*					0	a	a	a	a	a	a		100*				4	4	_	_
Cefuroxime	1.8	a	a	a	a	a	a	a	100*	100*				21.1	a	a	a	a	a	a		89*	96*			4	8	_	_
Ciprofloxacin	7	a	a	a	a	89*	94*							1.8	a	a	a		95*	98*						1	0.5	0.5	_
Clindamycin	5.3	a	a	a	91[a]*	96[a]*	*	98*	b	b	b	b	b	24.6	a	a	a	68[a]*	77*	*	86*	b	b	b	b	0.3			0.25
Daptomycin[#]	0	a	a	a	a	95[a]*	100[a]*	100*	100*					17.5	a	a	a	a	82[a]*	82*	82*	82*				0.5			0.5
Ertapenem[#]	3.5	a	a	a	a	98*[a]	98[a]*				100			1.8	a	a	a	a	97[a]*	97*						0.5	0.5		0.5
Erythromycin	1.8	a	a	a	a	a	99*	99*		b	b	b	b	15.8	a	a	a	a		82*	84*		b	b	b	1			1
Fosfomycin[#]	1.8	a	a	a	a	a	a	a	a	a	98[a]*	a	b	12.3	a	a	a	a	a	a	a	a	a	88[a]*		32			32
Fusidic Acid[a]	0							100*						10.5							89*					2			2
Gentamicin	7	a	a	a	a	a	93[a]*	98[a]*	100[a]*		b	b	b	22.8	a	a	a	a	a	79[a]*	98[a]*	98*		b	b	1	2		1
Imipenem	3.5	a	a	a	a	a	a	99[a]	99[a]	99	b	b	b	21.1	a	a	a	a	a	a	100[a]*	100*	100*	b	b	2	2		
Levofloxacin[#]	0	a	a	a	a	a	100[a]*	100[a]*		b	b	b	b	0	a	a	a	a	a	100[a]*	100*	b	b	b	b	1	1		
Linezolid[#]	0	a	a	a	a	21[a]*	64[a]	100[a]*	100*					15.8	a	a	a	a	21[a]*	37[a]*	82*	84				2		1	2
Meropenem[#]	1.8	a	a	a	a	a		100[a]*	100*	100*				21.1	a	a	a	a			100*	100*	100*			2	2		
Moxifloxacin[#]	0	a	a	a	a	100[a]*	100*	b	b	b	b	b	b	0	a	a	a	a	100*	100*	b	b	b	b	b	0.5	0.5		
Nitrofuratoin[#]	0	a	a	a	a	a	a	a	a	a	a	100*	b	0	a	a	a	a	a	a	a	a	a		91*	64	64		
Oxacillin	1.8	a	a	a	54[a]*	91[a]*	98[a]*	100[a]*						21.1	a	a	a	56[a]*	79[a]*	88[a]*	88*					0.3		0.5	0.25
Penicillin	36.8	61*	70*	70[a]*	70[a]*			76*						47.4	33*	40[a]*	53[a]*	54[a]*	*	*	63*							0.03	0.12
Rifampin[#]	0	a	a	a	a	100[a]*	100[a]*	100*	b	b	b	b	b	5.3	a	a	a	a	93[a]*	95*	95*	b	b	b	b	0.5	0.5		
Synercid	5.3	a	a	a	a	a	100[a]*	100*	100[b]*	b	b	b	b	19.3	a	a	a	a	a	75*	77*	82[b]*	b	b	b	1			1
Teicoplanin[#]	0	a	a	a	a	a	100[a]*	100[a]*	100[a]*	100[a]*	b	b	b	14	a	a	a	a	a	82[a]*	84[a]*	8[a]*	86*	b	b	1			1
Tetracycline	7	a	a	a	a	a	92[a]*	92[a]*	a					7	a	a	a	a	a	95[a]*	95[a]*					1	1	1	
Tobramycin[#]	3.5	a	a	a	a	a	98[a]*	98[a]*	100[a]*		b	b	b	7	a	a	a	a	a	93[a]*	98[a]*	98*		b	b	1	1		
Trimeth/Sulfa	0	a	a	a	a	a	100*	100*	100*					0	a	a	a	a		98*	98*	100*				1	1		
Vancomycin	0	a	a	a	0[a]*	27[a]*	86[a]*	100[a]*	100*	100*	b	b	b	31.6	a	a	a	7[a]*	18[a]*	54[a]*	67*	68*	81*	b	b	2	_	1	1

 # CLSI Human breakpoints M100-S25 used, antimicrobials with no superscript CLSI Veterinary breakpoints M31-A3used, * dilution ranges PM 32,

[a] Denote the susceptible MIC, [b] Denote resistant MIC, for antimicrobials with only [a] the unmarked fields are taken as resistant, for antimicrobials with [a] and [b] the unmarked fields in between denote Intermediate (CLSI M100-S25 & CLSI M31-A3).

Trimeth/Sulpha = Trimethoprim / Sulphamethoxazole; STA + = maltose positive *S. aureus*; STA - = maltose negative *S. aureus*; n = number of isolates.

Table 5.4 Minimum inhibitory concentration (MIC) results (CLSI M31-A3 2015 and CLSI M100-S25 2015) and EUCAST of maltose positive (shown as STA +) and maltose negative *Staphylococcus aureus* (shown as STA -) which showed resistance to more than one antibiotic.

Isolate	(n)		Products																									
	T	Class	AC	AM	AZ	CE	CT	FO	CX	CP	DA	DP	ET	E	FO	FA	G	IM	LZ	ME	MU	OX	P	R	SY	TEI	TE	TO	VA
STA-	2	1	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	S	S	S	S*	S	S*	S	S*	R	S	S	S	S	S	S
STA+	2	1	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
STA-	2	1	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
STA-	2	1	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
STA-	2	1	S	R	S	S	S	S	S	S	I	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
STA-	2	1	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
STA-	3	3	S	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
STA-	3	2	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	S	S	R	S*	S	S*	S	S*	R	S	S	S	S	S	S
STA-	3	2	S*	R	S	S*	S*	S	S*	R	S	S	N/A	S	S	S	S	S*	S	S*	S	S*	R	S	S	S	S	S	S
STA-	3	2	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S
STA+	3	2	S	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
STA+	3	2	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	S
STA-	4	3	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	R
STA-	3	3	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R
STA-	3	3	S	R	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S
STA-	3	3	S	R	S	S	S	S	S	S	R*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
STA+	4	3	S	R	R	S	S	S	S	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
STA-	4	3	S	R	S	S	S	S	S	S	I	S	S	S	S	S	R	S	S	S	S	S	R	S	I	S	S	S	R
STA-	4	3	S	R	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
STA-	5	3	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	S	S	R	S*	S	S*	S	S*	R	S	S	S	S	R	R
STA+	5	3	S	R	S	S	S	S	S	R	S	S	S	S	S	S	R	S	S	S	S	S	R	S	S	S	S	R	S
STA+	5	2	R	R	S	S	S	S	S	S	I	S	R	S	S	S	S	R	S	S	S	S	R	S	S	S	S	S	S
STA-	5	3	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	R	R	S	S	S	R
STA-	5	3	S	R	S	R	R	S	S	S	R	S	S	S	R	S	S	S	S	S	N/A	S	S	S	S	S	S	S	S
STA-	4	3	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	R	S	S	S*	S	S*	S	S*	R	R	S	S	S	S	S
STA-	5	3	S*	R	S	S*	S*	S	S*	S	S	S	N/A	S	S	S	R	S*	S	S*	S	S*	R	S	S	S	S	R	R
STA-	6	3	S	R	S	S	S	S	S	S	R	R	S	R	S	S	S	S	R	S	S	S	S	S	R	S	S	S	S
STA-	9	3	R	R	S	R	R	S	R	S	S	S	N/A	S	S	S	S	R	S	R	S	R	R	S	S	S	S	S	S
STA-	12	3	S	R	S	S	S	S	S	S	R	R	R	R	R	S	S	S	R	S	R	S	R	S	R	R	S	S	R
STA-	14	3	R	R	S	R	R	S	R	S	I	R	N/A	S	S	S	S	R	R	R	S	R	R	S	R	S	S	S	R
STA+	16	3	R*	R*	R	R*	R*	R	R*	S	R	S	R*	S	S	S	R	R*	S	R*	N/A	R	R*	S	R	S	S	R	S
STA-	16	3	R	R	S	R	R	S	R	S	R	R	N/A	I	S	R	S	R	R	R	N/A	R	R	S	R	R	S	S	R
STA-	18	3	R	R	S	R	R	S	R	S	R	R	N/A	R	S	R	S	R	R	R	R	R	R	S	R	R	S	S	R
STA-	20	3	R	R	R	R	R	S	R	S	R	R	N/A	R	S	R	S	R	R	R	N/A	R	R	R	R	R	R	S	R
STA-	21	3	R	R	S	R	R	S	R	S	R	R	N/A	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S	R
STA-	20	3	R	R	S	R	R	S	R	S	R	R	N/A	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R
STA-	21	3	R	R	S	R	R	S	R	S	R	R	N/A	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R

T = Total number of antimicrobial products resistant, Class = Number of antimicrobial classes that each isolate is resistant to, S = Susceptible, R = Resistant, I = Intermediate, N/A = Not applicable, (n) = number of isolates.

AC = Amox/K Clav, AM = Ampicillin, AZ = Azithromycin, CE = Cefepime, CT = Cefotaxime, FX = Cefoxitin Screen, CX = Cefuroxime, CP = Ciprofloxacin, DA = Clindamycin, ET = Ertapenem, E = Erythromycin, FO = Fosfomycin, FA = Fusidic Acid, G = Gentamycin, IM = Imipenem, LZ = Linezolid, ME = Meropenem, MU = Muriprocin, OX = Oxacillin, P = Penicillin, RI = Rifampin, SY = Synercid (Quinupristin-Daldopristin), TEI = Teicoplanin, TE = Tetracycline, TO = Tobramycin, VA = Vancomycin.

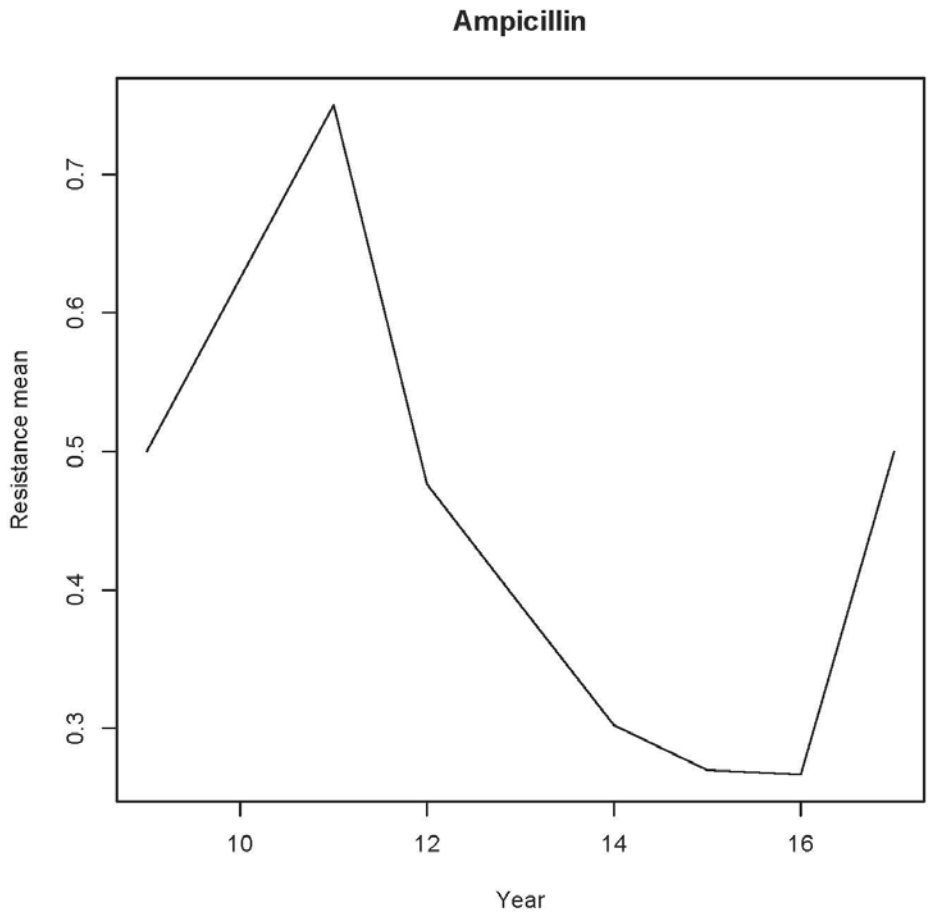

Figure 5.1 Trends of antibiotic resistance over time of maltose negative *S. aureus* for ampicillin (Kirby-Bauer, retrospective data), according to CLSI Vol. 32 No.3 (CLSI 2012) and (CLSI 2008a), with intermediate responses grouped with resistant responses (the previous system).
Resistance mean is the proportion of isolates per year.

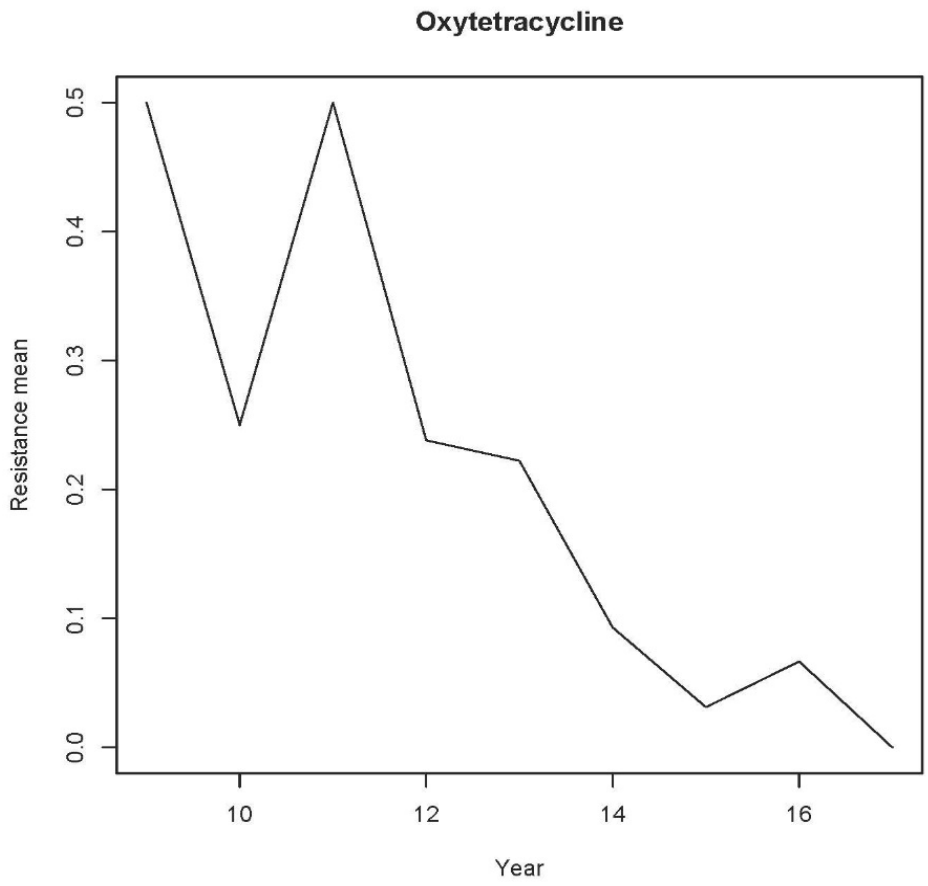

Figure 5.2 Trends of antibiotic resistance over time of maltose negative *S. aureus* for oxytetracycline (Kirby-Bauer, retrospective data) according to CLSI Vol. 32 No.3 (CLSI 2012) and (CLSI 2008a), with intermediate responses grouped with resistant responses (the previous system).

Resistance mean is the proportion of isolates per year.

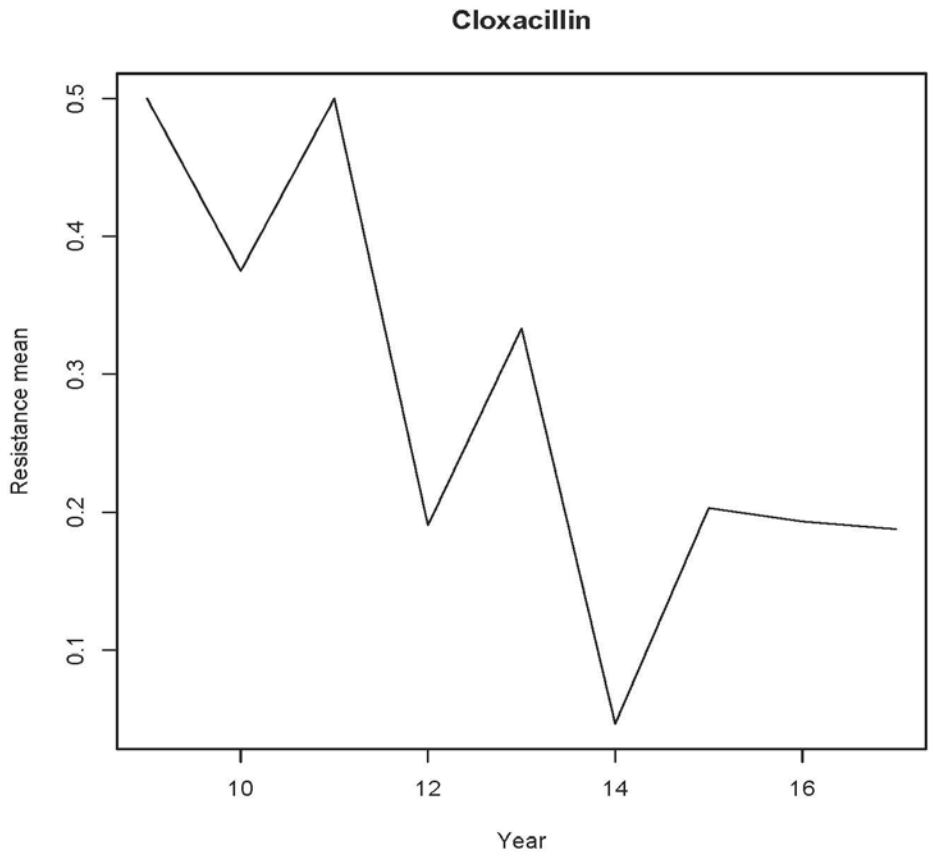

Figure 5.3 Trends of antibiotic resistance over time of maltose negative *S. aureus* for cloxacillin (Kirby-Bauer, retrospective data) according to CLSI Vol. 32 No.3 (CLSI 2012) and (CLSI 2008a), with intermediate responses grouped with resistant responses (the previous system).

Resistance mean is the proportion of isolates per year.

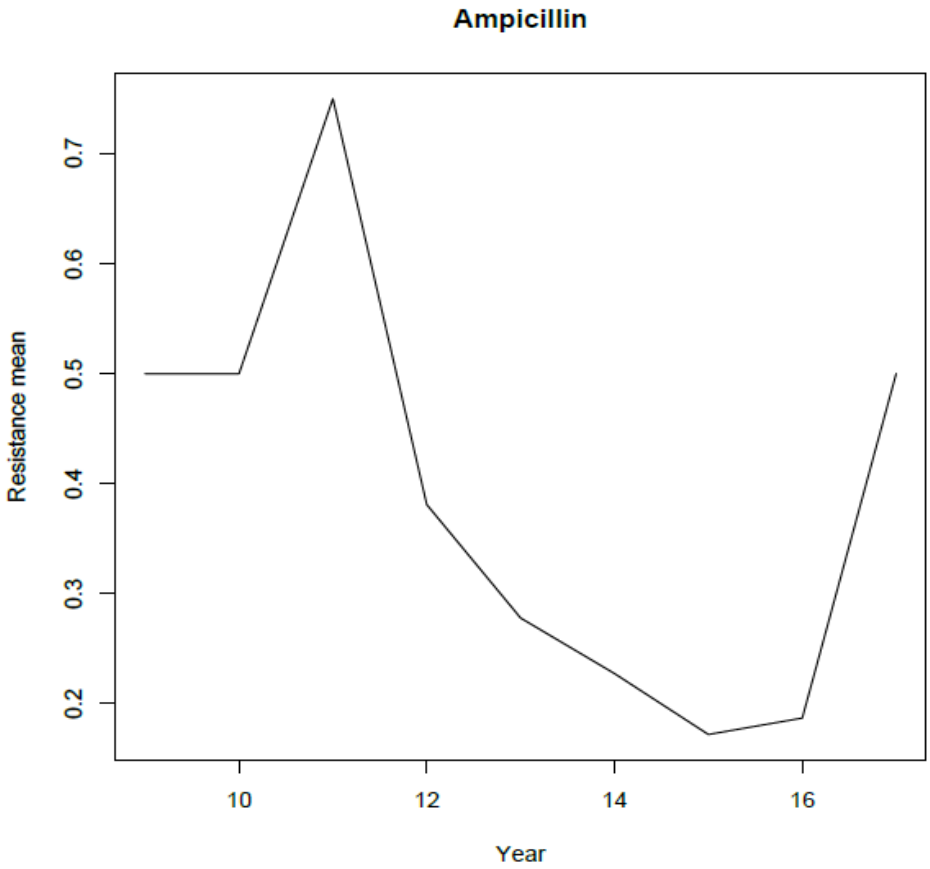

Figure 5.4 Trends of antibiotic resistance over time of maltose negative *S. aureus* for ampicillin (Kirby-Bauer, retrospective data), according to CLSI M31-A3 2015 and CLSI M100-S25 2015, with intermediate responses grouped with susceptible responses (the more recent system).

Resistance mean is the proportion of isolates per year.

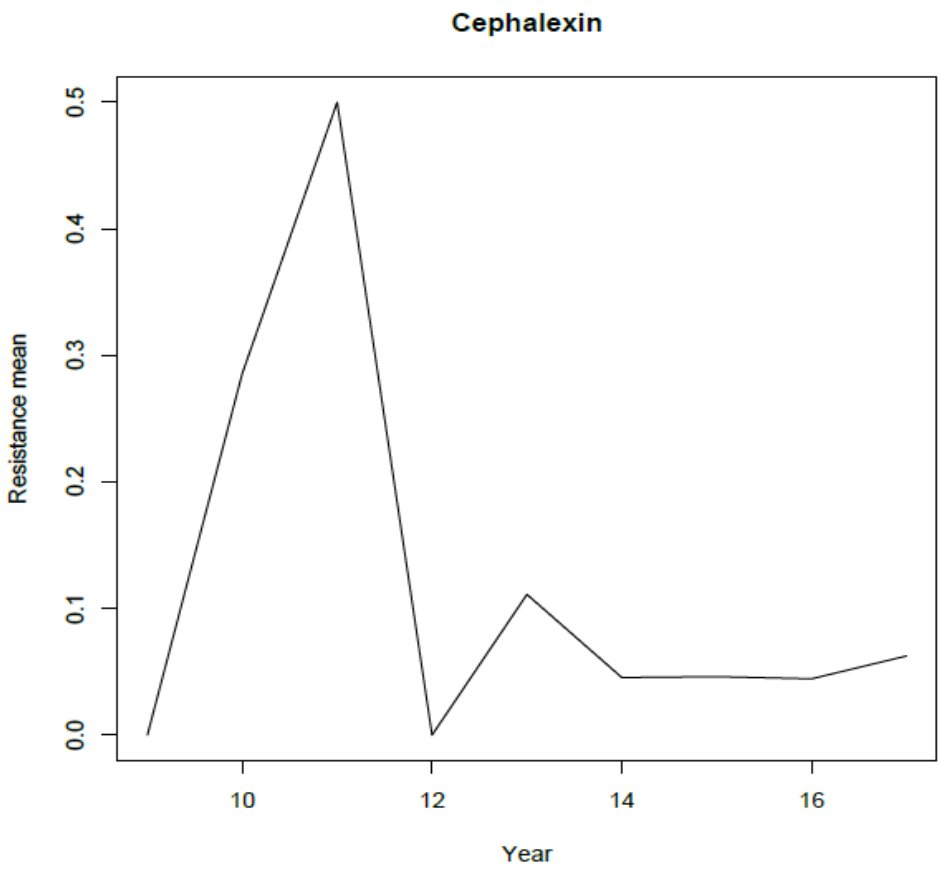

Figure 5.5 Trends of antibiotic resistance over time of maltose negative *S. aureus* for cephalexin (Kirby-Bauer, retrospective data), according to CLSI M31-A3 2015 and CLSI M100-S25 2015, with intermediate responses grouped with susceptible responses (the more recent system).

Resistance mean is the proportion of isolates per year.

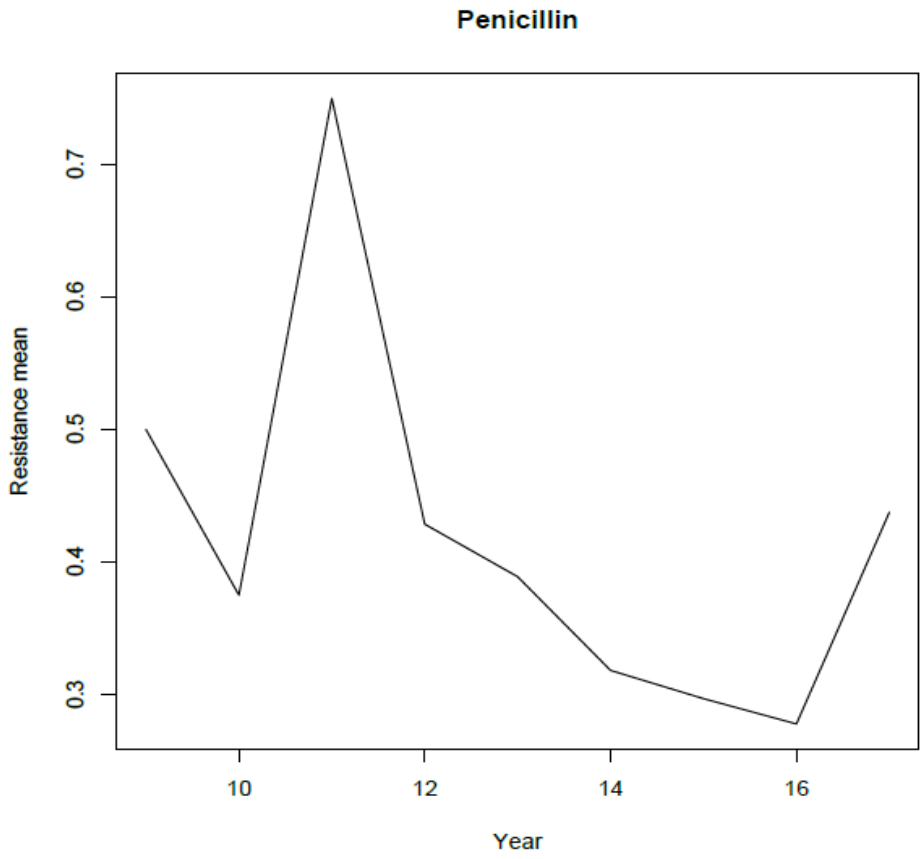

Figure 5.6 Trends of antibiotic resistance over time of maltose negative *S. aureus* for penicillin G (Kirby-Bauer, retrospective data), according to CLSI M31-A3 2015 and CLSI M100-S25 2015, with intermediate responses grouped with susceptible responses (the more recent system).
Resistance mean is the proportion of isolates per year.

Discussion

This study on the maltose negative *S. aureus* showed no significant differences of antibiotic resistance between the provinces, and only a limited significant difference related to seasons and SCC categories, whereas there were no significant interactions between any of the variables (Tables 5.1 & 5.2). The relationship of the occurrence of SCC category to antibiotic resistance was in accordance with a study in Denmark (Bennedsgaard et al. 2006), which also found high SCC to correspond with low antibiotic resistance and low SCC to correspond with high antibiotic resistance. This could be due to the SCC being more of an indicator of irritation and severity of the infection rather than an indicator of antibiotic resistance of the organism.

In contrast, the study on maltose positive *S. aureus* showed that of the provinces, the lowest prevalence of antibiotic resistance to the majority of the categories of antibiotics that were tested was present in KwaZulu-Natal during spring, except for cephalosporins which had the lowest levels of prevalence of bacterial resistance in Gauteng during winter (Karzis et al. 2019). Although, there were great differences in the numbers of herds and samples between the provinces, these differences were taken into account in the model, during the analysis.

Resistance patterns of the maltose positive *S. aureus* to the eight antibiotics varied in the different seasons and provinces, possibly because of the different weather conditions, as well as the action and spectrum of antibiotics (Karzis et al. 2019). There was one specific strain of maltose negative *S. aureus* (Chapter 4), originating in and found mostly in KwaZulu Natal, but also now present in all nine provinces of South Africa, albeit in small numbers.

The antibiotic resistance trends (Figures 5.1 to 5.6) were in agreement with those shown for the same antibiotics with coagulase negative staphylococci (Petzer & Karzis 2019), but in contrast to the trends shown for the maltose positive *S. aureus* over time (Karzis et al. 2018). The study on the maltose positive *S. aureus* showed a general increase in resistance over time except for the 20 well managed herds (part of the pro-active udder health programme), which showed a decrease in resistance over time (Karzis et al. 2018). However, due to the general increase in antibiotic resistance in recent years, there were some cases where there may not have been any effective products available and then the intermediate product/s would have been used at a higher concentration, which is why the intermediate responses should now be grouped with the susceptible responses. However, the trends of antibiotic resistance over time of maltose negative *S. aureus* evaluated with both the previous (intermediate responses grouped with resistant responses) (Figures 5.1, 5.2 & 5.3) and the more recent system (intermediate responses grouped with susceptible responses) have been similar for these organisms tested (Figures 5.4, 5.5 & 5.6).

The antibiotic resistance trends (Figures 5.1 – 5.6) and profiles (Tables 5.3 & 5.4), allow for informed treatment choices to be made without the need to wait for any antibiotic sensitivity test results. However, the results of antibiotic sensitivity tests (Kirby Bauer or MIC), are just an indication that the particular organism has the ability to be killed by a particular antibiotic *(in-vitro)*. In the udder, the situation may be very different from tests *in vitro*. This is because the site of the infection is very difficult to reach via very small arteries and lactiferous ducts and also due to udder pharmacodynamics (very few products are successful in a water and fat environment). As a result of this, the treatment success for mastitis in is not very high (27%)

as determined in a large study done in five European countries; France, Hungary, Italy, the Netherlands, and the United Kingdom (Swinkels et al. 2013) to begin with, and can lead to the development of antibiotic resistance by mastitis-causing organisms. Treatment success against *S. aureus* tends to be better in the dry period but this is nevertheless not ideal (Barkema et al. 2006, Roy et al. 2009). The focus should be on the prevention and monitoring through the pro-active udder health programme (Petzer et al. 2016) rather than only on the actual treatment.

The second part of this study concerned the MIC test on 57 maltose negative and 57 maltose positive *S. aureus* isolates. The MIC results of antibiotic resistance (Table 5.3) confirmed the Kirby Bauer results (Tables 5.1 & 5.2) for maltose negative *S. aureus* for the products tested. Antibiotics are grouped into three broad groups that are based on approved usage (US FDA): approved for human use only; approved for animal use only; or approved for the use in both humans and animals. The antibiotics studied for the retrospective data analysis for the maltose negative *S. aureus* are approved for the use in both humans and animals. However, from the antibiotics that were studied for the MIC testing (PM 32 panel), daptomycin, ertapenem, fosfomycin, meropenem, moxifloxacin, rifampin, teicoplanin and tobramycin are approved for human use only and the remainder of antibiotics in the panel (PM 32) are approved for both use in humans and animals (Tables 5.3 & 5.4). Antibiotics that are approved for human use only, create a reserve of unique antibiotics for humans. Antibiotics that are approved for animal use only, such as the ionophores, should not create a risk to human health.

Antibiotics contribute to animal care in four ways: to treat animals diagnosed with an illness; to control spread of illness within a herd or flock; to prevent illness in healthy animals when exposure is likely; and to ensure healthy growth by maintaining the right balance of bacteria for improved nutrient utilization (antibiotics approved for animal use only) (FAO 2009, Food and Agricultural Organization of the United Nations 2014). Antibiotics that are used in animals are important for global food security. Estimates have been made that nearly one billion people in the world do not get enough to eat each day and nearly three billion are trying to diversify their diet to include more meat, milk and eggs (FAO 2009, Food and Agricultural Organization of the United Nations 2014). Veterinary medicines that include antibiotics provide a valuable tool to help veterinarians and producers to deliver healthy animals to meet the growing need for safe, nutritious, affordable food, while making the most of limited natural resources in a sustainable manner. As the world population is expected to grow to nine billion by 2050, tools such as antibiotics which keep animals healthy will be essential to meet the increasing demand

for food (Kharas 2010, FAO 2009, Food and Agricultural Organization of the United Nations 2014).

Antibiotics that could be considered for routine testing by veterinary diagnostic laboratories, may be divided into four groups (CLSI document M31-A3):
Group A: Antibiotics with specific interpretive criteria for veterinary medicine;
Group B: CLSI approved interpretive criteria for human medicine;
Group C: No veterinary species-specific or human-specific interpretive criteria;
Group D: Supplemental to be tested selectively.
Ampicillin, oxacillin, erythromycin, penicillin and tetracycline are the corresponding antibiotics from the panel tested which are approved for the control of bovine mastitis specifically.

The distribution of the MIC test results of the antibiotics tested are summarized in Tables 5.3 and 5.4 for maltose positive and maltose negative *S. aureus* isolates respectively. One maltose positive and 12 maltose negative *S. aureus* isolates were resistant to oxacillin (Table 5.4). For erythromycin one maltose positive and nine maltose negative *S. aureus* isolates proved to be resistant, respectively. The MICs of clindamycin were two dilution steps lower (Table 5.4) than those of azithromycin, cefotaxime, erythromycin, gentamycin, linezolid, teicoplanin, tetracycline, tobramycin, trimeth/sulpha and vancomycin for maltose negative *S. aureus* isolates (Table 5.3). However these patterns differed for the maltose positive *S. aureus* isolates (Table 5.3). In general, the resistance rates of the maltose positive *S. aureus* obtained in this study, corresponded well to those reported in other studies (Salmon et al. 1998, Wallmann et al. 2004, Lüthje & Schwarz 2006).

The MIC 50 represents the MIC value at which ≥50% of the isolates in a test population are inhibited, and it is equivalent to the median MIC value. The MIC 90 represents the MIC value at which $\underline{\geq}$90% of the isolates in the test population are inhibited (Schmidt 1987). The MIC breakpoints (chosen concentration [mg/L] of an antibiotic which defines whether a species of bacteria is susceptible or resistant to the antibiotic) of certain antibiotics were susceptible for MIC 50 and MIC 90 for both the maltose negative and the maltose positive *S. aureus*, except for MIC 90 of maltose negative *S. aureus* (Table 5.3). The maltose negative *S. aureus* was more resistant to the amoxicillin clavulanic acid combination (used in human medicine), ampicillin and cefuroxime at MIC 90, and for clindamycin at MIC 50 (Table 5.3). However, maltose positive *S. aureus* was more resistant to oxacillin at MIC 90 and MIC 50 and to clindamycin at MIC 90 (Table 5.3). Infrequently found resistance patterns were found in 17 of the 57 maltose negative *S. aureus* isolates which were resistant to vancomycin and

one maltose positive and eight maltose negative *S. aureus* isolates which were resistant to oxacillin (CLSI M31-A3).

Overall in this study there are more multi-drug resistant maltose negative *S. aureus* than maltose positive *S. aureus* isolates and the same general interpretation applied for isolates resistant to two or more antibiotics of varying combinations in general (Table 5.4).

There have been many studies in both animal and human medicine that have identified multidrug resistant and pan-drug resistant *S. aureus* isolates (Magiorakos et al. 2012, Hiramatsu et al. 2014, Haran et al. 2012, Gopal & Divya 2017). However, most of these studies were done on traditionally identified coagulase positive, maltose positive *S. aureus*, since the maltose negative coagulase positive isolates were thought at that time to have been part of the *S. intermedius* group of isolates. Multi-drug resistant *S. intermedius* and *S. pseudintermedius* isolates have been described in dogs, cats and horses, mainly from skin infections (Griffeth et al. 2008, Vengust et al. 2006, Hanselman et al. 2007, Medleau et al. 1986, Kania et al. 2004, Abraham et al. 2007, Lilenbaum et al. 1999, Weese et al. 2010). Although a coagulase positive, maltose negative *S. aureus* strain was subsequently isolated from bovine mastitis (Johler et al. 2012), there appear to be no antibiotic susceptibility profiles of this organism yet. The maltose negative *S. aureus* in this study was originally phenotypically identified as *S. pseudintermedius* (Hajek 1976, Sasaki et al. 2007), but further MALDI-TOF MS and 16S r RNA sequence analysis on these isolates from dairy cattle, showed that these were in fact a strain of *S. aureus* (Chapter 4). Therefore it would make sense that these maltose negative *S. aureus* isolates in this study, have reacted in a way more like the classical *S. pseudintermedius* isolates which were identified in other species, reacted in other studies (Griffeth et al. 2008, Vengust et al. 2006, Hanselman et al. 2007, Medleau et al. 1986, Kania et al. 2004, Abraham et al. 2007, Lilenbaum et al. 1999, Weese et al. 2010).

Human nasal *S. aureus* colonization has previously been reported as being a risk factor for pig farming (Armand-Lefevre et al. 2005, Aubry-Damon et al. 2004) and *S. aureus* strains from pig farmers were found to be those present in pigs but had not been found in non-farmers (Aubry-Damon et al. 2004). However, little attention was paid to MRSA in pigs until ‘unexpected’ MRSA infection and colonization were identified in people that had been in contact with pigs in the Netherlands (Voss et al. 2005). In the light of this research in pigs, it is possible that in a similar way of transmission of some strains of these resistant *S. aureus* (predominantly maltose negative strains) isolated from dairy cattle in South Africa could be from people. Previous studies done in KwaZulu Natal (Schmidt et al. 2015), have indicated

anthroponosis ("reverse zoonosis") of *S. aureus* in South Africa, with one of the strains identified as the same maltose negative *S. aureus* strain in Chapter 4. These maltose negative strains of *S. aureus* seem to have completely different antibiotic resistance trends and severity of antibiotic resistance, when compared to the traditionally identified maltose positive *S. aureus*.

Resistance to antimicrobial agents, such as doxycycline and trimethoprim-sulfamethoxazole is very uncommon in *S. aureus.* In this study the MIC 90 of trimethoprim-sulfamethoxazole was the same for both the maltose negative and the maltose positive *S. aureus*. The reason for these uncommon resistance profiles of maltose negative *S. aureus* isolates, which have also shown to be resistant to antibiotics that are only used in human medicine, (e.g. carpapenems like imipenem and ertapenem) which had 10 resistant isolates each (Table 5.4), remains questionable. These are antibiotics which are not used at all in animal medicine. Anthroponosis seems to be a strong possibility, because these isolates have been shown to be present on the skin of humans that come into close contact with dairy cattle. This would be similar to the findings of the studies with the dogs in Brazil (Machado et al. 2017) and pigs in Germany (Armand-Lefevre et al. 2005, Aubry-Damon et al. 2004) and the Netherlands (Voss et al. 2005) respectively. Further studies need to be done to explore the origin of such resistant isolates of maltose negative *S. aureus*. Future work is also necessary to determine the resistance genes present in resistant maltose negative *S. aureus* strains.

A study done with dairy cows in Tennessee which was similar to this study also showed that there was a variation of prevalence of antibiotic resistance of *S. aureus* within and among farms over time, with an increasing trend in tetracycline resistance (Abdi et al. 2018). This was also in accordance with the report which showed that the predominant antibiotic groups used in animal health management in South Africa from 2014 to 2015, were the growth promoters (animal-use-only antibiotics) (62%) followed by tetracyclines (17%) and macrolides (11%) (National Department of Health 2018). In this study five isolates were resistant to tetracyclines and ten isolates were resistant to macrolides (represented by erythromycin) and only one of each of these isolates were maltose positive, whereas the rest were maltose negative *S. aureus* (Table 5.4).

The use of antibiotics in South Africa in 2015, has been stated to be 21 149 standard units per 1000/population (IMS Health 2015) (Note: 1 standard unit is equivalent to 1 tablet, or injection), and this was significantly higher than most other countries in the world. Broad-spectrum penicillin usage in humans in South Africa was 1.3 to 3.3 times more than that used

in other countries and 0.8 times that used in the United Kingdom or the USA (National Department of Health 2018). It is by no means clear if the high number of maltose negative *S. aureus* isolates resistant to penicillin and ampicillin (Table 5.4) might solely be attributed to the high antibiotic usage.

Conclusion

The antibiotic resistance trends found were similar for both the previous CLSI system of analyses (intermediate responses grouped with resistant responses) and the more recent system (intermediate responses grouped with susceptible responses) of analyses. These trends for ampicillin, cephalexin, cefalonium, cloxacillin, oxy-tetracycline and penicillin peaked (at highest level) in 2011 and for tylosin in 2013 and then decreased again. These antibiotic resistance trends over time, showed a closer comparison with analysis of similar data for coagulase negative staphylococci than for the maltose positive *S. aureus*. Antibiotic resistance trends over time also differed between maltose negative and maltose positive *S. aureus*. Antibiotic resistance of maltose negative *S. aureus* showed no significant differences between provinces (using both the previous and also the more recent interpretation) and there were only limited differences between seasons for ampicillin, penicillin, tylosin and cefalonium (using the more recent interpretation system). High SCC corresponded with low antibiotic resistance and were significantly different from low SCC which to corresponded with high antibiotic resistance, for cloxacillin and cefalonium. The MIC results of antibiotic resistance for maltose negative *S. aureus* confirmed the results of the Kirby Bauer tests. The results of the MIC method also showed more resistance in general for maltose negative than for the maltose positive *S. aureus* isolates for most of the products that were used. The MIC breakpoints were susceptible for MIC 50 and MIC 90 for both maltose negative and maltose positive *S. aureus*, except for MIC 90 of maltose negative *S. aureus*, which showed more resistance.

Acknowledgements

The authors thank the milk producers for allowing them to use the data and the samples obtained on their farms. Thanks are also due to the staff of the Milk Laboratory, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, at Onderstepoort, for the laboratory analyses namely Mrs J.C. Watermeyer, Miss R. Badenhorst, Mrs E.F. Konaite, Mr K.S. Malekane, Miss O.P. Nchako and Miss O.I. Memma. The authors also thank Prof Nontombi Mbelle and Mrs Dina Pombo of Tshwane Academic Division,

Microbiology, NHLS for the use of their facilities and Mr Thomas M. Rafundisani of Beckman Coulter for his help with the Microscan testing and analysis.

References

Abdi, R.D., Gillespie, B.E., Vaughn, J., Merrill, C., Headrick, S.I., Ensermu, D.B., D'Souza, D.H., Agga, G.E., Almieda, R.A., Olivier, S.P., Kerro Dego, O., 2018. Antimicrobial Resistance of *Staphylococcus aureus* Isolates from Dairy Cows and Genetic Diversity of Resistant Isolates. Foodborne Pathogens and Disease 15(7), 449-458. doi:10.1089/fpd.2017.2362

Abraham, J., Morris, D., Griffeth, G., Shofer, F., Rankin, S., 2007. Surveillance of healthy cats and cats with inflammatory skin disease for colonization of the skin by methicillin-resistant coagulase-positive staphylococci and *Staphylococcus schleiferi ssp. schleiferi.* Veterinary Dermatology 18, 252–259.

Armand-Lefevre, L., Ruimy, R., Andremont, A., 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerging Infectious Diseases 11, 711–714.

Aubry-Damon, H., Grenet, K., Sall-Ndiaye, P., Che, D., Cordeiro, E., Bougnoux, M., Rigaud, E., Le Strat, Y., Lemanissier, V., Armand-Lefe` vre, L., Delzescaux, D., Desenclos, J., Lie´ nard, M., Andremont, A., 2004. Antimicrobial resistance in commensal flora of pig farmers. Emerging Infectious Diseases 10, 873–879.

Barkema, H.W., Schukken, Y.H., Zadoks, R.N., 2006. Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. Journal of Dairy Science 89(6): 1877-1895.

Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M., 1966. Kirby Bauer method antimicrobial susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology 45(4), 493–496. doi:10/1093/ajcp/45.4 ts.493

Bemis, D. A., Jones, R.D., Videla, R., Kania, S.A., 2012. Evaluation of cefoxitin disk diffusion breakpoint for detection of methicillin resistance in *Staphylococcus pseudintermedius* isolates

from dogs. Journal of Veterinary Diagnostic Investigation 24, 964- 967. doi:1040638712452112;10.1177/1040638712452112

Bennedsgaard, T.W., Thansborg, S.M., Aarestrup, F.M., Enevoldsen, C., Vaarst, M., Christoffersen, A.B., 2006. Resistance to penicillin of *Staphylococcus aureus* isolates from cows with high somatic cell counts in organic and conventional dairy herds in Denmark. Acta Veterinaria Scandinavica 48, 24. doi:10.1186/1751-0147-48-24

Clinical and Laboratory Standards Institute (CLSI) formerly NCCLS General Susceptibility testing (SCM03). Clinical and Laboratory Standards Institute 2012, 32(3), from www.techstreet.com/CLSI, accessed 1 August 2017.

Clinical and Laboratory Standards Institute (CLSI), Performance Standards for Antimicrobial Susceptibility Testing: Twentieth Information Supplement. CLSI document M100-S20. Wayne, P.A., Clinical and Laboratory Standards Institute, 2008a.

Clinical and Laboratory Standards Institute (CLSI), 2015. Performance standard for antimicrobial susceptibility testing. Twenty fifth information supplement. CLSI document M100-S25. Wayne, P.A., Clinical and Laboratory Standards Institute, 2015.

Clinical and Laboratory Standards Institute (CLSI), 2015. Performance standards for antimicrobial disc and dilution susceptibility tests for bacteria isolated from animals. Approved Standard. 3rd Edition. CLSI document M31-A3, Wayne, P.A., Clinical and Laboratory Standards Institute, 2015.

Food and Agricultural Organisation (FAO)., 2009. How to feed the world in 2050. Rome, 12-13 October, 2009, 1-6.

Frazee, B., Lynn, J., Charlebois, E., Lambert, L., Lowery, D., Perdreau-Remington, F., 2005. High prevalence of methicillin-resistant *Staphylococcus aureus* in emergency department skin and soft tissue infections. Annals of Emergency Medicine 45, 311–320.

Gopal, S., Divya, K.C., 2017. Can methicillin-resistant *Staphylococcus aureus* prevalence from dairy cows in India act as potential risk for community-associated infections? A review. Vet World 10(3), 311–318. doi:10.14202/vetworld.2017.311-318

Griffeth, G.C., Morris, D.O., Abraham, J.L., Shofer, F.S., Rankin, S.C., 2008. Screening for skin carriage of methicillin-resistant coagulase-positive staphylococci and *Staphylococcus schleiferi* in dogs with healthy and inflamed skin. Veterinary Dermatology 19, 142–149.

Hajek, V., 1976. *Staphylococcus intermedius*, a new species isolated from animals. International Journal of Systematic Bacteriology 26, 401–408.

Hanselman, B.A., Kruth, S., Weese, J.S., 2007. Methicillin-resistant staphylococcal colonization in dogs entering a veterinary teaching hospital. Veterinary Microbiology 126, 277–281.

Haran, K.P., Godden, S.M., Boxrud, D., Jawahir, S., Bender, J.B., Sreevatsan, S., 2012. Prevalence and Characterization of *Staphylococcus aureus*, Including Methicillin-Resistant *Staphylococcus aureus*, Isolated from Bulk Tank Milk from Minnesota Dairy Farms. Journal of Clinical Microbiology 50(3), 688-695. doi:10.1128/JCM.05214-11

Hiramatsu, K., Katayama, Y., Matsuo, M., Sasaki, T., Morimoto Y., Sekiguchi, A., Baba T., 2014. Multi-drug-resistant *Staphylococcus aureus* and future chemotherapy. Journal of Infection and Chemotherapy 20(10), 593-601. doi:10.1016/j.jiac.2014.08.001

Johler, S., Moser, M., Engl, C., Tasara, T., Corti, S., Chen, J., Stephan, R., 2012. A Coagulase- and α-Glucosidase- Negative Variant of *Staphylococcus aureus*: a Challenge for routine Microbiological diagnostics. Journal of Clinical Microbiology 5(50), 1827-1828. doi:10.1128/JCM.06345-11

Kania, S., Williamson, N., Frank, L., Wilkes, R., Jones, R., Bemis, D., 2004. Methicillin resistance of staphylococci isolated from the skin of dogs with pyoderma. American Journal of Veterinary Research 65, 1265–1268.

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., 2018. Proactive udder health management in South Africa and antimicrobial resistance monitoring of *Staphylococcus aureus* in dairy herds from 2001 to 2010. Journal of South African Veterinary Association 89(0), a1490. doi.10.4102/jsava v89i0-1490

Karzis, J., Petzer, I.M., Donkin, E.F., Naidoo, V., Etter, E.M.C., 2019. Climatic and regional antibiotic resistance patterns of *Staphylococcus aureus* isolated from milk samples of dairy

herds in South Africa between 2000 and 2010. Onderstepoort Journal of Veterinary Research 86(1), a1674. doi:10.4102/ojvr.v86i1.1674

Kharas, H., 2010. The emerging middle class in developing countries. Global development outlook. OECD development centre. Working paper no. 285, 1-61.

Klevens, R.M., Morrison, M.A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., Craig, A.S., Zell, E.R., Fosheim, G.E., McDougal, L.K., Carey, R.B., Fridkin, S.K., Investigators, A.B.C.s.A.M., 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. Journal of the American Medical Association 298, 1763–1771.

Kwon, N., Park, K., Jung, W., Youn, H., Lee, Y., Kim, S., Bae, W., Lim, J., Kim, J., Kim, J., Hong, S., Park, Y., 2006. Characteristics of methicillin resistant *Staphylococcus aureus* isolated from chicken meat and hospitalized dogs in Korea and their epidemiological relatedness. Veterinary Microbiology 117, 304–312.

Lactodata, 16(2) November 2013, accessed 07 June 2017, from, www.milksa.co.za/category/all-reports/industry-information.

Lilenbaum, W., Esteves, A.L., Souza, G.N., 1999. Prevalence and antimicrobial susceptibility of staphylococci isolated from saliva of clinically normal cats. Letters in Applied Microbiology 28, 448–452.

Machado, A.B., Machado, M.F.R., Picoli, S.U., 2017. An investigation of methicillin-resistant Staphylococcus pseudintermedius (MRSP) in domestic and shelter dogs in Montenegro (RS-Brazil). Revista Brasileira de Saúde e Produção Animal 18(4), 542-548. doi:10.1590/s1519-99402017000400005

Magiorakos, P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Webber, J.T., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance**.** Clinical Microbiology and Infection 18(3), 268-281. doi:10.1111/j.1469-0691.2011.03570

Medleau, L., Long, R.E., Brown, J., Miller, W.H., 1986. Frequency and antimicrobial susceptibility of Staphylococcus species isolated from canine pyodermas. American Journal of Veterinary Research 47, 229–231.

National Department of Health, 2018. Surveillance resistance and consumption of antibiotics in South Africa.

National Mastitis Council (NMC), 2004. Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality, 4th edn., NMS, accessed 01 August 2017, from www.nmconline.org

National Mastitis Council (NMC), 2017. Staphylococci. In Laboratory Handbook on Bovine Mastitis, 3rd ed.; National Mastitis Council: Verona, WI, USA, 2017, 43–52.

Nickerson, S.C., Owens, W.E., Boddie, R.L., 1995. Symposium: Mastitis in Dairy Heifers: Initial Studies on Prevalence and Control. Journal of Dairy Science 78, 1607-1618.

Petzer, I.M., Karzis, J., 2019. Virulence, Antibiotic Resistance and Diagnostics of CNS Isolated from Milk of SA Dairy Cows. RuVasa Congress, Umhlanga Rocks, 20-22 May 2019.

Petzer, I.M., Karzis, J., Donkin, E.F., Webb, E.C. 2016. A pathogen-specific approach towards udder health management in dairy herds: Using culture and somatic cell counts from routine herd investigations**.** Onderstepoort Journal of Veterinary Research 83(1), 12 doi:10.4102/ojvr.v83i1.1146

Petzer, I.M., Karzis, J., Watermeyer, J.C., van der Schans, T.J., Badenhorst, R., 2009. Trends in udder health and emerging mastitogenic pathogens in South African Dairy Herds. Journal of South African Veterinary Association 80(1), 17-22.

Petzer, I.M., Karzis, J., Watermeyer, J.C., Badenhorts, R., van der Schans, T.J., 2012. Trends of mastitogenic bacteria from 2008 to 2012 in South African dairy herds. International Dairy Federation, World Dairy Summit and exhibition, Cape Town, South Africa, IDF Press.

Roy, J.P., DesCôteaux, L., DuTremblay, D., Beaudry, F., Elsener, J., 2009. Efficacy of a 5-day extended therapy program during lactation with cephapirin sodium in dairy cows

chronically infected with *Staphylococcus aureus.* Canadian Veterinary Journal 50(12): 1257–1262.

Salmon, S.A., Watts, J.L., Aarestrup, F.M., Pankey, J.W., Yancey, R.J. Jr., 1998. Minimum inhibitory concentrations for selected antimicrobial agents against organisms isolated from the mammary glands of dairy heifers in New Zealand and Denmark. Journal of Dairy Science. 81(2), 570–578.

Sasaki, T., Kikuchi, K., Tanaka, Y., Takahashi, N., Kamata, S., Hiramatsu, K., 2007. Reclassification of phenotypically-identified Staphylococcus intermedius strains. Journal of Clinical Microbiology. 45, 2770–2778.

Schmidt, L.H., 1987. The MIC_{50}/MIC_{90}: assessments of in vitro activities of antimicrobial agents that facilitate comparative agent-agent and agent-species susceptibility comparisons. Antimicrobic Newsletter 4(1), 1-8.

Schmidt, T., Kock, M.M., Ehlers, M.M., 2015. Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. Journal of Dairy Science 98, 6256-6269. doi:10.3168/jds.2015-9715

Sol, J., Sampimon, O.C., Snoep, J.J., Schukken, Y.H., 1997. Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by *Staphylococcus aureus*. Journal of Dairy Science 80(11), 2803-2808.

Staneck, J.L., Allen, S.D., Harris, E.E., Tilton, R.C., 1985. Automated Reading of MIC Microdilution Trays Containing Fluorogenic Enzyme Substrates with the Sensititre Autoreader. Journal of Clinical Microbiology 22, 187-191.

Swinkels, J.M., Cox, P., Schukken, Y.H., Lam, T.J., 2013. Efficacy of extended cefquinome treatment of clinical *Staphylococcus aureus* mastitis. Journal of Dairy Science 96(8), 4983-92. doi:10.3168/jds.2012-6197

Vengust, M., Anderson, M., Rousseau, J., Weese, J., 2006. Methicillin resistant staphylococcal colonization in clinically normal dogs and horses in the community. Letters in Applied Microbiology 43, 602–606.

Voss, A., Loeffen, F., Bakker, J., Klaassen, C., Wulf, M., 2005. Methicillin resistant *Staphylococcus aureus* in pig farming. Emerging Infectious Diseases 11, 1965–1966.

Wallmann, J., Kaspar, H., Kroker, R., 2004. The prevalence of antimicrobial susceptibility of veterinary pathogens isolated from cattle and pigs: national antibiotic resistance monitoring 2002/2003 of the BVL. Berliner und Münchener tierärztliche Wochenschrift 117(11-12), 480-492.

Weese, J.S., van Duijkeren, E., 2010. Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. Veterinary Microbiology 140(3-4), 418-29. doi:10.1016/j.vetmic.2009.01.039

Chapter 6: Thesis summary

6.1 Antibiotic resistance of coagulase positive staphylococci isolated from milk of South African dairy herds.

The research recorded in this Thesis includes an extensive and thorough literature review of mastitis in dairy cows and the many factors relating to its incidence, research over many decades, and treatment strategies.

The dedicated udder health diagnostic programme, as part of the pro-active udder health programme run by the Milk Laboratory in the Faculty of Veterinary Science at the University of Pretoria has led to the establishment of an extensive antibiotic resistance surveillance data base. The surveillance data are continually recorded, but for this specific research project, the data and isolates analysed were from a period of nineteen years. This information on antibiotic sensitivity testing (Kirby Bauer) of routine diagnostic samples, is extremely valuable for the treatment and management of mastitis, because the treatment should be specific for the infective organisms. The treatment and control of mastitis should take into account the ever changing dynamics of the bacteria identified. This continued monitoring of antibiotic resistance in dairy cows in South Africa will provide a resource for many future studies. This will be most important in terms of the One Health concept and for food security.Such extensive data about the antibiotic resistance of bacteria in dairy cows when dealing with these important food producing animals is also significant in terms of the One Health concept and food security. The contributions of this antimicrobial resistance research and the discovery of unique maltose negative *S. aureus* strain (ST 2992), will have an impact on the One Health concept, by direct impact on animal health. However, the origin of this unique strain may be from the environment or human origin. Also the antimicrobial resistance of this *S. aureus* ST 2992 in products used only in human health implies an anthroponosis. Thus this research shows the collaboration of the animal-human-environmental interfaces of the One Health concept.

In the pro-active udder health programme, maltose negative staphylococci were first isolated from one South African dairy herd in 2005. Subsequently, from these isolates, a large number of coagulase positive staphylococci at that time identified as *S. aureus* were found in milk samples with SCC < 100 000 cells/ml. This low SCC, which is unusual for *S. aureus,* led to

the initial identification of the coagulase positive and maltose negative *Staphylococcus* strain. This was followed by the correct identification of this emerging pathogen using molecular tests. This strain had originally been identified in KwaZulu-Natal, but has now been shown to be present in all nine provinces of South Africa, albeit in small numbers. Such information is very helpful to producers and veterinarians in the field, as this organism reacts differently from the previously identified maltose positive *S. aureus* and therefore it needs to be managed and treated differently in practice.

6.1.1 Proactive udder health management in South Africa and monitoring of antibiotic resistance of coagulase positive staphylococci in dairy herds from 2001 to 2010

The information included in the first two published manuscripts forming part of this Thesis, were obtained from two datasets derived from a total of 5942 antibiotic sensitivity tests of *S. aureus* (maltose positive) that were conducted over an eleven-year period in all nine provinces of South Africa. The results of this surveillance study have provided information which can be utilised by producers and veterinarians when making decisions about the treatment and management of *S. aureus* infections (as identified previously) in dairy cows. Such antibiotic susceptibility profiles, allow for informed treatment choices to be made without the need to wait for any antibiotic sensitivity test results. It should be noted that the results of antibiotic sensitivity tests (the Kirby Bauer or Minimum Inhibitory Concentration, MIC tests), are only an indication that the particular organism can be killed by a particular antibiotic *in vitro*. However, the situation in the udder differs from *in vitro* testing, because of the udder pharmacokinetics (very few products are successful in a water and fat environment) and because the site of the infection may be very difficult for the antibiotic to reach via very small arteries and lactiferous ducts. The continuous secretion of milk also dilutes and washes antibiotics out. As a result treatment for mastitis is not very successful (<50%). It is therefore clear that this is for several reasons, apart from the development of antibiotic resistance by mastitis-causing organisms. Therefore is why the focus should be on the prevention and the monitoring through the pro-active udder health programme rather than only on the actual treatment. The application of this pro-active udder health programme has shown a reduced incidence of antibiotic resistance through the management of *S. aureus* infections in these 20 well-managed herds studied over an eleven-year period.

The limited range of antibiotics available as intramammary remedies in South Africa and the fact that some of these remedies can be obtained without a prescription “over the counter” under the Stock Remedies Act, might point to potentially contributing factors causing antibiotic

resistance. However the focus generally has been for many years on the prudent use of antibiotics.

6.1.2 Climatic and regional antibiotic resistance patterns of *S. aureus* in South African dairy herds.

This second published manuscript described a few significant differences in antibiotic resistance between the different seasons within the provinces of South Africa. Such information is useful for producers and veterinarians when deciding on the best available treatment in specific provinces of South Africa at particular times of the year. Although the reasons for these differences are mostly unexplained, it could be due to different weather conditions, different actions and spectrums of antibiotics and factors such as biofilm of the bacteria produced under the differing weather conditions.

With the exception of cefuroxime, it is a concern that all of the tested antibiotics, showed a predicted prevalence of resistance of above 50% in most provinces. The lowest predicted prevalence of resistance for all antibiotics exceptt for cephalosporins, was in KwaZulu-Natal during spring. For cephalosporins the lowest predicted prevalence of resistance was in Gauteng during winter. The reasons for these differences are obscure.

6.1.3 Challenging the conventional identification of coagulase positive staphylococci

The coagulase positive, maltose negative *S. aureus* strain was identified as an emerging pathogen, and is quite distinct from the previously classified maltose positive *S. aureus*.
Even though the conventional identification procedure was not accurate in diagnosing this organism, it was still a useful, fast and cost effective method to use to differentiate between maltose positive and maltose negative *S. aureus* in practice. This differentiation is important, since maltose negative *S. aureus* is less pathogenic than maltose positive *S. aureus*. Even though maltose negative *S aureus* appears to be less contagious (no repeat cases or chronic cases isolated), it still shows a higher antibiotic resistance and thus requires different management and treatment in practice. This information will assist producers and veterinarians to make the correct decisions for the management and treatment of this emerging pathogen which is different from the traditionally classified maltose positive *S. aureus*. Although this study was done on a limited number (n = 23) of confirmed maltose negative *S. aureus* isolates from 2009 to 2018, there was a complete correlation between the method of identification using the MALDI-TOF MS (Matrix Assisted Laser

Desorption/Ionisation Time-of-Flight, Mass Spectrometry) and the 16S rRNA method. The MLST (multi-locus sequence typing) further confirmed that this organism is a single strain which has been isolated repeatedly over time and in different provinces of South Africa.
The phenotypic difference between the maltose positive and maltose negative *S. aureus* strains was investigated through the identification of the *malA* and *malR* genes of maltose negative *S. aureus* ST 2992 compared to previously classified maltose positive *S. aureus* strains. This led to the discovery of a stop codon at base pair 844 of the *malA* gene, caused by a cytosine to thymine transition which probably causes early termination of the α-glucosidase protein. This is the reason why this maltose negative *S. aureus* strain had a different phenotype in spite of the presence of the *malA* and *malR* genes.

6.1.4 Surveillance of antibiotic resistance of maltose negative *Staphylococcus aureus* in South African dairy herds

This was a two-part study. In the first part, the data of a total of 271 antibiotic sensitivity tests on maltose negative *S. aureus* isolates from 2010 to 2017 were analysed. The results of this surveillance study and the availability of antibiotic resistance profiles for the maltose negative *S. aureus* will allow for informed decisions to be made on treatment and management of this emerging pathogen in practice. These results of antibiotic sensitivity tests (Kirby Bauer or MIC) are merely an indication *in vitro* of possible treatment success but not necessarily in the udder due to specific pharmacodynamics and other factors which can also limit successful treatment with antibiotics.

The further MIC testing of both maltose negative and maltose positive *S. aureus*, confirmed the antibiotic resistance results of the retrospective data analysis (Kirby Bauer- disc diffusion method). This showed that the Kirby Bauer method, in addition to being a relatively quick and cost-effective method, is also accurate enough for routine veterinary diagnostics for antibiotic sensitivity testing in dairy cows.

6.2 Advantages of the complete study

One of the key outcomes of this study included the importance of the pro-active approach to udder health, by identifying the antibiotic resistance profiles of pathogens responsible for causing the highest percentage of mastitis cases in South Africa. Such pathogens would be the maltose positive *S. aureus* and the emerging pathogen maltose negative *S. aureus*. The

accurate identification of antibiotic resistance trends and correct treatment, should also be likely to reduce the risk of developing multi-drug resistant organisms Such information about the antibiotic resistance trends should enable dairy farmers to deal with these organisms in a more effective manner in the different regions of southern Africa and during different seasons, without having to perform extensive testing each time. The retrospective (Kirby Bauer) antibiotic resistance data was also confirmed by the MIC analyses.

This whole pro-active udder health programme is essential in the context of the general public health, by facilitating a safe dairy industry, controlling the incidence of antibiotic resistance in dairy cows and contributing to food security. This might include the need to take account of the possibility of zoonosis (transmission from animals to humans) and anthroponosis (transmission from humans to animals) of antibiotic resistant bacteria.

Mastitis and antibiotic treatment have great financial implications for the milk producer when assessing the cost of the treatment and also the cost incurred in discarding the milk for the duration of the withdrawal period. Thus more information on the effective and prudent use of antibiotics and the effective management of mastitogenic pathogens by intramammary treatments should reduce expenses for the producer and improve their profitability and sustainability.

The differentiation of the phenotypic characteristics and pathogenicity between the conventionally identified maltose positive *S. aureus* and maltose negative *S. aureus* is helpful for the management and the appropriate treatment of these organisms in practice. The discovery of the stop codon at position 844 of the *malA* gene caused by a cytosine to thymine transition and early termination of the α-glucosidase protein of the *S. aureus* ST 2992 maltose negative strain, is genetic proof of the phenotypic difference between maltose positive *S. aureus* and the maltose negative *S. aureus* ST 2992 strain. This discovery has beneficial applications in udder health management because of the difference required in management and treatment strategies in practice needed to control such organisms. In the case of the maltose negative *S. aureus* ST 2992, only the clinical cases need to be treated, but care should be taken to treat with effective antibiotics, as these organisms generally showed a high incidence of antibiotic resistance. This contrasts with the much more serious implications of maltose positive *S. aureus* infections, which are highly pathogenic and require strict compliance to the pro-active udder health programme.

6.3 Limitations of this research study

The MIC (Microscan 40 Walkaway system, Beckman Coulter, California, USA) testing was done on only a limited number of samples and not on the entire data set. In addition, the antibiotics used for MIC testing were limited to combinations of available commercial panels which are formulated for human use and not specifically for veterinary use.

6.4 Future research envisaged

The following are some of the envisaged or suggested research topics that could follow on and amplify this current research project.

The incidence of antibiotic resistance in dairy herds in South Africa should be evaluated for other mastitis causing pathogens, such as: non-aureus staphylococci; *Streptococcus uberis*; *Streptococcus agalactiae; Streptococcus dysgalactiae*; Gram negative bacteria. The ability of bacterial strains to produce biofilm and the conditions that might enhance this defensive mechanism need to be studied under South African conditions needs further investigation.

Future research is necessary to determine the antibiotic resistance genes present in maltose negative *S. aureus*, including multi-resistant isolates. Research on the resistance of *S. pseudintermedius* (phenotypically identified) from humans in South Africa, which would assist in the investigation of the origin of the maltose negative *S. aureus* ST 2992 strains causing increased resistance, is also deemed to be very important.

The whole genome sequencing of one of these maltose negative *S. aureus* isolates would establish if there are any other mechanisms in addition to the stop codon in the *malA* gene which have contributed to this unusual biochemical profile and its expression in practice.

6.5 General Conclusion

The initial study of this research showed the importance of good udder health management and the application of the pro-active udder health programme which led to a reduced incidence of antimicrobial resistance of *S. aureus* infections in 20 well-managed herds studied over an 11-year period (Isolates: n= 5942).

In subsequent studies this research showed that additional factors such as, environmental factors, climatic and regional also had an effect on antimicrobial resistance and the efficacy of antimicrobial drugs. However, there are many more factors which need to be considered in future research, e.g. biofilm formation under challenging environmental conditions which cause increased antimicrobial resistance.Thus this research shows the importance of the collaboration of the animal-human-environmental interfaces of the One Health concept.

The subsequent discovery of coagulase positive and maltose negative staphylococci with doubtful species identification, was confirmed by two molecular methods. MLST further identified this strain of single origin as maltose negative *S. aureus* ST 2992 in which the α-glucosidase gene present was not being expressed due to the abnormally placed stop codon discovered. This highlights the importance of individual organisms in antimicrobial resistance which is shown by the higher antimicrobial resistance of maltose negative *S. aureus* ST 2992 compared to that of maltose positive *S. aureus*. This research and the discovery of the unique maltose negative *S. aureus* strain will contribute to the application of the One Health concept in dairy herds.