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Incidence of *Staphylococcus aureus* in cows' milk following mucosal and parenteral administration of autogenous *S. aureus* vaccine during the dry period

by

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Declaration

I, Sarah M. Kotzé declare that apart from the acknowledgements indicated and the advice and guidance by my supervisors, this dissertation is my own original work.

This full dissertation or any part of it will not be submitted for another degree at this or any other university.

This dissertation is presented in partial fulfilment for the requirements of a Master of Science in Veterinary Science in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science University of Pretoria.

Signed.....

Date.....

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Table of contents

List of Tables

List of Graphs

Abbreviations

Ag:	Antigen
APC:	Antigen presenting cell
BB:	Blocking buffer
BTA:	Blood Tryptose Agar
C:	Cows
CB:	Coating buffer
CD:	Cluster of differentiation
Cf A:	Clumping factor A
CFU:	Colony forming units
CMT®:	Californian Mastitis Test
Cna:	Collagen binding protein
Cl:	Column
DNA:	Deoxyribonucleic acid
DU:	Dry-up
Ebp:	Elastin binding protein
EL:	In lactation at the end of the project (regardless of month in lactation)
ELISA:	Enzyme linked immunosorbant assay
Fc:	Fragment crystallizable
FnBP:	Fibronectin binding protein

HRPO:	Horseradish peroxidise
H:	Hour
Ig:	Immunoglobulin
IgA:	ImmunoglobulinA
iELISA:	Indirect Enzyme linked immunosorbent assay
IM:	Intra-muscular
Jc:	Joining chain
L:	Lactation
MALT:	Mucosal associated lymphoid tissue
MHC:	Major Histocompatibility Complex
mℓ	mille litre
MVV:	Mucosal Vaccination Vaginal
MVU:	Mucosal Vaccination Udder teat canal
Neg:	Negative for *S. aureus* on bacterial culture of milk sample
NV:	Not Vaccinated
OD:	Optical Density
OPD:	Ortho Phenylene Diamine
PAMP:	Pattern associated molecular pattern
PAMPS:	Pathogen associated molecular patterns
PBS:	Phosphate buffered saline
PBS T:	Phosphate buffered saline Tween

Pos:	Positive for *S. aureus* on bacterial culture of milk sample
PP:	Periparturient
PV:	Parenteral vaccination
R:	Row
PRR:	Pattern recognition receptor
S. aureus:	*Staphylococcus aureus*
SU:	Steam–up
Th:	T helper cell
TLR:	Toll-like receptors
µℓ	micro litre
UV:	Ultra violet
V:	Vaccinate

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by

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Abstract

A prospective study was performed for a 12 month period during which a group of 90 cows from two different dairy farms were vaccinated with herd-specific autogenous *Staphylococcus aureus* vaccines. The IgA response as well as the incidence of *S. aureus* in the milk was evaluated. Each herd was clinically evaluated before vaccination and the data recorded as a base line for udder health and group diversity. Vaccination was administered on the mucosal surfaces of the vagina and teat canal as well as intra-muscularly (IM). The first vaccination was done at dry-up via the intra-teat and IM routes and the second vaccination at steam-up via the intra-vaginal and IM routes. Pre-vaccination (at dry-up), and post calving (at monthly intervals) milk samples were tested for *S. aureus* specific IgA with ELISA. The same milk samples were also cultured for *S. aureus*. The vaccinated and un-vaccinated groups were further stratified according to the presence or absence of *S. aureus* in milk

cultured at dry-up. Data was analysed over time according to lactation stage. The fate of all cows was recorded throughout the study.

The incidence of *S. aureus* in milk from vaccinated and un-vaccinated groups was evaluated using McNemars Test. Although the incidence of new cases were insignificant in both groups the risk for development of new cases of *S. aureus* was lower in the vaccinated group, 24% ,than in the un-vaccinated group,36%. Comparison of IgA titres between vaccinated and un-vaccinated groups on the respective farms was only done for cows that did not culture any *S. aureus* for the duration of lactation. There were no significant differences for IgA titres between the two groups except between the fourth and fifth month of farm 2 where the vaccinated group showed a higher titre. The interpretation of serum conversion was complicated by the diversity of the groups and presence of natural *S. aureus* infection.

KEY WORDS: Autogenous *Staphylococcus aureus* mastitis vaccine, mucosal vaccination, *S. aureus* incidence

Chapter 1

General introduction

Background

It is well documented that mastitis control is unsatisfactory and economically draining in dairy herds (Sordillo & Streicher 2002).The estimate of the economic loss is related to the incidence of mastitis in a herd. *Staphylococcus aureus* is known to be the primary pathogen of concern, especially because of chronic infected animals that must be culled to prevent further spread in the herd (Gronlund, Johannisson & Waller 2006; Pellegrino 2008; Petzer 2001). Other direct losses include: a loss in milk production during current and subsequent lactations and an increased need of replacement heifers (Bagley 1997; Shkreta, Talbot, Diarra & Lacasse 2004). Indirect losses associated with dairies suffering from a high level of mastitis include: laboratory testing for mastitis pathogens, costs of antimicrobial therapy, veterinary costs and labour costs. An efficacious vaccine can reduce direct as well as indirect losses incurred by dairy farmers.

The damage of the teat canal by the milking machine together with the sharing of teat holsters between cows are risks for the exposure of the udder to bacterial pathogens (Petzer 2004). To address these risks, teat ends must be protected from potential pathogens and the resistance of the cow to infection must be improved (Jones, Bailey & Robertson 1998). In the past commercial bacterin vaccines against *S. aureus* have only been partially protective (Talbot & Lacasse 2005). Recently, however, a vaccine that contains inactivated bacteria that strengthens the immunity and that reduced the number of cows with mastitis and also the severity of those that had mastitis has been introduced on the market (STARTVAC® European Medicines Agency).

Autogenous bacterin vaccines have also been shown to give promising results (Leitner, Yadlin, Glickman, Chaffer & Saran 2003).

This study involved the administration of an autogenous vaccine composed of strains of *S. aureus* that were prevalent in the herd. The vaccine was administered intra- muscularly as well as on mucosal surfaces via the intra-teat and vaginal routes during the dry period. A second vaccination was given four weeks after the first vaccination. The IgA responses to vaccination were measured in milk samples taken at monthly intervals up to six months post vaccination. These samples were also cultured to determine the presence of *S. aureus.*

Research Questions:

What effect will the use an autogenous *S. aureus* mastitis vaccine have on the incidence of *S. aureus* cultured from milk samples?

Will the mucosal and parenteral administration of an autogenous *S. aureus* vaccine in the dry period of the cow elicit an IgA response in the milk?

First hypothesis:

H_0: There will be a significant number of new cases in the vaccinated group

There will be an insignificant number of new cases in the un-vaccinated group

H_1 There will be an insignificant number of new cases in the vaccinated group

There will be a significant number of new cases in the un-vaccinated group

Second hypothesis:

H_0 There will be no significant increase in the IgA levels in the milk of cows vaccinated with an autogenous *S. aureus* vaccine via the mucosal and parenteral routes as compared to un-vaccinated cows.

H_1 There will be a significant increase in the Ig A levels in the milk of cows vaccinated with an autogenous *S. aureus* vaccine via the mucosal and parenteral routes as compared to un-vaccinated cows.

Chapter 2

Literature Review

2.1 Introduction

Mastitis is defined as a condition of inflammation and infection in the mammary gland caused by organisms of different aetiological origin, usually bacteria (Talbot & Lacasse 2005). It leads to interruption of lactation and invariably to reduced production in subsequent lactations. In fact, mastitis can result in a 20% to 80% loss of milk production (Vakanjac , Pavlovic & Pavlovic 2006). This will increase the cost of milk production on the farm and can, if prevalent in a country, result in milk shortages and high milk prices (Jones & Bailey 2009).

The most important mastitis causing pathogen is *Staphylococcus aureus* which causes variable grades of contagious mastitis (Castagliuolo, Piccini, Beggiao, Palu, Mengoli, Ditadi, Vicenzoni & Zecconi 2006). Contagious mastitis is distinguished from mastitis caused by environmental pathogens in that it spreads from udder to udder as opposed to new infections from the environment (Jones & Baily 2009). *S. aureus* induced mastitis may range from acute to sub-acute cases which may regress into chronic cases that become infectious carriers in the herd (Bagley 1997; Gronlund *et al.,* 2006). Control of mastitis is generally unsatisfactory due to incorrect or insufficient treatment as well as the development of drug resistant strains and the persistence of organisms in mammary abbesses (Bagley 1997; Wilson, Gonzalez, Case, Garrison & Grohn 1999). The control of mastitis should therefore be focused on prevention, and the development of an effective vaccine will be an important contribution. It is well established that pathogenic strains involved in mastitis are subject to continuous change, leading to differences in antigenicity and pathogenicity (Hui & HuoChun 2006). Autogenous vaccines overcome the challenge of changing strains.

2.2 MASTITIS

Udder inflammation is usually a response to bacterial invasion of the teat canal (Talbot & Lacasse 2005). When these bacteria multiply, they produce toxins that damage milk secreting tissue (Jones & Bailey 2009). Mastitis usually presents in one of two forms; either clinical, which is characterized by abnormal flaky milk and swollen sensitive quarters or subclinical, in which case the milk appears macroscopically normal, but the milk composition is altered and production is markedly decreased (Jones 1998; Jones & Bailey 2009). Clinical mastitis can present as per-acute, acute or chronic of which the latter seems to be the most prevalent, especially in the case of *S. aureus* mastitis (Pellegrino 2008).

There is a correlation between the efficacy of the defence mechanisms in the mammary gland and the functional transitions in the udder as the cow moves through the different stages of lactation (Sordillo 2005). The sudden physiological changes in the udder during the periparturient period (PP) leads to an increased demand for oxygen and subsequently to an increase in oxygen derived reactants referred to as ROS (Gitto, E., Reiter, Karbownik, Tan, Gitto, P., Barberi, S. & Barberi, I. 2002). This could lead to an imbalance in the anti-oxidant defence mechanisms with the potential danger of tissue damage and an increased risk to the development of mastitis (Miller, Brzezinska-Slebodzinska & Madsen 1993). The stress hormones produced during calving and the concurrent introduction to the milking parlour can greatly influence the immune status of the cow (Sordillo 2005). An excess of corticosteroids will have an immune suppressive effect by decreasing the number and the migration of leucocytes (Burton & Erskine 2003).

The delicate balance between nutrition and the physiological changes during lactation can have a significant impact on the immune status of the cow. Extra care must be taken to prepare heifers for the first lactation by supplying sufficient energy in the steam-up period. Heifers also require a higher protein level to support growth and udder development (Meglia 2004). There is therefore an

increased risk for the development of metabolic disturbances during the periparturient period (Persson 2000). An increased demand for glucose in high producing cows can lead to ketoses with subsequent decreased responses in both lymphocytes and neutrophils (Sartorelli , Paltrinieri & Agnes 1999).

Genetic factors may also have an influence on the prevalence of mastitis. There seems to be an indirect correlation between selection for high producing cows and udder defence (Heringstad, Klemetsdal & Steine 2003). Selection specifically for efficacious immune responses may greatly influence the prevalence of mastitis in the herd (Sordillo 2005).

The teat end and the Furstenburch rosette form the first line of defence in the udder and proper sphincter function can significantly reduce the number of bacteria that enter the udder (Zecconi, Hamman, Bronzo, Moroni, Giovannini & Piccinini 2000). The use of a milking machine can cause trauma to the teat ends and is one of the primary causes for iatrogenic introduction of pathogens into the udder (Jones, Bailey & Robertson 1998; Petzer 2004; Sordillo & Streicher 2002; Sordillo 2005).

Petzer (2004) reported a positive correlation between bacterial colonization and swelling of the teats after machine milking. Gravitation and the vacuum present during machine milking enhances the inflow of blood into the teat while the vacuum and cuff around the teat restricts the venous draining leading to accumulation of blood with resultant swelling (Petzer 2004). The negative pressure generated by the milk machine in the teat can overcome the barrier provided by the Furstenburg rosettes at the entrance of the teat canal. Milking an infected cow can contaminate the holsters with bacteria which can infect the teat canal of the next cow (Petzer 2001).

The diagnosis and control of mastitis is complex and often unsuccessful (Jones *et al*., 1998). The control of mastitis should include multiple aspects such as proper hygiene, maintenance of equipment, correct milking procedures and sound nutritional programmes (Jones *et al.,* 1998; Petzer

2001; Ruegg 2001). A program for the monitoring of udder health should be part of the milking procedure (Mongeon 2006). This program must include daily clinical observations, monitoring milk yield, strip cup on pre-milk and the California Mastitis Test® (CMT®) on suspected mastitis cases. The CMT® is still regarded as one of the best indicators of the degree of inflammation in the udder and is a very useful aid in the diagnosis of acute mastitis (Jones 1998). However, cows suffering from chronic mastitis may have milk that appears normal on macroscopic examination and test negative in the CMT® (Petzer 2001). Bacterial culture for *S. aureus* in the milk has varying sensitivity ranging from sixty percent to eighty seven percent according to Buelow, Goodger, Collins, Clayton, Nordlund and Thomas, (1996a). They advised that quarter samples are taken separately and that 0.1mℓ is used as inoculum volume. The sensitivity can be as high as ninety one percent depending on the interval of sampling and repeated sampling increases the sensitivity of bacterial culture of milk samples (Buelow, Thomas, Goodger, Nordlund & Collins, 1996(b); Sandholm & Sears, 1998 in Petzer, 2001)

Bacterial culture can be performed on samples from bulk tanks or from individual cows but the latter is less sensitive (Jones 1998; Petzer 2001). Long term treatment of mastitis does not always result in clinical cure, even with accurate diagnosis and treatment (Vakanjac, Pavlovic,V. & Pavlovic, N. 2006). This is one of the main reasons for the culling of Staphylococcus positive cows as a last resort to eliminate chronic carries in the herd (Jones *et al.,* 1998; Petzer 2004).

2.3 Etiology: *Staphylococcus aureus*

S. aureus is regarded as the most important mastitis pathogen because of its contagious nature and the difficulty to successfully cure infected cows (Jones *et al*. 1998). It is a facultative anaerobic, Gram-positive coccus, which presents as grape-like clusters in milk (Ryan & Ray 2004). *S. aureus* forms fairly large gold coloured colonies on artificial culture media and is often β-haemolytic on blood agar. The bacteria are coagulase positive and resistant to polymixin B (Ryan & Ray 2004).

2.4 Pathogenesis: *Staphylococcus aureus*

S. aureus is known to express several virulence factors of which some facilitate tissue invasion and others actively counteract the body's defence mechanisms (Todar 2008).

The production of extracellular proteins enhances invasion of the host's tissues in different ways and *S. aureus* secretes twenty-five to thirty such proteins (Boyd & Hoerl 1991) Laminin and fibronectin are proteins in the basement membrane and extra cellular matrix that play an important role in the architecture of all vertebrate tissues. *S. aureus* can express proteins on its surface that promote attachment to these host proteins whereby colonization of host tissues is promoted. Some of the most prominent adhesion molecules include clumping factor A, fibronectin-; collagen- and elastin binding protein (Foster & Hook 1998). After entering the host *S. aureus* can change its phenotype from adhesive to invasive to facilitate spread in the host (Lowy 1998). During increased bacterial density and change in the growth phase of the bacteria, the genes for producing adhesions are down regulated and those for producing toxins and enzymes are up regulated (Arvidson & Tegmark 2001).

S. aureus can secrete several toxins like leukocidin, alpha toxin and beta toxin that can damage cell membranes and milk producing tissues (Jones *et al.* 1998; Petzer 2001; Quinn, Carter, Markey & Carter 1999). Gamma-toxin, also known as gamma-hemolysin, produced by most *S. aureus* isolates, is a potent haemolysin and leucotoxin and plays a role in the development of gangrenous mastitis in cows (Petzer 2001). *S. aureus* can produce coagulase, an extracellular protein that binds to prothrombin to form the fibrin activator staphylothrombin (Boyd & Hoerl1991). The local activation of fibrin may assist in the evasion of phagocytes. On the other hand, the secretion of staphylokinase will activate plasminogen into plasmin, a potent fibrinolytic enzyme that will disrupt the fibrin meshwork to enhance the spread of the organism in the host (Quinn *et al*., 1999; Todar 2008).

Defencins are anti-microbial peptides secreted by leucocytes and epithelial cells. They can perforate microbial cell walls and act as chemotoxins. Staphylokinase can bind and neutralise alpha-defencins (Ganz 2003; Jin, Bokarewa, Fosre, Mitchell, Higgens & Tarkowski 2004).

The evasion of phagocytosis is one of several immune evasive strategies deployed by *S. aureus.* A micro-capsule consisting of a surface polysaccharide inhibits the binding of C1q and C3b to the antibody bound to the pathogen and prevents the activation of complement thereby evading opsonisation and phagocytosis (Abbas & Lichtman 2003;Todar 2008). *S. aureus* can also express Protein A on its surface. Protein A will non-specifically bind to the FC region of antibodies thereby blocking the effector functions of specific antibody (Todar 2008).

S. aureus can survive and multiply within macrophages. This stimulates a cell-mediated immune response that leads to the formation of encapsulated granulomas. Theses deep foci of infection lead to the development of chronic carrier cows that continuously shed bacteria (Petzer 2001).

2.5 Udder immunity

The high prevalence of mastitis and the apparent inability of the immune system to cope with specifically *S. aureus* udder infections have instigated research into the immune mechanisms of the bovine udder (Leitner *et al.*, 2000). These mechanisms are traditionally classified under the innate and adaptive immune system.

The innate immune mechanisms are not antigen-specific, are present from birth and cannot mount an anamnestic response (Halliwell & Gorman 1989; Tizard 1996). It represents the first line of defence in the recognition and elimination of invaders in the body (Halliwell & Gorman 1989). This system includes physical barriers to the host's environment, like the Furstenburg rosettes at the entrance of the teat canal, mucosal surfaces as well as humoral and cellular responses (Halliwell & Gorman 1989; Sordillo & Streicher 2002).

The cellular responses include phagocytic cells, cells that release inflammatory mediators (eg mast cells) and natural killer cells, while acute-phase proteins and the complement system are important components of the humoral responses (Halliwell & Gorman 1989). During inflammation the most important phagocytic cells in the udder are neutrophils and macrophages (Sordillo & Streicher 2002). These innate cells play an important role in recognition of invading microbial organisms, the so-called sense of danger. Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system being able to recognize structurally conserved molecules from microbes (Janeway, Travers, Walport & Schlomchik 2001). They are pattern recognition receptors (PRR) being able to identify pathogen-associated molecular patterns (PAMP) and can be located on the cell surface or in the intracellular compartments of phagocytes (Cornelie, Hoebeke, Schacht, Bertin, Vicogne, Capron & Riveau 2004; Meglia 2004). PAMPs are usually widespread in many different microorganisms and are often essential for the survival of the organism and these pattern recognition receptors (PRRs) recognize molecules that are constantly associated with threats and are highly specific, for instance bacterial DNA which is now proven to be a potent stimulus for immune cells (Cornelie *et al.,* 2004; Hansson & Edfeldt 2005).

The recognition and processing of pathogens by the innate system is an important first step in the development of the subsequent adaptive response.

The adaptive immune response is a very specific reaction to a particular invading pathogen and also involves the development of memory (Halliwell & Gorman 1989). Memory responses are stronger, longer lasting and more effective than innate responses (Sordillo & Streicher 2002). The most important cells in the adaptive immune system are the B- and T lymphocytes and the antigen presenting cells (APCs) (Halliwell & Gorman 1998). The APCs present endogenous or exogenous antigens and include macrophages, dendritic cells and B lymphocytes (Halliwell & Gorman 1998). The adaptive immune mechanisms are also classified as humoral and cellular. The humoral responses are mediated by the various antibodies that are produced by the B lymphocytes. The cell

mediated responses are a function of the T lymphocytes (Tizard 1996). T Lymphocytes can be divided into two groups, αβ T lymphocytes which include $CD4^+$ and $CD8^+$ cells and γδ T cells (Tizard 1996).

Lymphocytes have specialised and highly specific antigen receptors on their cell surface. $CD8^+$ and $CD4^+$ lymphocytes can only recognise antigen that is presented through MHC class I and MHC class II molecules respectively (Tizard 1996). The T helper (Th) cells determine the type of immune response.

Th 1 cells are mainly responsible for a cellular response and lead to the production of cytotoxic $CD8^+$ T cells, activate macrophages and proliferate production of opsonising antibodies IgM and IgG2 (Tizard 1996).

Th 2 cells are responsible for a humoral response and stimulate B cell proliferation with the production of IgG1 and IgE (Tizard 1996). Th 3 cells are involved in mucosal immunity and promote class switching to IgA (Chen, Jin, Hardegen, Lei, Li, Marinos, McGrad & Wahl 2003). Th 17 has an anti-microbial effect at mucosal surfaces and epithelial linings with the production of antimicrobial proteins and is involved in auto-immune disease. (Harrington, Hatton, & Mangan 2005; Korn, Betteli, Oukka & Kuchroo 2009)

$CD8^+$ cells are cytotoxic T cells and play a role in the elimination of host cells that harbour intracellular pathogens that are presented in association with MHC class I molecules (Sordillo & Streicher 2002).

The γδ T cells can be activated in a non-MHC-restricted way (Mackay & Hein 1991). Gamma-delta T cells play a more prominent role in the defence of mucosal surfaces and are more prevalent in the udder parenchyma than in the blood circulation (Sordillo & Streicher 2002). The γδ T cells are more common in ruminants and account for 60% of the T cell population (Tizard 1996).

Local mucosal immunity is critical in the protection against ascending infections in the teat and is mediated by mucosal associated lymphoid tissue (MALT). Mucosal immunity is driven by antigen that is actively sampled from the mucosal surfaces (Crivellaro, Vacca & Ribatti 2004). Both macrophages and dendritic cells are involved in mucosal searching and are both professional antigen presenting cells (APCs) that present antigen via MHC II molecules to $CD4^+$ lymphocytes (Janeway, Travers, Walport & Schlomchik 2001; Meglia 2004). Mature dendritic cells migrate to the supra mammary and inguinal lymph nodes, the so called inductive sites, and here they present antigen to naive $CD4^+$ lymphocytes (Janeway *et al.,* 2001). Lymphocytes become activated when they bind to specific antigen. B lymphocytes require the assistance of $CD4^+$ lymphocytes that were also activated by the same antigen. Under the influence of specific cytokines from the $CD4^+$ lymphocytes, the B lymphocyte will proliferate and differentiate into plasma or memory cells (Brandtzaeg & Reinhard 2004)

Primed memory B- and T-lymphocytes migrate from the MALT and the lymph nodes via the peripheral blood circulation to the various mucosal barriers. This is controlled by chemokines and vascular adhesion molecules (Brandtzaeg & Reinhard 2004). Here B lymphocytes differentiate into plasma cells which secrete immunoglobulins (Ig) (Meglia 2004). All B cells start by making IgM whereafter they switch to use either the Cγ, Cα or Cε genes to produce the corresponding Ig isotype as dictated by immune mediatory cytokines from the Th cells and other factors as discussed elsewhere (Tizard 1996).

IgA functions primarily as a neutralizing antibody which prevents the adhesion of bacteria to mucosal surfaces (Sordillo & Streicher 2002). IgA di-mers are actively transported through the mucosal cells after binding to the polymeric immunoglobulin receptor on the laminapropria side of secretory epithelial cells (Johansen, Braathen & Brandtzaeg 2001). Brandtzaeg and Reinhard (2004) have suggested that antigens within the laminapropria can complex with these IgA molecules in

transit and in this way be expelled. Since IgA is a poor activator of complement and opsonises weakly, it does not usually lead to an inflammatory response (Halliwell & Gorman 1998).

IgG1, IgG2 and IgM are present in udder secretions during infection and enhance bacterial phagocytosis by neutrophils and macrophages through opsonisation (Avery & Gordon 1991; Sordillo & Streicher 2002). IgM is the second most abundant Ig in serum and of more importance during a primary immune challenge (Tizard 1996). IgG is the Ig found in the highest concentration in the blood and due to its small size it can move with ease into the inter-cellular spaces of inflamed tissues (Halliwell & Gorman 1998). IgG binds rapidly to antigen causing clumping, opsonisation, phagocytosis and activation of complement (Tizard 1996). IgG also binds to and neutralises toxins (Halliwell & Gorman 1998). Ruminants have two sub-classes IgG1 and IG2 (Tizard 1996). IgG1 is the predominant Ig in cow's milk as opposed to the predominant Ig, IgA, in the other domestic animals and constitutes fifty percent of the IgG in blood serum. ImmunoglobulinG2 levels in serum vary between individuals and are highly heritable (Tizard 1996).

Bovine phagocytes have Fc receptors that are specific for IgG1 and will therefore be more efficient in phagocytosis of antigens bound by IgG1. Immunoglobulin G1 activates the classical pathway of complement in the ruminant (Howard, Taylor & Brownlie 1980; Sordillo & Streicher 2002). During complement activation the formation of C3a and C5a are pro inflammatory as both have an anaphylatoxin activity increasing vascular permeability and smooth muscle contraction. Immunoglobulin G1 is selectively transported into the udder during infection (Sordillo & Streicher 2002). Mammary macrophages seem to express fewer Fc receptors and have reduced phagocytic activity during infection. Immunoglobulin G2 agglutinates particular antigen and bovine neutrophils have specific Fc receptors for IgG2 (Halliwell & Gorman 1998).

A systemic immune response will result in both IgG1 and IgG2 levels to be similar in the infected and healthy quarters (Sordillo & Streicher 2002). With a local response as is the case with IgA the infected quarter will have higher concentrations of IgA (Sordillo & Streicher 2002).

The immune response in the udder is thus clearly extremely complex and greatly influenced by environmental factors.

2.6 Vaccines

Vaccination against mastitis has been researched for the last forty years and has been very challenging due to the insufficient and poor memory immune response during and after udder infection (Ruegg 2001; Talbot & Lacasse 2005). An effective vaccine may be described as one that reduces the severity and preferably also the frequency of clinical mastitis and assists in clearing infected cows in subsequent lactations (Ruegg 2001).

A potential side effect of the vaccination of the udder is an increase in the somatic cell count (SCC). A high SCC is undesirable as this is generally regarded as an indication of inflammation or infection of the udder with a subsequent drop in price paid by dairy companies (Ruegg 2001). The continual strive towards higher producing cows poses a formidable challenge to the cow to generate sufficient immunity in these unnatural conditions (Detilleux, Kehrli, Stabel, Freeman & Kelley 1995). The large volume of milk will dilute the available number of immune cells and antibodies, while at the same time presents an ideal medium for bacterial growth (Ruegg 2001).

The route of vaccine administration can have a significant influence on the type of immune response (Gomez, Garcia, Gherardi, Cerquetti & Sordelli 1998).Parenteral vaccination can either be intra-vascular, intra-muscular or sub-cutaneous (Tizard 1996). Vaccines administered on mucosal membranes (at the site of natural infection) usually offer a more effective and longer lasting immunity (Halliwell & Gorman 1998). Intramammary vaccination has been shown to be successful

(Gomez *et al.,* 2002). This however is not true mucosal vaccination because it is not administered directly on a mucosal membrane. The following routes have been described for mastitis vaccination: sub-cutaneous, intra-muscular, intra-mammary, intra-peritoneal and mucosal surfaces of the respiratory and gastrointestinal tracts (Celer & Cerny 1977; Chang 1980; Sheldrake, Husband, Watson, & Cripps 1985).

Different commercial mastitis vaccines are available and include primarily *S. aureus, E. coli* and *Streptococci* species (Ruegg 2001; Talbot & Lacasse 2005). These vaccines consist of bacterial extracts from the most common serotypes or purified antigens with or without carriers (Talbot & Lacasse 2005). Varying degrees of success is reported and some of these vaccines certainly reduce the severity of the clinical signs and others claim to reduce the number of clinical cases as well (see examples below). Vaccines incorporating cell surface associated proteins essential for bacterial growth were successful to some extent in rendering protection in the case of *Streptococcus uberis* (Fontaine *et al*., 2002). Perez *et al*., (2009) recorded that a *S. aureus* vaccine of bacterins surrounded by slime, conferred protection in a study on sheep. Prenafeta *et al*., (2010) also conducted a study on the extracellular components (slime) from *S. aureus* to determine whether antibodies against slime associate antigenic complex (SAAC) renders protection against *S. aureus*. Autogenous vaccines using homologous strains to those causing mastitis on a farm have been used with some success in *Staphylococcus aureus* (Leitner *et al.,* 2003b). Some success has been claimed with the use of DNA vaccines especially for *S. aureus* (Talbot & Lacasse 2005).

First generation vaccines are whole-organism vaccines and consist of live or live attenuated organisms which usually give a strong and lasting immunity. These vaccines have several disadvantages which include the danger of residual virulence or the reversion to virulence; are sensitive to temperature fluctuations, UV radiation and antimicrobial drugs. The following are examples of live *S. aureus* vaccines in the literature:

- Gomez *et al*., (1997) reported a live attenuated *S. aureus* vaccine that was successful in protecting mice after intra-mammary vaccination,
- Pellegrino *et al*., (2008) used an avirulant mutant of *S. aureus* in heifers and vaccinated them subcutaneous thirty and ten days prior to calving and it induced an IgG response in milk and blood. Post vaccination challenge by the virulent strain of the *S. aureus* at ten days post calving were better handled in the vaccinated group as compared to the unvaccinated group (Pellegrino *et al*., 2008),
- Buzzola, Barbagelata, Caccuri and Sordelli (2006) used an attenuated *S. aureus* aroA mutant as a live vaccine in a mouse model and elicited Th 1 and Th 2 responses and afforded significant protection against the parental wild-type and a heterologous strain from a mastitis cow.

Inactivated vaccines are safer especially when used in immune compromised animals. These vaccines did not induce good cell-mediated immunity and required more regular booster vaccinations (Alarcon, Waine & Mc Manus 1999).

- Startvac®*Escherichia coli* J5 inactivated, Staphylococcus *aureus* **(**CP8) strain SP 140 inactivated, expressing Slime Associated Antigenic Complex (SAAC). This vaccine contains inactivated bacteria and is an emulsion for injection. The vaccine strengthens the immunity of whole herds or of individual cows in herds known to have mastitis problems. The vaccine reduced the number of cows with mastitis and also the severity of those that had mastitis (STARTVAC® European Medicines Agency);
- Commercial mastitis vaccine for *S. aureus* available in USA is a bacterin marketed under two different names Somato- Staph® and Lysigen® and includes different groups of bacteria and antigen containing phage types 1, 11, 111 and 1V. The vaccine is known to reduce the severity of new cases;

- Hongsheng, Jie, XinPu, JinYin, JiYing, LiHua, FuCun, FuJie and YuYing (2007) described an inactivated polyvalent-vaccine that include strains of *S. aureus, Streptococcus agalactiae* and *Streptococcus dysgalactia*. It was highly immunogenic and protective and resulted in a decrease in the incidence of clinical mastitis.

Second generation vaccines incorporate sub-units, for example toxoids. The immunity induced by these vaccines is similar to that induced by killed organisms - primarily humoral and short-lasting (Alarcon, Waine & Mc Manus 1999). The following are examples of second generation *S. aureus* vaccines in the literature:

- Fattom, Horwith, Fuller, Propst and Naso (2004) vaccinated with a mixture of cell wall polysaccharides of *S. aureus* alone or bound to bacterial toxins and it seemed to render some protection in humans;
- Schennings, Heimdal, Coster and Flock (1993) used vaccines consisting of the different adhesions including Fibronectin binding protein (FnBP), clumping factor A (CfA), Collagen binding protein (Cna), elastin binding protein (Ebp) and EAP, a non specific binding protein produced from recombinant proteins combined with adjuvants and induced partial protection.

The development of third generation vaccines was instigated when Wolf *et al.,* (1990) administered a recombinant bacterial plasmid DNA in an attempt to express a beta-galactosidase gene in mice (Dhama, Mahesh, Gupta & Rai 2008). Plasmid expression DNA vaccines require a bacterial plasmid vector and have to be carefully designed. The plasmid should include a gene encoding the target antigen under the trans-scriptural control of an effective viral/eukaryotic promoter, along with a poly-adenylation signal sequence and a bacterial origin of replication (Gurunathan, Klinman & Seder 2000). These vaccines can only accommodate a limited amount of DNA. Nonetheless DNA vaccines

have been constructed to contain several mini genes and thus express multiple antigens, each antigen being specific for a particular virulence associated epitope (An & Whitton 1999).

The plasmid-vector DNA vaccines are not influenced by maternal antibodies, are stable, have lower production costs and don't require a cold chain (Dhama *et al.,* 2008). The demerits of DNA vaccines are theoretical, with integration into host genome, activation of proto-onco genes, inactivation of tumour suppressor genes and the possibility of generating anti-nuclear antibodies being listed as possible risk factors (Dhama *et al.,* 2008). The following are examples *S. aureus* DNA vaccines in the literature:

- Kerro-Dego, Prysliak, Potter and Perez-Casal (2006) used DNA vaccination specifically against GapB and GapC proteins of *S. aureus* with or without a booster of recombinant proteins and it rendered insufficient immune response for protection;
- Shkreta, Talbot, Diarra and Lacasse (2004) reported a DNA and protein vaccine with adhesion molecules fibronectin binding protein (Fnbp) and clumping factor A (ClfA) as targets and were partially protective. The vaccine was used on heifers who received two vaccinations with the DNA vaccine in the dry period at three week intervals followed with a booster at ten weeks with the recombinant proteins (Shkreta *et al.,* 2004);
- Adel, Nour, Shkreta, Talbot, Diarra & Lacassa attempted to block the primary stages of *S.aureus* with a DNA immunization of dairy cows with the clumping factor A and the vaccinated group showed a strong and specific antibody response. Three months later after a protein boost a good response was again elicited and *S. aureus* pre-incubated with milk or serum from the vaccinated cows showed a reduced ability to adhere to MAC-T cells.

The need for autogenous vaccines arose because in some instances the registered product does not contain the local pathogenic strain or because of continual antigenic variation of the pathogen in

question. These vaccines are considered herd specific and they include locally selected field isolates (Leitner, Lubashevsky, Glickman, Winkler, Sanan & Trainin 2003b).

- Leitner *et al*., (2003b) reported an autogenous type vaccine composed of different field strains from one area in a mixture included in a *S. aureus* vaccine and it was fairly successful to provide protection against mastitis in the dairy were it was used.

The development of an efficacious mastitis vaccine remains a challenge. In view of the severe damage caused by *S.aureus* and the unsuccessful treatment of these mastitis cases this particular route of vaccination was chosen in order to attempt to elicit an IgA response. The rationale behind this was to bind the pathogen before it actually enters the udder tissue were it seems difficult for the defence mechanism to eliminate the pathogen.

Chapter 3

Materials and methods

3.1 Study design

A prospective study on a cohort of ninety animals was performed over a twelve month period. Cows from two previously identified farms were randomly assigned to vaccinated and un-vaccinated groups. Herd-specific autogenous *Staphylococcus aureus* vaccines were used on the respective farms. Each herd was clinically evaluated before vaccination and the data recorded as a base line for udder condition and group diversity. Vaccination was administered on the mucosal surfaces of the vagina and teat canal as well as intra-muscularly (IM). The first vaccination was done at dry-up via the intra-teat and IM routes and the second vaccination at steam-up via the intra-vaginal and IM routes. Pre-vaccination (at dry-up), and post calving (at monthly intervals) milk samples were tested for *S. aureus* specific IgA with iELISA. The same milk samples were also cultured for *S. aureus*. The incidence of *S. aureus* in milk from vaccinated and un-vaccinated groups was evaluated using McNemars Test (Fleiss 1981).

Throughout the study positive (Pos) refers to cows positive for *S. aureus* on bacterial culture for milk sample and negative (Neg) refers to cows negative for *S. aureus* on bacterial culture for milk sample and dry-up (DU) refers to the time when cows enter the study.

A comprehensive McNemars matrix (Table 3.1.2) will be used to present the *S. aureus* culture results from milk samples for the duration of the study. The vaccinated and un-vaccinated groups will be presented separately.

Table 3.1.1 represents a simplified version of the McNemars test matrix. The test was used to assess the significance of the difference between cows negative (D) before vaccination and new positive cases after vaccination (E) (Bland 2000; Fleiss 1981).

	Lactation Negative	**Lactation Positive**	**Row Total**
DU Negative	D	E	D+E=B
DU Positive	F	G	F+G=C
Column Total	D+F	E+G	A=B+C

Table 3.1.2 represents the comprehensive McNemars matrix for cows before and after vaccination. Cows were categorised as negative or positive when entering the project. Cows that were negative at DU either remained negative throughout lactation or became positive. Positive cows at DU either remained positive throughout or become negative. The table presents different proportions and percentages.

DU	**Lactation**		
	Negative	Positive	Total B+C
Negative	**D** Cows remaining Negative	**E** Cows becoming Positive	**B**
	D^1	E^1	B^1
	D^2	E^2	
	D^3	E^3	
Positive	**F** Cows becoming Negative	**G** Cows remaining Positive	**C**
	F^1	G^1	C^1
	F^2	G^2	
	F^3	G^3	
Total	**H**	**I**	**A=B+C (n)**
	H^1	I^1	A^1 (%)

A represents the total number of cows in the study.

D^1, E^1, F^1 and G^1 represent percentages of the total population

B represents the negative cows in the study at DU.

D^2 and E^2 represent the percentages of the negative population

C represents the positive population at DU.

F^2, and G^2 represent the percentages of the positive population.

D^3 and F^3 represent the percentages of negative cows in lactation

E^3 and G^3 represent the percentages of positive cows in lactation

3.2 Study Population

Two dairy herds with predominantly *Staphylococcus aureus* mastitis were identified. They were screened three months prior to the trial to determine the prevalence of different *S. aureus* strains on each farm. A total of ninety pregnant animals irrespective of their number of lactations were included. Thirty-six of these animals were from farm 1 and 54 from farm 2. Cows were randomly divided into two equal vaccinated and un-vaccinated groups per farm by selecting each alternate animal as they came through the crush. The composition of the herds on the two respective farms is presented in Table3.2.1.

Farm 1 is the smaller and "younger" of the two farms. All adult cows were bought in and replacement heifers only entered the milking parlour in the months just after the end of the project. No cows were bought in during the study. This farm uses a semi zero grazing system with cows in nearby pastures receiving a complete ration.

Farm 2 is the larger farm and this herd has a closed system with regards to adult cows and only heifers are bought in every year. This farm uses a zero-grazing system. Cows are kept in soil enclosures close to the dairy with no access to pasture. Cows are totally dependent on the supplied ration.

Table 3.2.1 The total population of the two farms from which the study population was chosen is presented indicating their origin and stage of lactation.

	Cows in herd	Replacement heifers	Animals bought in	Cows in lactation	Dry cows	Heifers bred
Farm 1	150	Zero: all sold	All cows	100	50	30
Farm 2	480	100 in a year	40 heifers	300	180	60

The cows entered the study over a period of four months and all cows that dried-up within this time were used. Cows were dried-up at monthly intervals.

Table 3.2.2 represents the study populations from both farms.

	Vaccinated Group	**Un-vaccinated group**
	Number of cows	Number of cows
Farm 1	18	18
Farm2	27	27
Total	45	45

3.3 Udder health of cows entering the study

Udder evaluation was performed by a veterinarian on cows entering the study by palpating empty udders for general consistency of the parenchyma and distinct masses which could either be abscesses or old fibrotic hardened scar tissues. Teats were also examined with special attention to the teat orifice. The udders were scored according to the parameters represented in table 3.3.1 and table 3.3.2 (Kotzé 2009, Personal Communication)

Table 3.3.1 represents the adapted scoring system (Kotzé 2009, Personal Communication) that was used to evaluate the udders of cows as they entered the study. One indicates a healthy udder and 5 indicates an udder with very poor prognoses. The clinical assessment of the udder is based on the presence of inflammation and palpable abscesses. An estimated functionality of the udder is expressed as a percentage. The scoring identifies chronic carriers that are potential sources of infection in the herd.

		Lesions	**Mastitis**					
Udder	**Quarter**	**Minor skin etc.**	**Acute**	**Subclinical**	**Chronic**	**Abscesses**	**Functional**	**Prognoses**
0	Dead, underdeveloped, inactivated							NA
1	Normal udder	Absent	Absent	Absent	Absent	Absent	100%	Excellent
2	Functional udder with minor lesions	Possible	Absent	Possible	Absent	Absent	85%	Good
3	Udder with current inflammation or infection	Possible	Possible	Possible	Possible	Absent	70%	Treatable
4	Udder starting to develop chronic lesions	Possible	Possible	Possible	Present	Possible	55%	Poor
5	Chronic carrier cow to be slaughtered	Possible	Possible	Present	Present	Present	0%	Zero

Table 3.3.2 represents the adapted scoring system (Kotzé 2009, Personal Communication) that was used to evaluate the teats of cows as they enterd the study. One indicates a healthy teat and 5 indicates a teat with very poor prognoses. The clinical assessment is based on the damage at the teat orifice and the functionality of the sphincter. Cows with permanent damage to the sphincter and protrusion of the mucosa are culled (Kotzé 2009, Personal Communication)

Score	Teat/Orifice	Lesions at orifice	Functional	Prognoses
0	Underdeveloped	NA	NA	NA
1	Normal	Absent	100%	Excellent
2	Functional	Mild	90%	Good
3	Damage to sphincter, inflammation at teat end	Cracks and minor wounds	80%	Treatable
4	Damage with emersion of mucosa and infection of teat end.	Cowpox, warts, cuts etc.	40%	Poor
5	Permanent damage, enablement of sphincter, exposed mucosa.	Open entrance with exposed mucosa.	0%	zero

3.4 Sample collection

Sampling procedures were performed approximately two hours after milking. Pre-vaccination milk samples were aseptically collected into 11 mℓ tubes (BD Vacutaner, Z no additive, Cat nr. 364915, Beliver Industrial Estate, Plymouth, UK) from each cow as they entered the study. Thereafter monthly post calving milk samples were collected. A minimum of 10 mℓ milk was collected as a combined sample from all four quarters. The tubes were appropriately identified with the date and the cow identification number.

3.5 Culture and Purification of *S. aureus*

In this study a combined sample from all four quarters were taken aseptically for each cow and cultured within four hours. The sample was taken two hours after milking to reduce the volume of milk in the udder and after physical palpation. Plates were observed for seventy-two hours and any growth was considered significant. Observation was done every twelve hours and single colonies were picked up for sub culturing to improve sensitivity of the bacterial culturing. Milk samples were cultured in the DVTD Bacteriology Laboratory according to standard operating procedures for general bacterial isolation from milk samples (QA/BS/SOP PRO 002, Jarvis, Kelerman, van Rensburg & Whitehead 1994; Quin, Carter, Markey & Carter 1994). Briefly a sterile bacteria loop of 0.1 mℓ litre was dipped into the sample and used to inoculate the plate. The sample was streaked out for single colonies and incubated in the 37 °C walk in incubator up to seventy-two hours. Plates were evaluated every twelve hours up to seventy-two hours. No growth after seventy-two hours was recorded as negative and growth within seventy-two hours was recorded as positive. Plates were sub-cultured to obtain pure cultures when necessary.

Once pure, a smear was prepared from al samples that showed positive growth and gram stain applied (QA/BS/SOP ST 002, DVTD, Onderstepoort, Jarvis *et al.,* 1994). All gram positive cocci in

clusters were subjected to *S. aureus* identification. *S. aureus* colonies typically are β-haemolytic on blood agar, larger than 3mm in diameter with positive growth on MacConkey agar. These Gram positive cocci are also Catalase positive, Coagulase positive, Ferments mannitol and maltose and are resistant to novobiocin and polymyxin B (QA/BS/SOP ID 030, DVTD, Onderstepoort, Jarvis *et al*., 1994; Quin *et al.,*1994).

All isolates were re-confirmed at another veterinary laboratory.

3.6 Autogenous *S. aureus* vaccine

An autogenous multivalent herd specific *S. aureus* vaccine was prepared for each farm. Isolates from a representative sample of twenty-five cows with either clinical or subclinical mastitis were used to determine the most prevalent strains on each of the two farms. A maximum of five of the most prevalent strains were selected for inclusion in the vaccine. The composition of the vaccine is confidential as described in the protocol and remains the IP of Melach Biotechnology.

3.7 Vaccination procedures

The first vaccination was administered when the cows entered the study (at dry-up) and the second vaccination was given thirty days later (during the steam-up phase) preferably done fourteen days before calving. Both the first and second vaccinations were administered via the parenteral and mucosal routes. Mucosal vaccination was either intra-teat or intra-vaginal. The first vaccination at dry-up was administered via the intra-teat route and the second vaccination during steam-up was administered via the intra-vaginal route.

Intra-teat administration was performed with a sterile one mℓ syringe by placing the tip of the syringe against the teat orifice of only one teat. One mℓ of vaccine was deposited in the teat canal at the Furstenburg rosette. This was done after milking and administration of the dry-cow remedy.

The dry-cow remedy is massaged properly into the udder before vaccination. All cows in the herd received the same dry-cow remedy according to table 3.11. Intra-vaginal vaccination was done with a pipette for artificial insemination connected to a 10 mℓ syringe and 1 mℓ of vaccine was deposited in front of the cervix.

The dose for parenteral administration was 1 mℓ intra-muscular and was administered in the tensor fasciae latae midway between the tuber coxae of the ilium and sub-iliac lymph node.

Farm 1 used several dry-cow preparations but all cows in the study received the same treatment. Farm 2 used only one dry-cow preparation during the project.

Table 3.7.1 represents the various dry cow remedies and program of application.

	Oct	**Nov**	**Dec**	**Jan**	**Feb**	**March**	**April**	**June**
Farm 1		A	A	B	C	C	C	C
Farm 2	C	C	C	C	C	C	C	C

A: Penstrep® Bayer AH Dry Cow Injection 1128 Procain penicillin G 3000 000iu; Dihidro-streptomycin 500mg, blue tracer dye 125 mg/5g

B: Cepravin® Intervet Schering AH Cephalonium 250 mg/3g (s4) Dry cow remedy, long acting

C: Ceph Udder® Intervet Schering AH Cephaprin 300mg/10mℓ Dry cow remedy

3.8 Indirect enzyme linked immunosorbent assay (ELISA) for detection of *S. aureus* specific IgA in milk.

The indirect ELISA (iELISA) was used to detect IgA levels in milk. This ELISA was adapted from methods that have been previously described by Leitner *et al*., 2000; Leitner, Lubashevsky, Glickman, Winkler, Saran & Trainin, 2003b; Wang, Taaffe, Parker, Solorzano, Cao, Garcia-Sastra,A. & Lu 2006; Wang, Zhang, Zhang, Li, Huang, Huang & Lu 2006)

The ELISA was standardized but not validated as it was developed separately for each herd and therefore only applicable for the *S. aureus* strains included in each crude antigen preparation. These antigens were the same as those included in the vaccine.

3.8.1 Preparation of antigen for coating of ELISA plates

The same *S. aureus* strains included in the respective vaccines for the two farms were used to prepare farm-specific batches of crude antigen for coating the ELISA plates.

S. aureus colonies from each strain were inoculated into a Colombia brain heart infusion broth (Oxoid CM 1135, pH7.4) and incubated overnight at 35 °C. The purity and viability of *S. aureus* was confirmed by a second culture on BTA and MacConkey agar. Forty millilitre of the broth was centrifuged at 896 G for fifteen minutes at 4 °C. The pelleted cells were washed three times in sterile phosphate buffered saline (0.15 M, pH 7.4) (PBS). The washed cells from the various strains were pooled per farm. The final suspension was adjusted with PBS up to an optical density of exactly two. This correlated with a Mac Farland standard of eight. Cells were inactivated in one percent formalin for forty-eight hours at 35 °C. Viability of Staphylococcus organisms was checked. The suspension was sonnicated for three cycles of 25 seconds at 45 Hertz with amplitude of 40 after which the antigen was aliquoted in 10 mℓ cryovials and stored at -20 °C.

3.8.2 Checkerboard titrations

Checkerboard titrations were performed for each of the antigens to determine the optimum antigen concentration for coating the ELISA plates. The optimum antigen dilution for both antigens was 1:6.

3.8.3 Preparation of test samples

Milk samples were centrifuged at 900 G for fifteen minutes. The cream layer was removed and the sample was taken from the lower third of the test tube. All samples were stored in appropriately labelled cryo-tubes at -20 °C. Samples from pre- mid- and post milking were compared and found to have similar levels of Ig.

3.8.4 ELISA procedure

Fifty µℓ antigen at a pre-determined concentration in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6, Sigma) was passively adsorbed overnight at 4 °C to NUNC Maxisorb ELISA plates (AEC Amersham, Cat. No 442404). Plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T) using a PW40 Biorad ELISA washer. Non-specific binding was reduced by blocking for one hour by adding 50 µℓ litres blocking buffer (PBS-T plus 3% casein) and placing plates in an incubator at 35 °C on an orbital shaker (Luckhams, Rotatest). Milk samples were pre-diluted (1:160) using blocking buffer and 50 µℓ of the sample and control were transferred to the respective ELISA plates. Plates were incubated for one hour on an orbital shaker on the bench and then washed as before. Fifty µℓ of horseradish-peroxidise (HRPO) conjugated to sheep anti-bovine IgA (Bethyl Laboratories, INC Catalogue No A10-121P) at a dilution of 1:5000 in blocking buffer was added to all the wells. The plates were incubated as before on the bench. Plates were washed and 50 µℓ of ortho-phenylenediamine (OPD) [Sigma] at a concentration of 0.4 mg/mℓ, and containing 0.05% H_2O_2 (30% v/v) was added to each well. Plates were developed for ten minutes at room temperature in the dark before stopping the colour development by the addition of 50 µℓ of 2 N H_2SO_4, (stopping solution) Plates were read after blanking using a BIOTEK EL 808 micro plate reader at a wave length of 490 nm.

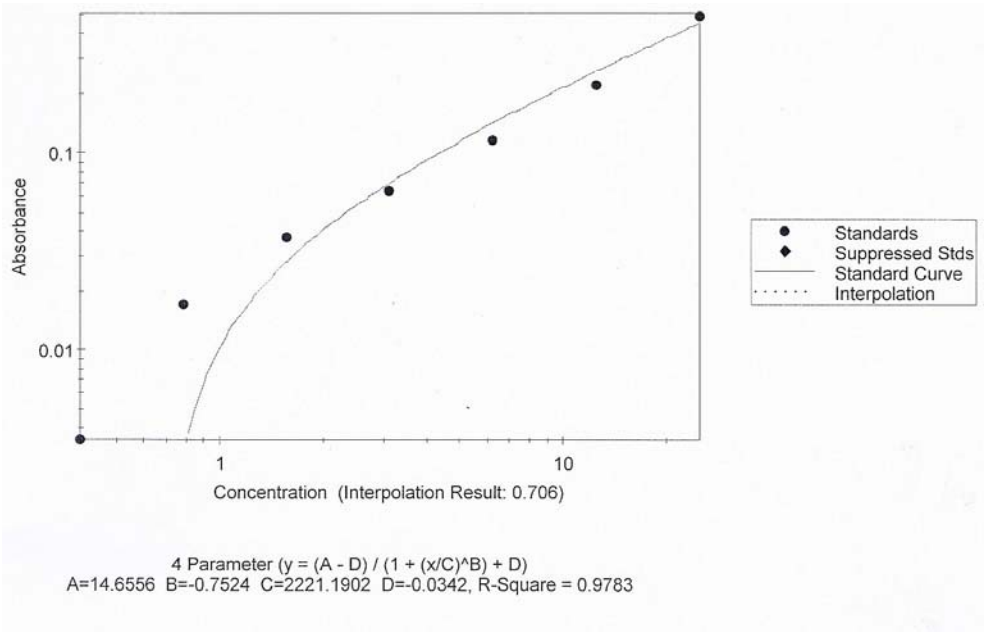
3.8.5 Standardization of ELISAs

A positive standard was prepared for each of the two herds. This was done by pooling milk samples from animals with a history of repeated pure growth of *S. aureus*. Standards were diluted 1:40 in blocking buffer and stored in 2 mℓ aliquots at –20 °C. Before use aliquots were thawed and used for a maximum of four days. The standards were titrated and standard curves were plotted for each ELISA. After titrations of samples at different concentrations a dilution of 1:160 was selected for testing the samples in the different iELISAs. Duplicate titrations of the appropriate standard were included on every ELISA plate to compensate for day to day variation of OD values. An arbitrary

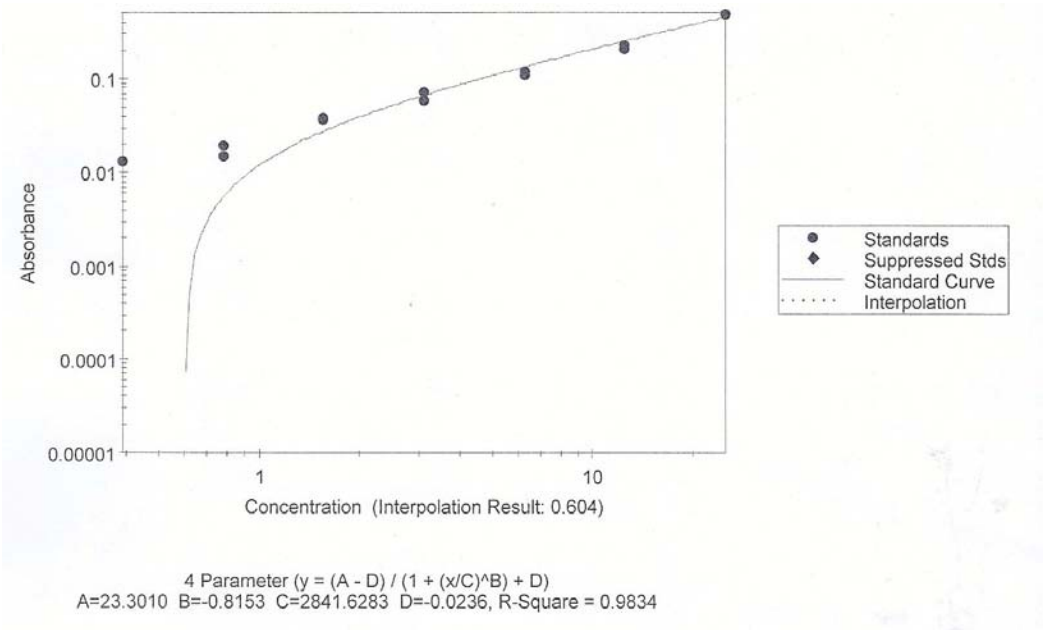
number of hundred units per mℓ were assigned to each standard and the strength of a single dilution of the test samples were calculated by curve-fitting using the four-parameter logistic-log model. Calculations were performed using KC Junior software® (BIO-TEK®).

Table 3.8.5.1 Plate lay out for the milk IgA iELISA. Forty samples were tested in duplicate on a plate. A standard was titrated in duplicate in column 11 and 12 of each plate. Column 11 indicates the dilution factor and column 12 the corresponding assigned unit value. H11 and H 12 were used as blanks.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Sample1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Standard	Standard
B	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	1:40	25
C	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	1:80	12.5
D	sample 11	sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	1:160	6.25
E	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	1:320	3.125
F	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	1:640	1.5625
G	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	1:1280	0.78125
H	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	Blank	Blank



Graph 3.8.5.1 represents the standard curve used for the milk IgA IELISA for farm 1. The curve was constructed using the four-parameter logistic-log curve-fitting method provided by the KC Junior software® (BIO-TEK®).



Graph 3.8.5 2 represents the standard curve used for the milk IgA IELISA for farm 2. The curve was constructed using the four-parameter logistic-log curve-fitting method provided by the KC Junior software® (BIO-TEK®).

Chapter 4

Results

4.1 Introduction

The study was conducted over a 12 month period and started with the screening of two dairy farms to determine the prevalence of *S. aureus* and to collect isolates from each farm to be included in the vaccine. Ninety cows were included in the project of which 36 were from the smaller farm. Cows that entered the study were clinically evaluated to determine the udder health status of the study group. The IgA response and the incidence of cows with positive *S. aureus* milk cultures were monitored in samples collected pre-vaccination (dry-up) and monthly thereafter for the duration of the study. The bacterial culture results were used to describe the *S. aureus* status of the population according to McNemars Test (Fleiss 1981).

4.2 Udder health of cows entering the study

Animals on farm 1 entered the study for the first time in November 2010 and the last samples were collected in June 2011. The study on farm 2 commenced in October 2010 and sampling continued until June 2011.

Udders and teats were categorised according to the highest score given to any quarter or teat.

In farm 1 there were a total of two cows with healthy udders in the entire group with the bulk of the group having udder scores of three and four. This farm did not have udders in category five. Farm 2 had a larger group with reasonable udder health but certain cows were scored five indicating very poor prognoses.

Table 4.2.1 Results of the clinical udder evaluation of cows as they entered the study. Udders were graded with a scoring system from 1 to 5 with 1 representing healthy udders and 5 representing udders with poor prognosis (Kotzé 2009, Personal communication)

Farm1		Number of Cows		
Udder score	**Quarter**	**Vaccinated**	**Un-vaccinated**	**Prognoses**
1	Normal udder	1	1	Excellent
2	Functional udder with minor lesions	2	2	Good
3	Udder with current inflammation or infection	9	8	Treatable
4	Udder starting to develop chronic lesions	6	7	Poor
5	Chronic carrier cow to be slaughtered	0	0	Zero
Farm2		Number of Cows		
Udder score	**Quarter**	**Vaccinated**	**Un-vaccinated**	**Prognoses**
1	Normal udder	0	1	Excellent
2	Functional udder with minor lesions	14	9	Good
3	Udder with current inflammation or infection	5	7	Treatable
4	Udder starting to develop chronic lesions	4	8	Poor
5	Chronic carrier cow to be slaughtered	4	2	Zero

Farm 1 and 2 had many cows with udders with current inflammation or infection at the beginning of the study. These cows have seventy percent functional udders and are considered treatable. Both groups have udders with poor prognoses and only fifty-five percent functional udder tissues remaining.

In farm 1 there was only one cow with a teat score of one. The majority of cows in both groups had a score of two or three. None of the cows had teats with permanent damage. In farm 2 teat evaluation indicated severe damage in all animals ranging from scores of two up to five.

Table 4.2.2 Results of the teat evaluation of cows as they entered the study. Teats were graded with a scoring system from 1 to 5 with 1 representing normal teats and 5 representing teats with poor prognosis (Kotzé 2009, Personal communication)

Farm 1		Number of Cows	
Teat score	**Teat/Orifice**	**Vaccinated**	**Un-vaccinated**
1	Normal	1	0
2	Functional	7	7
3	Damage to sphincter, inflammation at teat end	8	8
4	Damage with emersion of mucosa and infection of teat end	2	3
5	Permanent damage, enablement of sphincter, exposed mucosa	0	0
Farm 2		Number of Cows	
Teat score	**Teat/Orifice**	**Vaccinated**	**Un-vaccinated**
1	Normal	0	0
2	Functional	10	8
3	Damage to sphincter, inflammation at teat end	15	13
4	Damage with emersion of mucosa and infection of teat end	1	5
5	Permanent damage, enablement of sphincter, exposed mucosa	1	1

In both farms two groups of similar size are distinguished, those with functional teats and those with teats that showed damage to the sphincter and inflammation at the teat end. Only two cows from farm 2 presented with severe teat damage.

4.3 *S. aureus* bacterial culture in milk samples during the study

Animals from the two farms that entered lactation were combined and the incidence of *S. aureus* on milk culture for the duration of the study was independently determined for the vaccinated and un-vaccinated groups according to McNemars test.

4.3.1 Vaccinated group

Within the vaccinated group (n=38), 86% were negative for *S. aureus* when they entered the study and 13% had *S. aureus* cultured from milk. Sixty-five percent of the vaccinated cows remained negative throughout the study and 21% became positive for *S. aureus* in milk. Five percent of the vaccinated cows remained positive throughout the study and 8% became negative.

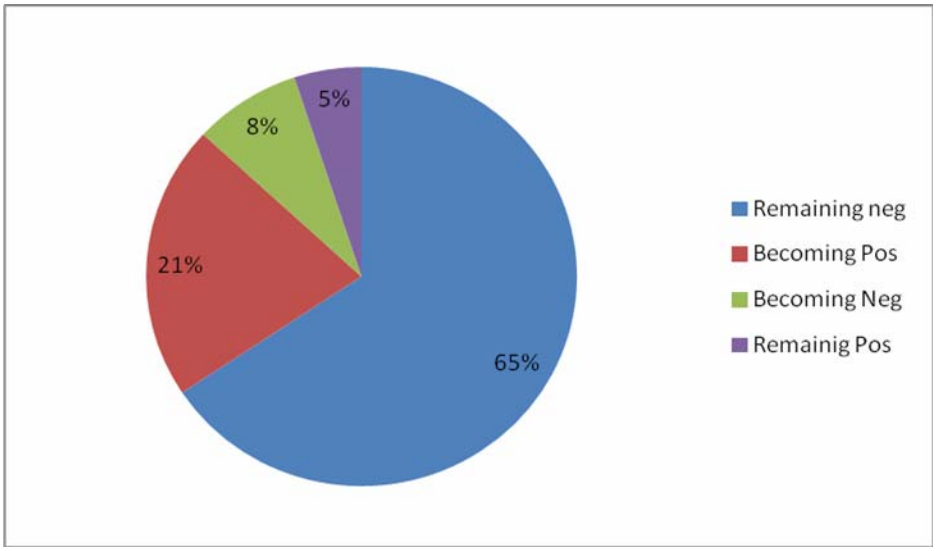
The incidence for *S. aureus* in milk was 24% (8/33) with an insignificant risk (p=0.1317).

From the final population of 38 cows, 74% (28/38) were negative and 26% (10/38) positive for *S. aureus* in milk at the end of the study.

Of the negative cows (n=28), 89% were negative from the beginning and 11% were positive cows that became negative for *S. aureus* culture in milk. Of the positive cows (n=10) 20% were positive from the beginning and 80% were negative that became positive for *S. aureus*.

Graph 5.3.1 shows the proportions in the lactating population for the vaccinated group of both farms combined. The negative population of 73% is divided into two groups, those cows that did not culture *S. aureus* at dry-up or during lactation (65%), and those that were positive at DU but became negative during lactation (8%), these cows were repeatedly negative on bacterial culture of milk samples.

The positive population of 26% is also divided into two groups. The smaller group of 5% that did culture *S. aureus* at dry-up and throughout lactation, these cows remained positive on repeated bacterial culture. A group of 21% represents new infections (cows that were negative at DU) and became positive on at least one bacterial culture of milk samples.



Graph 4.3.1 represents *S.aureus* milk culture results from vaccinated cows on both farms for the duration of the study.

Table 4.3.1 represents *S. aureus* culture results of the vaccinated group using the McNemars test matrix. Cows were categorised into negative or positive when entering the project according to bacterial culture of milk samples. The cows in the negative DU group either remain negative in lactation or became positive for *S. aureus*. The cows in the positive DU group either became negative or remain positive during lactation. The incidence of *S. aureus* in milk from cows that were negative is 24%

Table of Status DU by Status Lactation				
Status DU		**Status Lactation**		
		Negative	**Positive**	**Total**
Negative cows		25	8*	33
	%	65	21	86
Incidence: Risk factor	%	76	**24**	
	%	89	80	
Positive cows		3	2	5
	%	8	5	13
	%	60	40	
	%	11	20	
Total		28	10	38
	%	74	26	100

*P = 0.1317

4.3.2 Un-vaccinated group

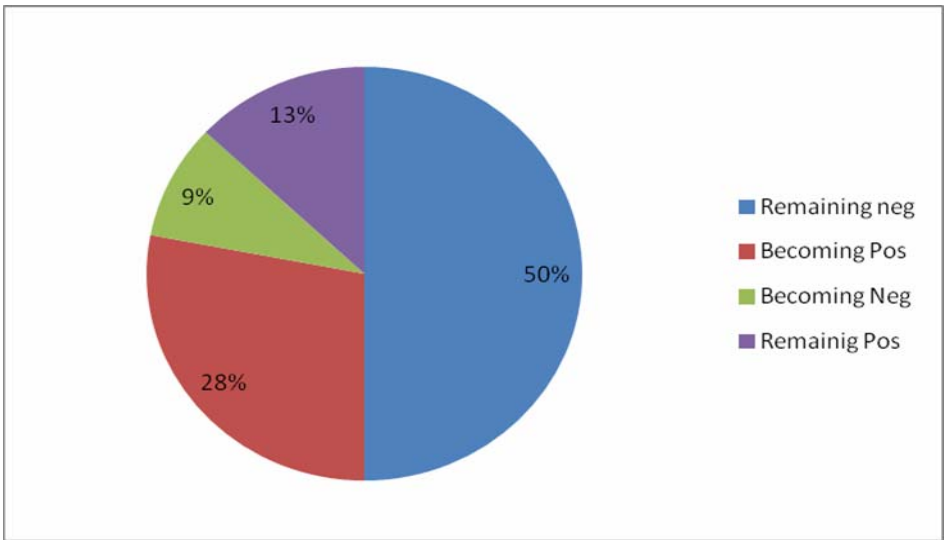
Within the un-vaccinated group (n=32), 78% were negative for *S. aureus* when they entered the study and 22% had *S. aureus* cultured from milk. Fifty present of the vaccinated cows remained negative throughout the study and 28% became positive for *S. aureus* in milk. Thirteen percent of the vaccinated cows remained positive throughout the study and 9% became negative. The incidence for *S. aureus* in milk was 36% (9/25) with an insignificant risk (p=0.0833).

From the final population of 32 cows, 59% (19/32) were negative and 41% (13/32) positive for *S. aureus* in milk at the end of the study.

Of the negative cows (n=16), 84% were negative from the beginning and 16% were positive cows that became negative for *S. aureus* culture in milk. Of the positive cows (n=9) 31% percent were positive from the beginning and 69% were negative that became positive for *S. aureus*.

The negative population of 59% is divided into two groups. Those cows that did not culture *S. aureus* at dry-up or during lactation (50%), and the smaller group (9%) that were positive at DU but became negative during lactation, they were negative on repeated bacterial culture.

The positive population of 41% is also divided into two groups. The smaller group of 13% that remained positive throughout the study, and a group of 28% that were negative at DU but became positive during lactation.



Graph 4.3.2 represents *S.aureus* milk culture results of un-vaccinated cows from both farms for the duration of the study

Table 4.3.2 represents *S. aureus* culture results of the un-vaccinated group using the McNemars test matrix. Cows were categorised into negative or positive when entering the project according to bacterial culture of milk samples. The cows in the negative DU group either remained negative during lactation or became positive for *S.aureus*. The cows in the positive DU group either became negative or remained positive.

Table of Status DU by Status Lactation				
Status DU		**Status Lactation**		
		Negative	**Positive**	**Total**
Negative cows		16	9*	25
	%	50	28	78
Incidence: Risk factor	%	64	**36**	
	%	84	69	
Positive cows		3	4	7
		9	13	22
	%	42	57	
	%	16	31	
Total		19	13	32
	%	59	41	100

*P = 0.0833

4.4 Detection of *S. aureus* specific IgA in milk samples.

The objective of this section was to determine whether an IgA response could be detected in cows vaccinated with an autogenous vaccine via the parenteral and mucosal routes during the dry period.

Due to the high prevalence of *S. aureus* in both study populations it was not possible to discriminate between the IgA responses from natural infection and that from vaccination; therefore we only present IgA data from cows in that remained negative on *S. aureus* milk culture throughout the study. It should be noted that a negative *S. aureus* culture from milk does not exclude natural exposure. The antigens used in the vaccines and iELISAs were unique for each of the two study populations therefore the results are presented separately. The titres are presented for the lactation stages starting at dry-up (DU), periparturient (PP), and one month after PP, two to three months after PP and finally three to four months after PP.

Farm1 dry-up titres

The DU titres had a median value of 3376 and 3444 in the vaccinated and un-vaccinated groups respectively. In the vaccinated group the upper confidence limit (UCL) was 18165 and the lower confidence limit (LCL) 3137. In the un-vaccinated group the UCL was 6475 and the LCL 3080.

Table 4.4.1 describes the DU titres of the vaccinated and un-vaccinated groups of farm 1. The DU titres in both groups indicate a large variation between the LCL and the UCL which is more pronounced in the vaccinated group.

	Vaccinated	Un-vaccinated
Number of cows	18	18
Median	3376	3444
Mean	10651	4778
95% LCL	3137	3080
95% UCL	18165	6475

LCL = lower confidence limit

UCL = upper confidence limit

Farm1 vaccinated group

The mean IgA titre for the seven animals in the vaccinated group that remained negative on monthly *S. aureus* culture increased from 366 at dry-up to 1433 one month into lactation, and stabilized at a titre of 1430 throughout the remainder of the lactation.

Table 4.4.2 Statistical description of *S. aureus* IgA iELISA titres for cows from farm 1 that remained negative for *S. aureus* culture in milk for the vaccinated group presented according to the different stages of lactation with median values and upper and lower quartiles.

Titre for *S. aureus* IgA						
Lactation stage	**Number of cows**	**Median**	**Lower Quartile**	**Upper Quartile**	**Minimum**	**Maximum**
DU	7	366	120	2576	98	11647
PP	7	544	248	1212	65	2228
1 month	7	1433	448	3432	326	3432
2-3 months	11	988	757	2002	120	3432
4-5 months	4	1430.5	1200.5	1803	1089	2057

DU = Dry-up

PP = periparturient

Farm1 un-vaccinated group

The five animals in this group had a higher DU titre than was present in the Vaccinated group. The group maintained a higher titre than the vaccinated group throughout the lactation stages with the exception of one month after the periparturient sample.

Table 4.4.3 Statistical description of *S. aureus* IgA iELISA titres for cows from farm 1 that remained negative for *S. aureus* culture in milk for the un-vaccinated group presented according to the different stages of lactation with median values and upper and lower quartiles

Titre for *S. aureus* IgA						
Lactation stage	**Number of cows**	**Median**	**Lower Quartile**	**Upper Quartile**	**Minimum**	**Maximum**
DU	5	4271	120	8072	80	8721
PP	5	1780	970	3880	799	4852
1 month	5	835	769	1554	679	2355
2-3 months	6	1645.5	1353	1911	508	2738
4-5 months	1	2506	2506	2506	2506	2506

DU = Dry-up

PP = periparturient

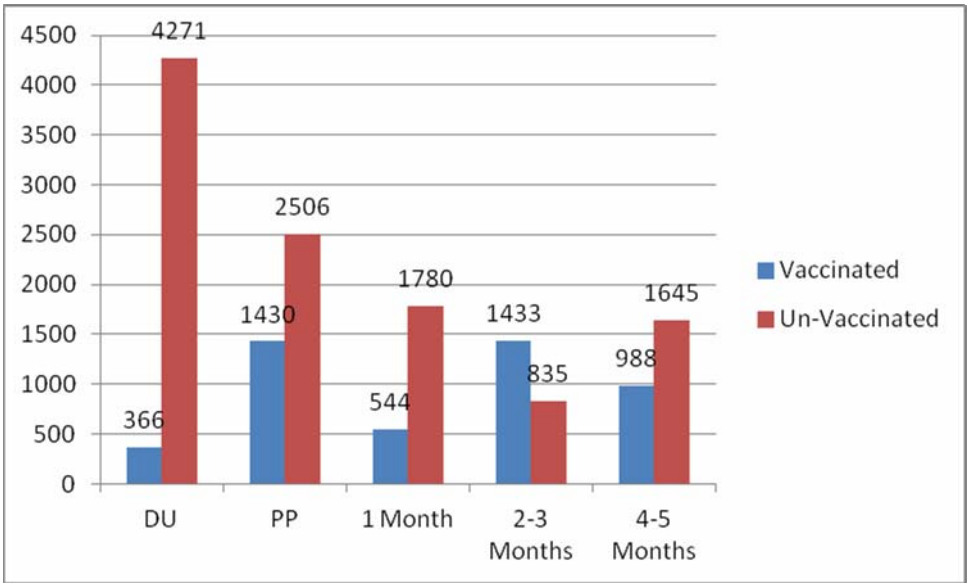
The titres of the vaccinated and unvaccinated groups were statistically compared using the Kruskal-Wallis Test but no significant differences could be shown.

Table 4.4.4 The titres of the vaccinated and unvaccinated groups from farm 1 were statistically compared using the Kruskal-Wallis Test. P-values for every stage of lactation are presented below. No statistical significant differences could be shown.

Kruskal-Wallis Test	
Lactation stage	P- value
DU	0.744
PP	0.0882
1 month	0.9352
2-3 months	0.3657
4-5 months	0.1573

DU = Dry-up

PP = Periparturient



Graph 4.3.1 represents IgA iELISA titres in milk for cows from farm 1 that were negative for *S. aureus* in milk during the study.

Farm2 dry-up titres

The DU titres had a median value of 333 and 384 in the vaccinated and un-vaccinated groups respectively. In the vaccinated group the Upper confidence Limit (UCL) was 1922 and the Lower Confidence Limit (LCL) 754. In the un-vaccinated group the UCL was 4430 and the LCL 1035

Table 4.4.5 describes the DU titres of the vaccinated and un-vaccinated groups from farm 2. The DU titre in both groups shows a moderate difference between the LCL and the UCL more pronounced in the vaccinated group.

	Vaccinated	Un-vaccinated
Number of cows	27	27
Median	333	384
Mean	1183	2732
95% LCL	754	1035
95% UCL	1922	4430

LCL = lower confidence limit

UCL = upper confidence limit

Farm2 vaccinated group

The mean IgA titre for the 18 animals in the vaccinated group that remained negative on monthly *S. aureus* culture had a DU titre of 328. This titre was not reached again during lactation. The PP titre of 106 increased to 251 two to three months later into lactation and fell again to 114 in the last four to five months. Serum conversion could not be displayed in this group as determined by DU titre. Although all the monthly milk samples for these cows were negative for *S. aureus* culture the possibility of natural exposure could not be completely ruled out.

Table 4.4.6 Statistical description of *S. aureus* IgA iELISA titres for cows from farm 2 that remained negative for *S. aureus* culture in milk for the vaccinated group presented according to the different stages of lactation with median values and upper and lower quartiles.

Titre for *S. aureus* IgA						
Lactation stage	**Number of cows**	**Median**	**Lower Quartile**	**Upper Quartile**	**Minimum**	**Maximum**
DU	18	328.5	273	600	82	8229
PP	18	114	71	150	20	2166
1 month	13	106	85	148	10	326
2-3 months	23	103	63	180	12	1134
4-5 months	7	251	123	397	102	894

DU = Dry-up

PP = Periparturient

Farm2 un-vaccinated group

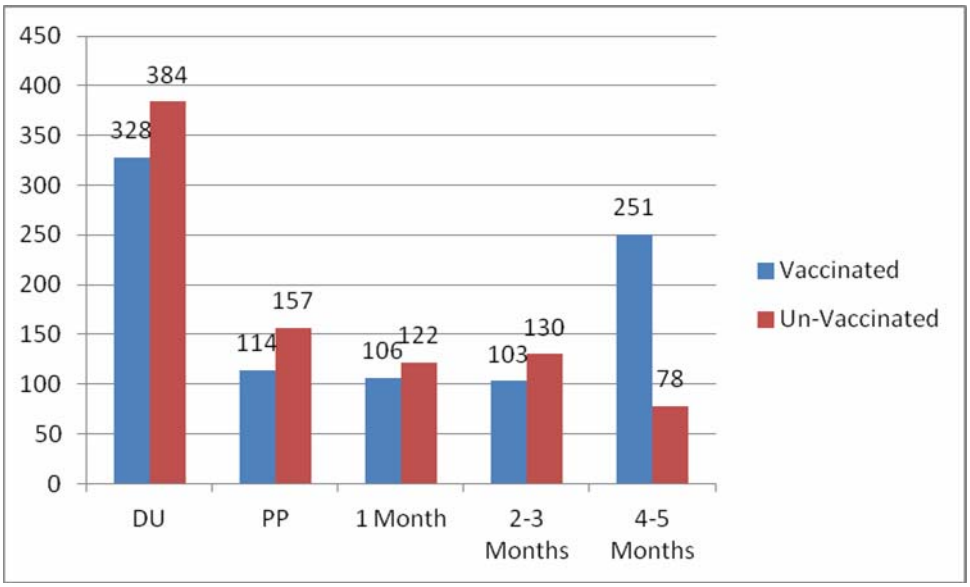
Eleven animals were in the *S. aureus* negative group and as with the vaccinated group the DU titre of 384 was not reached again and therefore serum conversion was not present. The PP titre showed a marked drop and thereafter the titre stabilised at 130 and decreased to 78 at the end of lactation.

Table 4.4.7 Statistical description of *S. aureus* IgA iELISA titres for cows from farm 2 that remained negative for *S. aureus* culture in milk for the un-vaccinated group presented according to the different stages of lactation with median values and upper and lower quartiles.

Titre for *S. aureus* IgA						
Lactation stage	**Number of cows**	**Median**	**Lower Quartile**	**Upper Quartile**	**Minimum**	**Maximum**
DU	11	384	262	886	98	1944
PP	11	157	84	371	3	1175
1 month	7	122	95	154	67	190
2-3 months	9	130	89	155	64	245
4-5 months	5	78	55	86	12	106

DU = Dry-up

PP = Periparturient



Graph 4.3.2 represents IgA iELISA titres in milk for cows from farm 2 that were negative for S. aureus in milk during the study.

The titres of the vaccinated and unvaccinated groups were statistically compared using the Kruskal-Wallis Test and significant difference could be shown during lactation four to five months after the periparturient stage (p=0.03485)

Table 4.4.8 The titres of the vaccinated and unvaccinated groups from farm 2 were statistically compared using the Kruskal-Wallis Test. P-values for every stage of lactation are presented below. Statistical significant differences could be shown during lactation four to five months after the periparturient stage.

Kruskal-Wallis Test	
Lactation stage	P- value
DU	0.9284
PP	0.1774
1 month	0.3621
2-3 months	0.5158
4-5 months	0.03458

DU = Dry-up

PP = periparturient

4.5 Fate of cows in the project

Farm1

Eight of the 18 cows in the vaccinated group did not complete the study, of which six were culled before they entered lactation and two during lactation. Two of the 18 cows from the un-vaccinated group were culled during lactation.

Farm 2

Two of the 27 cows in the vaccinated group were culled before they entered lactation. Eleven of the 27 cows in the un-vaccinated group did not complete the study of which 10 were culled before they entered lactation an one during lactation.

Chapter 5

Discussion and Conclusion

Mucosal immunity in the udder is not as well described in the literature as for other mucosal surfaces like the respiratory and intestinal tract (Brandtzaeg & Pabst 2004). Genital associated lymphoid tissue in non human primates has been described by Lehner, Panagiotidi, Bergmeier, Tao, Brookes, Gearing & Adams, (1999). The importance of the route of vaccination has been described by Lehner *et.al*. (1999) who used a combined oral and vaginal mucosal vaccination to elicit a Ig response including IgA and IgG in non human primates. They only detected an increase in specific $CD4^+$ T cells in the spleen and related genital lymph nodes thereby identifying genital associated lymphoid tissue. Lehner, Wang, Ping, Bergmeier, Mitchell, Cranage, Hall, Dennis, Cook, Doyel & Jones used mucosal vaccination via the oral, rectal or vaginal route to elicit secretory IgA and serum IgG as well as specific T cell responses. They reported that targeted iliac lymph node vaccination resulted in good mucosal antibody responses of regional mucosal surfaces, including the rectum and vagina. In this study we targeted the lymphoid tissues in the vagina and the Furstenburg rosette as well as the regional lymph nodes to elicit a local mucosal immunity.

5.1 Clinical evaluation of udders and teats before vaccination

Cows with chronic lesions in the udders also presented with permanent damaged teat sphincters. Severe teat damage is known to correlate with poor udder health (Petzer 2001). The palpation of udders and teat examination is described by Petzer (2001) who demonstrated poor prognoses in the presence of distinct fibrotic changes. The poor udder condition of herds where *S. aureus* is prevalent is well described in the literature and culling of cows with abscesses is often advised because of persistence of bacteria within these abscesses (Petzer, 2001). The udder and teat scoring system as

used by Kotze (2009) was used as a diagnostic tool to evaluate the herd and asses udder damage. Pronounced teat damage is often associated with machine milking (Petzer, 2004). The exposed mucosal surface at the damaged teat orifice provides a portal for pathogen entry (Jones & Baily, 1998; Petzer, 2001). The teat orifice serves as the entrance to the udder and the sphincter forms the first line of defence in the mammary gland (Jones & Bailey 2009). The Furstenburg rosette consists of mucosal ridges starting at the internal orifice of the teat canal and has a protective leucocyte population (Reece, W., 2009). Sordillo, Doymaz, & Oliver, (1990), described the presence of immunoglobulin bearing leucocytes (Ig G1 and Ig G2) in the Furstsenburg rosettes in cows with *S.aureus* present in the udder.

In both farms the marked udder and teat damage present at the beginning of the study emphasised the poor condition of udder health in in both vaccinated and un-vaccinated groups. This posed a problem as cows with marked udder damage and positive bacterial culture on milk samples at the time of entrance had a high titre for Ig A in the milk.

5.2 *S. aureus* bacterial culture in milk samples during the study

The diagnosis of mastitis is complicated and the isolation of the causative organism remains the gold standard of diagnosis for clinical, sub clinical and chronic cases (Jones *et al*., 1998; Bagley 1997; Gronlund *et al.,* 2006; Petzer, 2001)

The sensitivity and specificity of bacterial culture of milk samples for the isolation of *S. aureus* are discussed in the literature review. It is generally accepted that repeated sampling improves reliability. (Buelow *et al,* .1996b; Sandholm & Sears, 1998 in Petzer 2001). The milk samples from cows in this study were cultured with monthly intervals. A single positive culture was used to classify cows as positive. Sampling was performed after udder palpation on an empty udder to minimize the dilution effect of large volumes of milk. Stringent aseptical procedures were applied during sample collection and therefore external contamination was reduced to the minimum. Any growth was

considered significant and all colonies were sub-cultured. All results were re-confirmed by a second laboratory.

For discussion purposes cows that cultured *S. aureus* from a milk sample are referred to as positive and cows that remained negative on culture are referred to as negative cows.

The McNemar test analyzes a dichotomous population with a twofold outcome after an intervention like for instance vaccination. (Bland 2000, Fleiss 1981) Data from the two farms are combined but vaccinated and un-vaccinated groups are not statistically compared.

The vaccinated group started with 86% negative cows. At the end of the study 74% cows were negative which represented cows that remained negative (89%) and those that became negative (11%). The incidence of 24% was statistically insignificant (p= 0.1317). Twenty percent of the positive cows entered the study as positive indicating the persistence of *S.aureus.*

The un-vaccinated group started with 78% negative cows. At the end of the study 59% cows were negative which represented cows that remained negative (86%) and those that became negative (14%). The incidence of 36% was statistically insignificant (p=0.0833). Forty percent of the positive cows entered the study as positive indicating the persistence of *S. aureus.*

It was demonstrated that the number of new cases were statistically insignificant in the vaccinated group (8/25) with a P value of 0.1317.

There were however at the same time an insignificant number of new cases in the un-vaccinated group (9/16) with a P value of 0.0833. One would have preferred a significant number of new cases in the un-vaccinated group with a P value of less than 0.05.

The number of new cases in the un-vaccinated group was higher than in the vaccinated group with a resultant higher risk factor of 36% opposed to the risk factor in the vaccinated group of 24%.

Reduction in severity and the number of new cases was shown for several commercial vaccines (Ruegg 2001; European Medicines Agency Science Medicines Health STARTVAC®). Similar findings were reported by Leitner *et al*., (2003b) for an autogenous vaccine.

5.3 Elisa Titre for IgA in milk samples

S. aureus can multiply within macrophages and can survive within encapsulated granulomas. This causes deep foci of infection that lead to the development of chronic carrier cows that continuously shed bacteria (Petzer, 2001). The first line of defence would thus be to prevent adhesion and penetration before the organism enters udder tissue. The role of IgG1 and IgG2 has been described for *S. aureus* mastitis vaccines by Nordhaug *et al.,* (1994). Immunoglobulin A is regarded as a non inflammatory immunoglobulin that acts primarily as a neutralizing antibody preventing the adhesion of bacteria to mucosal surfaces (Sordillo & Streicher 2002).

The lymphoid tissue in the Furstenburg rosette and vaginal mucosa may function as udder associated lymphoid tissue to elicit a regional mucosal IgA response. We attempted to enhance this response by targeting the regional lymph node via the parenteral route.

The high prevalence of *S. aureus* in the study population prohibited the comparison of IgA titres between vaccinated and un-vaccinated groups. The contagious nature of *S. aureus* leads to continual exposure of cows to the organism during milking and especially on zero-grazing systems. This was probably the reason for the high Ig A titres at the onset of the study, especially in cows that were in their third or fourth lactation. To effectively evaluate sero-conversion in vaccinated cows the study should be repeated with sero-negative cows in a controlled environment with minimal risk of cross contamination between cows.

Cows that were positive for *S. aureus* culture in milk also displayed high IgA titres. The iELISA could not differentiate between the IgA responses from natural infection and that from vaccination;

therefore the observed differences in titres between the groups are difficult to interpret. IgA response in milk after intra-mammary vaccination in cows has been demonstrated (Plat-Sinnige *et al.*, 2009).

5.4 Fate of cows

The initial group size of 90 cows was compromised by the large number of cows (n=18) that were eliminated either by selling or by slaughter and two animals were still pregnant at the end of the project. This reduced the statistical significance of the study as the number of cows that entered lactation were reduced (n= 70). Most of the cows left the herd before they entered lactation with five animals eliminated during lactation of which three were in the un-vaccinated group. The large percentage of cows that did not complete lactation was alarming and this was mostly due to pre-existing problems in the cow at dry-up. To overcome the weak point only healthy animals with no previous history of *S. aureus* infection should be used. These conditions are however extremely difficult to achieve in the field.

5.5 Conclusion

This study was performed as a field trial and was subjected to all the challenges and stressors as experienced by farmers and cows on a day to day basis. Adverse weather conditions with very high rainfall were experienced during the study and caused extreme muddy conditions, especially on farm 2.

Taking the above conditions as well as the lowered statistical power due to reduction in sample size into consideration, the lowered risk in the vaccinated group as compared to the un-vaccinated group should be interpreted with caution.

The new cases in the vaccinated group should be further typed to determine whether they were in fact included in the vaccine or not. To further improve the vaccine the degree of cross protection between strains as well as the duration of protection should be determined. This study emphasised the need for further research into the role of IgA and mucosal vaccination in the control of *S. aureus* mastitis in cows.

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