Incidence of *Staphylococcus* species in bovine milk; their antimicrobial sensitivity in selected antibiotics and *Usnea barbata* lichen extracts

By

Emrobowansan Monday IDAMOKORO

A Dissertation submitted in fulfilment of the requirements for the degree of Master of Science in Agriculture (Animal Science)

Department of Livestock and Pasture Science

Faculty of Science and Agriculture

University of Fort Hare

Together in Excellence

November 2013

Alice, South Africa

Supervised by:

Prof. P. J. Masika

Prof. V. Muchenje

Dr. E. Green

i

Incidence of *Staphylococcus* species in bovine milk; their antimicrobial sensitivity in selected antibiotics and *Usnea barbata* lichen extracts

By

Emrobowansan Monday IDAMOKORO

A Dissertation submitted in fulfilment of the requirements for the degree of Master of Science in Agriculture (Animal Science)

Approved as to style and content by:

_____   _____

Prof V Muchenje   **Dr E Green**

(Co-supervisor)   **(Co-supervisor)**

_____

Prof P Masika

(Main supervisor)

November 2013

Abstract

Incidence of *Staphylococcus* species in bovine milk; their antimicrobial sensitivity in selected antibiotics and *Usnea barbata* lichen extracts

By

Emrobowansan Monday IDAMOKORO

This study was done in order to assess the incidence of *Staphylococcus* species from milk of cows with subclinical mastitis and their antimicrobial sensitivity in some selected antibiotics and *Usnea barbata* lichen extracts. The study was conducted in two different commercial dairy farms. *Staphylococcus* species isolates were identified using several biochemical tests which included Gram's staining test, catalase test and oxidase test. A commercial API$^{®}$ staph kit (bioMerieux, France) was used to confirm the bacterial organisms to their species level. The antimicrobial sensitivity of individual species was determined according to the Clinical Laboratory Standard Institute (CLSI) for the selected antibiotics. Agar well diffusion method and the broth micro-dilution technique were used to determine the sensitivity of *Staphylococcus* species in *U. barbata* extracts. A total of 467 milk samples were screened for bacterial identification from the two farms. Fifteen different *Staphylococcus* species isolates were identified from all milk samples that were examined. The most frequently isolated species included *Staphylococcus xylosus* (54.34%), *Staphylococcus hominis* (24.78%), *Staphylococcus aureus* (16.38%), *Staphylococcus saprophyticus* (16.12%) and *Staphylococcus haemolyticus* (11.63%). Most *Staphylococcus* species were resistant to Penicillin (75.35%), Nalidixic acid (72.55%) and Ampicillin (63%). Furthermore, the bacterial sensitivity evaluation of *U. barbata* lichen extracted with methanol and ethyl-acetate against selected *Staphylococcus* species isolates showed 92.31% and 53.85% susceptibility, respectively. The minimum inhibitory concentration (MIC) of the methanol and ethyl-acetate extracts ranged between 0.0390 to 10 mg/ml. There was a relatively high incidence of

Staphylococcus species identified in milk of cows with subclinical mastitis from both farms. Conversely, *Staphylococcus* species isolates were resistant to antibiotics (mostly penicillin and ampicillin) commonly used in the farms. Furthermore, the study investigated the antimicrobial sensitivity of *U. barbata* extract *in-vitro* which may validate its use in traditional medicine for treatment of cows with mastitis.

Key words: Antibiotic resistance, *Staphylococcus* species, subclinical mastitis, susceptibility, traditional medicine.

List of abbreviations

ADH – Argenine DiHydrolase

ANOVA – Analysis of Variance

API – Analytical Profile Index

CLSI – Clinical Laboratory Standard Institute

CNS – Coagulase Negative Staphylococci

CPS - Coagulase Positive Staphylococci

DMSO – Dimethyl Sulphoxide

MIC – Minimum Inhibitory Concentration

NMC – National Mastitis Council

SAS – Statistical Analysis System

URE – Urease

WHO – World Health Organization

Table of Contents

List of tables

List of Figures

Acknowledgements

Chapter 1: Introduction

A major udder health problem of cows is mastitis disease which could lead to the inflammation of cow udder in severe cases (Awale *et al*., 2012). It is a livestock disease that is spread among dairy herds and currently known to cause a serious challenge in the dairy sector nationwide (Gill *et al*., 2006). Several micro-organisms of common bacteria, fungi, mycoplasmas and algae cause mastitis in cows (Batavani *et al*., 2007). Major mastitis causing organisms include *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus bovis* (Pitkala *et al*., 2004; Tenhagen *et al*., 2006). They make the milk produced by lactating cows to be high in bacteria count and of low quality (Barbano *et al*., 2006; Oliver, 2012). Cows with mastitis have reduced milk yield, which in turn reduces farmers' profits (Halasa *et al*., 2009; Ampe *et al*., 2012). The disease is one of the most expensive diseases that affect dairy farms all over the world (Dufour *et al*., 2012). The financial loss suffered by farmers as a result of mastitis disease alone could be up to an estimate of about 70% of all avoidable losses incurred during milk production (Sumathi *et al*., 2008).

Staphylococcus species are the bacteria that are frequently isolated from milk samples of cows with subclinical and clinical mastitis (Pitkala *et al*., 2004; Tenhagen *et al*., 2006). They are grouped as Coagulase Negative Staphylococci (CNS) and Coagulase Positive Staphylococci (CPS) and have been linked with the cause of both clinical and subclinical mastitis (De Viegher *et al*., 2003; Taponen *et al*., 2006). Common species of CNS include *Staphylococcus Chromogenes*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Staphylococus hyicus* and *Staphylococcus haemolyticus* (Thorberg *et al*., 2009) while examples of CPS include *Staphylococcus aureus* and *Staphylococcus hyicus* (Awale *et al*., 2012). Lately, CNS has evolved in dairy farms as an

emerging mastitis causing organism (Simojoki *et al*., 2011). According to Petzer *et al*. (2009), CNS is a significant mastitis causing organism in South Africa. They are often isolated from cow udder teats, skin, udder duct and milk of cows that show clinical and subclinical signs (De Viegher *et al*., 2003; Taponen *et al*., 2006).

Most cases of CNS infections are subclinical and they lead to high culling rates, cow replacement and reduction in farmers' profits due to poor quality of milk (Sandgren *et al*., 2008). Raw milk can be contaminated by infected animals during milking (Kousta *et al*., 2010). Poorly managed milking machines, improper farm practices during milking could also cause high risk of infections among cows by mastitis pathogens (Philip *et al*., 2011). Presently, most dairy farms are becoming antibiotic dependant so as to improve cow productivity (Walther *et al*., 2008; Mc Kenna, 2011). Mastitis disease in cows is one of the sole reasons for the use of antibiotics in dairy farms (Awandkar *et al*., 2009). Antibiotics are used as therapeutics, growth promoters and prophylactics in livestock production (Sawant *et al*., 2005). However, mastitis pathogens are developing resistance to antibiotic drugs (Febler *et al*., 2010). This is raising a serious alarm as the rate of resistance is increasing yearly in various countries of the world (WHO, 2000). The complex inter relationship among antibiotics, bacteria and the immediate surroundings of animals gave rise to the evolution and spread of antibiotic resistance in farms (Mc Dermott *et al*., 2002; Taponen and Pyorala, 2009).

Some of the antimicrobial agents used in the treatment of mastitis disease include beta-lactams, macrolides and lincosamides. Conversely, several *Staphylococcus* species including CPS and CNS isolated from bovine milk are currently becoming resistant to these antimicrobial agents (Gentilini *et al*., 2002; Pitkala *et al*., 2004; Luthje *et al*., 2006; Sawant *et*

al., 2009); thus, increasing the search for other effective options and/or antibiotics to control and treat cows with mastitis (Pieterse and Todorov, 2010). A lot of small scale farmers currently use other cheaper antibiotic agents (plants) as an alternative against the conventional antibiotic agents to treat their animals (Masika *et al*., 2000). Africa, as a continent is in the lead in the use of plants for treating both humans and animals (Ndip *et al*., 2007). One of the plants that have been investigated to be active against mastitis causing pathogens is *U. barbata* (Rankovic *et al*., 2012). *Usnea barbata* lichen, locally known as old man's beard are epiphytes that grow on trees, rocks and soils with low fertility (Vrablikova *et al*., 2006).

The *U. barbata* extracts have been shown to possess inhibitory activity against most Gram positive bacteria and some Gram negative bacteria (Madamombe and Afolayan, 2003; Rankovic *et al*., 2012). To our knowledge, information on the use of the lichen extracts against the selected *Staphylococcus* species in the current study is sparse. More so, Petzer *et al*. (2009) in their study reported that CNS together with other *Staphylococcus* species including *S. aureus* is increasingly becoming a significant mastitis causing pathogen in South Africa.

1.1.Justification

Udder health disease caused by mastitis pose a severe threat in the dairy sector (Bradley, 2002). There is an emergence of several *Staphylococcus species* especially CNS linked with the cause of mastitis on a daily basis in dairy farms all over the world. Increase in the rate of antimicrobial resistance by *Staphylococcus* species to antibiotic drugs is also becoming a huge global concern. Prevalence of *Staphylococcus* species bacteria among dairy herd in a farm, may lead to intra mammary infections among the herd, high culling rate and increase in

milk somatic cell counts (Barbano *et al.*, 2006). The study by Petzer *et al.* (2009) gave a general prevalence of *S. aureus* and CNS isolated in milk samples from dairy herds over a period of eleven years in South Africa. Yet, not much information about the incidence of CNS at the species level and their reactions to antibiotics has been reported in South Africa. There is need to identify the specific individual CNS (not as a group) in bovine milk and their antimicrobial sensitivity pattern to conventional antibiotics. Also, by extension it is essential to evaluate the sensitivity of the selected *Staphylococcus* species bacteria in *U. barbata* extracts. This is because the lichen is a commonly used medicinal plant for the treatment of mastitic cows by local farmers in the Eastern Cape Province of South Africa. The findings from the current study may help in getting a better understanding about the prevalence of the bacteria species in dairy farms and their response to antibiotics. Most dairy farmers in the Eastern Cape Province of South Africa do not engage in antimicrobial susceptibility testing as part of their routine practices. The result obtained from the current study may give useful information to farmers on the importance of antimicrobial susceptibility testing as part of their routine practices in their farms. In addition, the information on the *in-vitro* outcome of *U. barbata* inhibitory activities against some isolated *Staphylococcus* species could give a cheaper alternative as an antimicrobial agent in traditional medicine.

1.2. Objectives

The broad objective of the study was to isolate and identify different *Staphylococcus* species from bovine milk and to establish antimicrobial sensitivity pattern.

The specific objectives were to:

1. Determine the incidence of *Staphylococcus* species in bovine milk from two farms over a period of time.

2. Determine the antimicrobial sensitivity of some *Staphylococcus* species isolated from bovine milk; and

3. Validate the *in vitro* inhibitory activity of *U. barbata* extracts from methanol and ethyl-acetate against some selected bovine *Staphylococcus* species.

1.3 Hypothesis

1. There is no incidence of different *Staphylococcus* species in commercial dairy farms.

2. The antimicrobial sensitivity pattern of *Staphylococcus* species to conventional antibiotics is the same.

3. Extracts from *Usnea barbata* lichen do not inhibit the growth of *Staphylococcus* species *in-vitro*.

1.4 References

Ampe, B., Goethals, K., Laevens, H. and Duchateau, L. 2012. Investigating clustering in intervals-censored udder quarter infection times in dairy cows using a gamma frailty model. *Journal of Preventive Veterinary Medicine* **106:** 251-257.

Awale, M.M., Dudhatra, G.B., Kumar, A., Chauhan, B.N., Kamani, D.R., Modi, C.M., Patel, H.B. and Mody, S.K. 2012. Bovine Mastitis: A threat to Economy. *Open Access Scientific Reports*, 1: 295. doi: 10.4172/scientificreports.295.

Awandkar, S.P., Khode, N.V., Sardar, V.M. and Mendhe, M.S. 2009. Prevalence and Current Antibiogram Trend of Mastitis Agents in Udgir and its Visinity, Maharashtra State, India. *International Journal of Dairy Science* **4:** 117-122.

Barbano, D.M., Ma, Y. and Santos, M.V. 2006. Influence of raw milk on fluid milk shelf life. *Journal of Dairy Science* **89:** 15-19.

Batavani, R.V., Asri, S. and Naebzadeh, H. 2007. The effect of subclinical mastitis on milk composition in dairy cows. *Iranian Journal of Veterinary Research* **8:** 205-211.

Bradley, A.J. 2002. Bovine mastitis: An evolving disease. *The Veterinary Journal* **164:** 116-128.

De Vliegher, S., Laevens, H., Devriese, L.A., Opsomer, G., Leroy, J.L., Barkema, H.W. and de Kruif, A. 2003. Prepartum teat apex colonization with *Staphylococcus chromogenes* in dairy heifers is associated with low somatic cell count in early lactation. *Veterinary Microbiology* **92:** 245-252.

Dufour, S., Dohoo, I.R., Barkema, H.W., Descoteaux, L., De Veries, T.J., Reyher, K.K., Roy, J.P. and Scholl, D.T. 2012. Epidemiology of coagulase- negative staphylococci intramammary infection in dairy cattle and the effect of bacteriological culture misclassification. *Journal of Dairy Science* **95:** 3110-3124.

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

Gentilini, E., Denamiel, G., Betancor, A., Rebuelto, M., Fermepin, M.R. and De Torres, R.A. 2002. Antimicrobial Susceptibility of Coagulase-Negative Staphylococci Isolated from Bovine Mastitis in Argentina. *Journal of Dairy Science* **85:** 1913-1917.

Gill, J.J., Pacan, J.C., Carson, M.E., Leslie, K.E., Griffiths, M.W. and Sabour, P.M. 2006. Efficacy and Pharmacokinetics of Bacteriophage Therapy in Treatment of Subclinical *Staphylococcus aureus* Mastitis in Lactating Dairy Cattle. *Antimicrobial Agents and Chemotherapy* **50:** 2912- 2918.

Halasa, T., Nielen, M., De Roos, A.P.W., de Jong, G., Lam, T.J.G.M., Van werven, T. and Hogeveen, H. 2009. Production loss due to new subclinical mastitis in Dutch dairy cows estimated with a test-day model *Journal of Dairy Science* **92:** 599-606

Kousta, M., Mataragas, M., Skandamis, P. and Drosinos, E.H. 2010. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. *Journal of Food Control* **6:** 805-815.

Luthje, P. and Schwarz, S. 2006. Antimicrobial resistance of coagulase- negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. *Journal of Antimicrobial Chemotherapy* **57:** 966-969.

Madamombe, I.T. and Afolayan, A.J. 2003. Evaluation of Antimicrobial Activity of Extracts from South Africa *Usnea barbata*. *Pharmaceutical Biology* **41:** 199-202.

Masika, P.J., Van Averbeke, W. and Sonandi, A. 2000. Use of herbal remedies by small-scale farmers to treat livestock diseases in central Eastern Cape Province, South Africa. *Journal of South African Veterinary Association* **71:** 87-91.

Mc kenna, M. 2011. More MRSA in milk: A new strain in cows and humans. http://www.wiredscience/2011/06/mrsa-milk-new/. (Accessed 22 July, 2012).

McDermott, P.F., Zhao, S., Wagner, D.D., Simjee, S., Walker, R.D. and White, D.G. 2002. The food safety perspective of antibiotic resistance. *Journal of Animal Science and Biotechnology* **13:** 71-84.

Ndip, R.N., Tarkang, A.E.M., Mbullah, S.M., Luma, H.N., Malongue, A., Ndip, L.M., Nyongbela, K., Wirmum, C. and Efange, S.M.N. 2007. *In vitro* anti- *Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. *Journal of Ethinopharmacology* **114:** 452-457.

Oliver, S.P. 2012. Best management practices to reduced mastitis and improve milk quality www.extension.org/pages/22418. (Accessed 10 October, 2012).

Petzer, I.M., Karzis, J., Watermeyer, J.C., van der Schans, T.J. and van Reenen, R. 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. *Journal of the South African Veterinary Association* **80:**17-22.

Philip, S.R., Penny, M.A. and Collin, D. 2011. Cattle medicine. Manson Publication Limited. London, Great Britain. London.

Pieterse, R. and Todorov, S.D. 2010. Bacteriocins- Exploring Alternatives to Antibiotics in Mastitis Treatment. *Brazilian Journal of Microbiology* **41:** 542-562.

Pitkala, A., Haveri, M., Pyorala, S., Myllys, V. and Honkanen-Buzalski, T. 2004. Bovine mastitis in Finland 2001- Prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science* **87:** 2433-2441.

Rankovic, B., Kosanic, M., Stanojkovic, T., Vasiljevic, P. and Manojlovic, N. 2012. Biological Activities of *Toninia candida* and *Usnea barbata* Together with Their Norstictic Acid and Usnic Acid Constituents. *International Journal of Molecular Sciences* **13:** 14707- 14722.

Sandgren, C.H., Waller, K.P. and Emanuelson, U. 2008. Therapeutic effects of systemic or intra mammary antimicrobial treatment of bovine subclinical mastitis during lactation. *The Veterinary Journal* **175:** 108-117.

Sawant, A.A., Gillespie, B.E., Oliver, S.P. 2009. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. *Veterinary Microbiology* **134:** 73-81.

Sawant, A.A., Sordillo, L.M. and Jayarao, B.M. 2005. A survey on antibiotic usage in dairy herds in Pennsylvanian. *Journal of Dairy Science* **88:** 2991-2999.

Simojoki, H., Salomaki, T., Taponen, S., Livanainen, A. and Pyorala, S. 2011. Innate immune response in experimentally induced bovine intramammary infection with *Staphylococcus simulans* and *S. epidermidis*, *Veterinary Research* **42:** 49-58.

Sumathi, B.R., Veeregowda, B.M. and Gomes, A.R. 2008. Prevalence and antibiogram profile of bacterial Isolations from clinical bovine mastitis. *Veterinary World* **1:** 237-238.

Taponen, S. and Pyorala, S. 2009. Coagulase negative staphylococci as cause of bovine mastitis- not so different from *Staphylococcus aureus*? *Veterinary Microbiology* **134:** 29-36.

Taponen, S., Simojoki, H., Haveri, M., Larsen, H.D. and Pyorala, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase- negative staphylococci identified with API or AFLP. *Veterinary Microbiology* **115:** 199-207.

Tenhagen, B-A., Koster, G., Wallman, J. and Heuwieser, W. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *Journal of Dairy Science* **89:** 2542-2551.

Thorberg, B.M., Danielsson-Tham, M.L., Emaneulson, U. and Waller, K.P. 2009. Bovine subclinical mastitis caused by different types of coagulase- negative staphylococci. *Journal of Dairy Science* **92:** 4962-4970.

Vrablikova, H., McEvoy, M., Solhaug, K.A., Bartak, M., Guaslaa, Y. 2006. Annual variation in photoacclimation and photoprotection of the photobiont in the foliose lichen *Xanthoria parietina. Journal of Photochemistry and Photobiology B: Biology* **83:** 151-162.

Walther, C., Rossano, A., Thomann, A. and Perreten, V. 2008. Antimicrobial resistance in lactococcus species from bovine milk: Presence of a matured multi-drug transporter mdt (A) gene in susceptible lactococcus garvieae strains. *Veterinary Microbiology* **131:** 348-357.

World Health organization, 2000. Overcoming antimicrobial resistance. World Health Organization report on infectious diseases 2000. http://www.who.int/infectious-disease-report/2000/index.html. (Accessed 22 January 2013).

Chapter 2: Literature review

2.1. Introduction

Mastitis disease in dairy herds negatively affects quality of milk (Barbano *et al*., 2006). High incidence rate of mastitis hampers the welfare of cows (Kemp *et al*., 2008; Hogeveen *et al*., 2011; Sundrum, 2012). Apart from *Staphylococcus aureus* several other staphylococci are reported to cause mastitis. In many countries globally, CNS have gained recognition (Pyorala and Taponen, 2009). This is because they cause mastitis in cows (Taponen *et al*., 2007). They are often isolated from milk of cows causing intra mammary infection (Pyorala and Taponen, 2009). The use of antimicrobials for the treatment of *Staphylococcus* species has been reported (Moon *et al*., 2007; Bengtsson *et al*., 2009; Frey *et al*; 2013). Despite this, they have also been several instances of these bacteria species becoming resistant to the antimicrobials used against them (Taponen *et al*., 2009).

In the case of CNS, they are often treated as a group even though they vary in both their phenotypic and genotypic characteristics (Layer *et al*., 2007; Onni *et al*., 2010). For easy identification of the different species of CNS, several methods including API staph 32 ID and the Staph Zym test have been adopted. The prevalence at the species level is necessary so as to ascertain the impact that they play in causing mastitis in dairy farms (Pyorala and Taponen, 2009). This chapter gives a review of the incidence of *Staphylococcus* species (especially CNS) in cows with subclinical mastitis and their antimicrobial susceptibility pattern in antibiotics.

2.2. *Staphylococcus* species *as mastitis pathogens in dairy farms*

The issue of mastitis is a serious challenge despite strategies put in place to control the prevalence in dairy farm environment. The resultant effect of mastitis infection is poor performance of cows in terms of milk production, milk quality and increased treatment cost of affected cows (Hunderra *et al.*, 2005). Mastitis is subclinical in most cases and it causes udder health problems. Of late, CNS has been shown to cause subclinical mastitis and in some other cases they cause a mild form of clinical mastitis (Taponen *et al.*, 2009). Their involvement in causing udder health problems is a concern to farmers, veterinarians and researchers all over the world.

Some bacteria pathogens are said to be major causes of mastitis. Others are referred to as minor pathogens because they have insignificant or no impact in causing mastitis in cows. Among bacteria pathogens grouped as minor pathogens is CNS. But recently, several reports have indicated that they are involved in mastitis infection (Pitkala *et al.*, 2004; Rajala-Schultz *et al.*, 2004). *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus chromogenes*, *Staphylococcus xylosus* and *Staphylococcus haemolyticus* are some examples of CNS pathogens (Aarestrup *et al.*, 1999; Taponen *et al.*, 2006). Major mastitis pathogens include *Streptococci agalactiae*, *Streptococcus dysgalactiae*, *Escherichia coli*, *Streptococcus uberius* and *Staphylococcus aureus* (Petersson-Wolfe *et al.*, 2010; Philip *et al.*, 2011). Factors such as mammary gland physiology, health of cow, management practices, cow immunity and dairy farm environment have been linked with the incidence of mastitis causing organisms (Waage *et al.*, 2000; Zadoks *et al.*, 2002; Oviedo-Boyso *et al.*, 2007).

2.3. Species of CNS: infection pathogenesis and prevalence

Staphylococcus species is a group of bacteria that is classified based on their ability to coagulate blood plasma. They are grouped as coagulase positive staphylococci (CPS) on one hand and coagulase negative staphylococci (CNS) on the other hand. An estimate of 50 *Staphylococcus* species and subspecies has been characterized under this group (Pyorala and Taponen, 2009). Mammary gland of cows becomes swollen in critical cases as a result of infection caused by bacterial pathogens that are associated with mastitis (Dufour *et al*., 2012). Though, CNS are indicted to be pathogens of less importance in bovine mastitis, they play a relevant role in intra mammary infection leading to mastitis in cows (Pyorala and Taponen, 2009). Intra mammary infection is caused mostly by bacteria including CNS found in subclinical mastitis (Djabri *et al*., 2002). Isolation of CNS from cows with subclinical and clinical mastitis has been reported (Jorun, 1991; Myllvs, 1995; Tenhagen *et al*., 2006; Koivula *et al*., 2007).

The ability of CNS to cause mastitis warrant that they should be studied as major mastitis causing pathogens and not just as minor pathogens of cow skin micro flora (Taponen *et al*., 2009). According to Andersen *et al*. (2010), researchers are yet to agree on the right definition for intra mammary infection caused by different bacteria on cows that result in mastitis. Information on the effect of CNS in relation to mastitis is rare (Thorberg, 2008).

Infection of cows by CNS to cause mastitis is usually at different stages of lactation. Primiparous cows are infected with CNS at their early stage of lactation whereas multiparous cows are infected at their late stage of lactation (Grohn *et al*., 2004; Taponen *et al*., 2007). The CNS species that infect heifers are not the same as those that infect cows; infection of cows by CNS varies based on their age (Pyorala and Taponen, 2009). Among the frequently

isolated CNS species from mastitis are *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus hyicus*, *Staphylococcus xylosus*, *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* (Trinidad *et al*., 1990; Matthews *et al*., 1992; Thorberg *et al*., 2006; Frey *et al*., 2013). Persistent intra mammary infections by CNS continue for a long period of time throughout lactation when cows are not detected and treated (Aarestrup *et al*., 1999; Oliver *et al*., 2003; Gillespie *et al*., 2009).

Mastitis prevalence and spread caused by CNS varies from country to country. Change in season also contributes to the proportion of CNS pathogens that causes mastitis (Makovec and Ruegg, 2003; Osteras *et al*., 2006). Type of housing system, level of farm production, parity, lactation stage, variation in sampling techniques and method of species identification are other factors that influence the prevalence of mastitis caused by *Staphylococcus* species (Thorberg, 2008). In most cases CNS occurs more in subclinical mastitis than in clinical mastitis (Pyorala and Taponen, 2009). Lack of awareness by farmers on the potential of CNS to cause mastitis may be reason for mastitis problem; and information on the prevalence of CNS is helpful in the control of mastitis spread caused by CNS (Pyorala and Taponen, 2009).

Table 2. 1 Proportion of CNS isolated from subclinical mastitis in different countries as reported by literature

Country of origin	%	Source
Germany	35	Tenhagen *et al*.(2006)
USA (Tennessee)	28(Herds with high SCC) 12-41 (Herd prevalence)	Roberson *et al*.(2006)
Netherland	6	Poelarends *et al*. (2001)
Estonia	16	Haltia *et al*. (2006)
Canada and USA	15	Dingwell *et al*. (2004)
France	13.7	Botrel *et al*. (2010)
Finland	24-50	Pitkala *et al*. (2004); Koivula *et al*. (2007)
Norway	16	Osteras *et al*. (2006)
South Africa	61	Petzer *et al*. (2009)

SCC= Somatic cell count

Isolation of CNS from cows with clinical mastitis has also been reported in some countries. Seasons determine the prevalence of CNS mastitis in different countries. Study by Koivula *et al*. (2007) in Finland and Osteras *et al*. (2006) in Norway indicated that there is seasonal influence on the occurrence of CNS mastitis in cows. In the case of intra mammary infection in dairy herds, there is higher prevalence of CNS in heifers when compared with older cows (Sampimon *et al*., 2009a). The knowledge of CNS distribution within specific regions and their species-specific prevalence will help to combat the issue of mastitis related to CNS.

Table 2. 2 Proportion of CNS isolated from clinical mastitis from different countries as reported by literature

Country of origin	%	source
Wisconsin (USA)	17.5	Makovec and Ruegg, (2003)
Israel	9	Shpigel *et al*. (1998)
Finland	18	Koivula *et al*., (2007)

2.4. Seasonal variation of CNS pathogen

In the dairy industry the rate of mastitis incidence in a dairy herd is one of the parameters used for determining udder health (Olde-Riekerink *et al*., 2007). Individual cow and bulk tank somatic cell counts are other parameters used to determine udder health (Olde-Riekerink *et al*., 2007). Incidence rate of mastitis is affected by season (Morse *et al*., 1988; Olde- Riekerink *et al*., 2007). Cows are more prone to high incidences of clinical mastitis in the fall (December) than in summer (Olde-Riekerink *et al*., 2007). Inter-play of specific mastitis disease causing organisms in cows in relation to season also exist (Osteras *et al*., 2006). High prevalence of *Streptococcus dysgalactiae* and CNS was reported in winter than in other seasons (Osteras *et al*., 2006). According to Hogan *et al*. (1989), Makovec and Ruegg (2003) the incidence rate of mastitis was high for Streptococci and coliforms in summer. Conversely, mastitis prevalence caused by *Staphylococcus aureus* and *Streptococcus uberis* was high in summer compared to other seasons (Osteras *et al*., 2006). The adaptive and persistent nature of some *Staphylococcus* species to the mammary glands of cows is a reason for variation in the incidence of the bacteria in milk (Piesssens *et al*., 2012).

2.5. Colonization of CNS species on cow udder quarters and their protective role

Coagulase negative staphylococci (CNS) are minor pathogens having little or no impact in causing mastitis in cows (Pyorala and Taponen, 2009). Recent studies indicated that some CNS species show inhibitory attributes against major mastitis causing pathogens thereby acting to protect to cow udders against potential mastitis causing pathogenic species (Reyher *et al*., 2012a). *Staphylococcus chromogenes* is an example of CNS that inhibits other major mastitis causing organisms from infecting the udder of cows (De Vliegher *et al*., 2004). According to some literatures, there are contradictory reports as to whether CNS actually play a protective role or increase the risk of infection on udder quarters of cows (Green *et al*., 2002; Reyher *et al*., 2012b). In a report by Zadoks *et al*. (2001), CNS species do not show a protective effect against udder quarters of cows and they also do not expose them to risk of infection by major mastitis pathogens. Matthews *et al*. (1990) and Lam *et al*. (1997) indicated that cow quarters that were infected with CNS are likely to show resistance to any further infections by *Staphylococcus aureus* and Streptococci species.

An *in-vitro* study carried out by De Vliegher *et al*. (2004) showed the inhibition of *Staphylococcus aureus*, *Streptococcus uberis*, *and Streptococcus dysgalactiae* by *Staphylococcus chromogenes*. Dos Santos Nascimento *et al*. (2005) also reported the inhibition of *Staphylococcus agalactiae* a major mastitis pathogen by some strains of CNS. The production of antibacterial peptides by CNS is a possible mechanism involved in the inhibition of major mastitis (Dos Santos Nascimento *et al*., 2005; Sawant *et al*., 2009). In the study of De Vliegher *et al*. (2004), *Staphylococcus chromogenes* secreted substances that inhibit the growth of *Staphylococcus aureus* and other streptococci. Contradicting studies by Compton *et al*. (2007) and Parker *et al*. (2007) indicated that CNS do not show any protective effect on udder quarters of cows against major mastitis. In order to properly understand the

protective and risk factor effect of CNS bacteria on udder quarter of cows factors such as age, lactation stage, immunity level, anatomy of cows are to be considered (Reyher *et al* ., 2012b).

2.6. Antimicrobial resistance by bovine CNS mastitis

There is high pressure on farmers to increase individual cow productivity. As a result of this, farmers have become more reliant on antibiotics (McKenna, 2011). Antibiotics are used for treatment and prevention of bovine mastitis and metritis (Walther *et al*., 2008). They are also used for promoting growth in animals, as therapeutics and prophylactics in livestock production (Sawant *et al*., 2005). Some of the antibiotics used in farms include Penicillin, Oxacillin, Cefazoloin, Clindamycin, Tobramycin, Ciprofloxacin, Tetracycline, Erythromycin and beta lactams (Sawant *et al*., 2005; Gao *et al*., 2012). Recent studies show specific differences in resistance to antibiotics by different CNS at their species level (Luthje and Schwardz, 2006; Waller *et al*., 2011; Saini *et al*., 2012). In a study by Sawant *et al*. (2009), *Staphylococcus epidermidis* was resistant against Erythromycin, Methicillin and Pirlimycin but other CNS species including *Staphylococcus simulans*, *Staphylococcus hyicus* and *Staphylococcus chromogenes* were susceptible. Resistance against Ampicillin by *Staphylococcus hyicus*, *Staphylococcus epidermidis* and *Staphylococcus chromogenes* was also indicated by Sawant *et al*. (2009). According to Pitkala *et al*. (2004) the resistance pattern of some antibiotics for *Staphylococcus aureus* and CNS including Erythromycin, Streptomycin, Gentamycin and Oxacillin was between 0 -5.1% and 0-9.96% respectively. In that study, resistance to Penicillin was high for *Staphylococcus aureus* and CNS (52.1% and 32% respectively).

The prevalence of antimicrobial resistant CNS bacteria such as *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus chromogenes* and *Staphylococcus simulans*

against penicillin was 70%, 33%, 18% and 0%, respectively (Sampimon, 2009). Resistance against Oxacillin, Tetracycline, Sulfonamides, Trimethoprim, Clindamycin, Methicillin and Kanamycin by CNS has also been indicated (Gentilini *et al*., 2002; Moon *et al*., 2007; Bengtsson *et al*., 2009; Frey *et al*., 2013). Apart from dairy cows, bacteria in raw milk of other livestock animals have been reported to show resistance to antibiotics. Lollai *et al*. (2008) indicated resistance in isolates of milk samples gotten from sheep against some antibiotics by CNS. Resistance to penicillin in sheep is lower compared to cattle (Lollai *et al*., 2008). Factors such as incidence of disease, cost of treatment, type of antibiotics, farm management practices and level of education influence the use of antibiotics for treatment of mastitis (Grave *et al*., 1999; Sawant *et al*., 2005). The continual use of antibiotics in livestock production has led to increase in resistance to several antibiotics by bacteria. When a brand of antibiotic is used for a long period without prescriptions by a trained expert or veterinarian, resistant to antibiotics by bacteria may result (Carlet *et al*., 2012). Other causes of resistance to antibiotics by bacteria also include the indiscriminate use of antibiotics for treatment of cows and inappropriate dosage of the antibiotic drugs to animals (Alanis, 2005; Rusell and Houlihan, 2006).

2.7. Virulence characteristics of CNS

The role CNS play in the infection of cow mammary glands cannot be over emphasized. Though, antibiotics have been used to treat cows that show mastitis and persistent intra mammary infections caused by CNS species (Taponen *et al*., 2006; Simojoki *et al*., 2009); treatment has always been done as a group. There is difference in the virulence determinates of CNS. In most cases CNS are often not identified at the species level but as a group. This is because the diagnostic relevance of CNS species in dairy management has not yet been critically looked into (Zadoks and Watts, 2009).

It is essential to identify mastitis pathogens at the species level including CNS (Rossito *et al*., 2002; Pitkala *et al*., 2004). Mastitis causing pathogens possess different levels of pathogenicity and virulence characteristics (Zhang *et al*., 2000; Waller *et al*., 2011). According to Zhang and Maddox. (2000) the severity of infection caused by *Staphylococcus chromogenes* in cows is much higher than other CNS. *Staphylococcus chromogenes* known as a minor mastitis pathogen can cause as much damage to cow mammary glands when compared to *Staphylococcus aureus* (Myllys *et al*., 1994). Information on species-specific differences in terms of their virulence characteristic will help in effective mastitis control programmes and management on dairy farms.

2.8. Identifying CNS from milk samples

Coagulase negative staphylococci are a group of *Staphylococcus* species that are often isolated from milk of cows with clinical mastitis, subclinical mastitis and even in normal milk. They are known to be pathogens that are becoming dominant in the cause of bovine mastitis. About 39 different species of CNS have been characterised. Identification of CNS in most cases is treated as a group and not as individual species. Recently it was reported that different species of CNS exhibit different virulence characteristics (Zhang and Maddox, 2000; Waller *et al*., 2011). Identifying CNS as a group may not be sufficient in effective therapy and mastitis control programmes.

The standard for identifying CNS at the species level is the use of conventional biochemical tests. This includes growth of species in various prepared media, morphology of species in colonies, gram staining, catalase production and coagulase test among others. The use of conventional biochemical tests is demanding, expensive and time consuming (Couto *et al*.,

2001). Several commercial biochemical kits have been developed to identify CNS at the species level phenotypically. Staph-zym system, ID 32 staph test and ATB 32 staph differentiation system are some commercial biochemical test kits that have been used to identify CNS at the species level (Deinhofer and Pernthaner, 1995; Thorberg and Brandstrom, 2000; Capurro *et al*., 2009). The use of ID 32 staph test for identifying CNS is efficient and also cheap when compared to staph-zym test (Thorberg and Brandstrom, 2000; Sampimon *et al*., 2009b). There is limitation in the use of commercial biochemical kits for identification of all species of CNS from animal source because the kits were originally designed to identify CNS in humans (Bes *et al*., 2000). The use of molecular method for identifying CNS species still remains the best to date (Zadoks and Watts, 2009).

2.9. Risk management practices and spread of CNS species during milking

Coagulase negative staphylococci pose a threat to the mammary gland in a poorly managed milking parlour and farm environment (Hundera *et al*., 2005). They are contagious and can persist in mammary glands and cow teat canal of infected cows thereby increasing the risk of infection to uninfected cows during milking (Peterrson-Wolfe *et al*., 2010). Good management practices and hygiene in the milking parlour are vital in preventing the spread of bacteria during milking (Yuen *et al*., 2012). The risk of infection for contagious and environmental mastitis pathogens including CNS increases when the milking machine is poorly managed (Philip *et al*., 2011). Unethical practices in the milking parlour expose cows to mastitis causing organism (Yuen *et al*., 2012). Some practices such as using the same towel to clean cows' udders and improper drying of cow teats during milking of cows will increase the spread of mastitis causing organisms (Jones, 2010).

2.9.1. Managing dairy cows against possible spread of bacteria in the milking parlour

Proper cleaning of cow teats with disinfectants before milking, dipping of teats with chemicals, proper hygiene, culling of chronically infected cows and effective cow therapy are practices employed to reduce the load of mastitis causing organisms around cow udder during milking (Barkema *et al*., 2006). In a standard dairy farm according to Food standards agency Scotland (2007), proper washing of hands before and during the milking process, wearing of clean clothes by milking operators and the use of physically healthy milking personnel should be maintained when milking cows. This helps to cub the possible spread of mastitis causing organism from one cow to another and also prevent milk from bacterial contamination.

According to Dufour *et al*. (2012) wearing of gloves by milking personnel during the milking process reduces intra mammary infection by *Staphylococcus aureus*. Regular cleaning and sanitation of milking equipment, proper cleaning of vacuum system, clean milking environment, right use of cleaners and sanitizer during milking prevents the contamination of raw milk with bacteria (NIANR, 2006). Fore stripping of cows and regular screening of fore milk samples before attaching milking clusters to udders are also effective measures used to reduce the spread of bacteria during milking (Oliver, 2012).

2.10 References

Aarestrup, F.M., Larsen, H.D. and Jensen, N.E. 1999. Characterization of *Staphylococcus simulans* strains isolated from cases of bovine mastitis. *Veterinary Microbiology* **66**: 165-170.

Alanis, A.J. 2005. Resistance to Antibiotics: Are we in the Post-Antibiotic Era? *Archives of Medical Research* **36**: 697-705.

Andersen, S., Dohoo, I.R., Olde-Riekerink, R., Stryhn, H. and Mastitis research worker's conference. 2010. Diagnosing intra-mammary infections: Evaluating expert opinions on the definition of intra mammary infection using conjoint analysis. *Journal of Dairy Science* **93**: 2966-2975.

Barbano, D.M., Ma, Y. and Santos, M.V. 2006. Influence of raw milk quality on fluid milk shelf life. *Journal of Dairy Science* **89**: 15-19.

Barkema, H.W., Schukken, Y.H. and 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.

Bengtsson, B., Unnerstad, H.E., Ekman, T., Artursson, K., Nilsson-ost, M. and Waller, K.P. 2009. Antimicrobial susceptibility of udder pathogens from cases of acute clinical mastitis in dairy cows. *Veterinary Microbiology* **136**: 142-149.

Bes, M., Guerin-Faublee, V., Meugnier, H., Etienne, J. and Freney, J. 2000. Improvement of the identification of staphylococci isolated from bovine mammary infections using molecular method. *Veterinary Microbiology* **71**: 287-294.

Botrel, M.A., Haenni, M., Morignat, E., Sulpice, P., Madec, J.Y. and Calavas, D. 2010. Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhone-Alpes, France. *Foodborne Pathogens and Disease* **7**: 479-487.

Capurro, A., Arturrsson, K., Persson Waller, K., Bengtsson, B., Ericsson-Unnerstad, H. and Aspan, A. 2009. Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and *tuf* gene sequence-based genotyping for species- level identification of coagulase- negative staphylococci isolated from cases of bovine mastitis. *Veterinary Microbiology* **134**: 327-333.

Carlet, J., Jarlier, V., Herbarth, S., Voss, A., Goossens, H., Pittet, D. and the participants of the 3ʳᵈ World Health Care-Associated Infections Forum. 2012. *Antimicrobial Resistance and Infection Control* 1:11 doi: 10.1186/ 2047-2994.

Compton, C.W., Heuer, C., Parker, K. and Mc Dougall, S. 2007. Risk factors for peripartum mastitis in pasture grazed dairy heifers. *Journal of Dairy Science* **90**: 207-218.

Couto, I., Pereira, S., Miragaia, M., Sanches, I.S. and de Lencastre, H. 2001. Identification of clinical staphylococcal isolates from humans by internal transcribed spacer PCR. *Journal of Clinical Microbiology* **39**: 3099-3103.

Deinhofer, M. and Pernthaner, A. 1995. *Staphylococcus Spp*. as mastitis-related pathogens in goat milk. *Veterinary Microbiology* **43**: 161-166.

De Vliegher, S., Opsomer, G., Vanrolleghem, A., Devriese, L.A., Sampimon, O.C., Sol, J., Barkema, H.W., Haesebrouck, F. and de Kruif, A. 2004. In vitro growth inhibition of major mastitis pathogens by *Staphylococcus chromogenes* originating from teat apices of dairy heifers. *Veterinary Microbiology* **101**: 215-221.

Dingwell, R.T., Leslie, K.E., Schukken, Y.H., Sargeant, J.M., Timms, L.L., Duffield, T.F., Keefe, G.P., Kelton, D.F., Lissemore, K.D. and Conklin, J. 2004. Association of cows and quarter- level factors at drying-off with new intra mammary infections during the dry period. *Journal of Veterinary Medicine* **63**: 75-89.

Djabri, B., Bareille, N., Beaudeau, F. and Seegers, H. 2002. Quarter milk somatic cell count in infected dairy cows: A meta-analysis. *Journal of Veterinary Research* **33**: 335-357.

Dos Santos Nascimento, J., Fagundes, P.C., de Paiva Brito, M.A., dos Santos, K.R. and do Carmode Freire Bastos, M. 2005. Production of bacteriocins by coagulase-negative staphylococci involved in bovine mastitis. *Veterinary Microbiology* **106**: 61-71.

Dufour, S., Dohoo, I.R., Barkema, H.W., Descoteaux, I., De Vries, T.J., Reyher, K.K., Roy, J.P. and Scholl, D.T. 2012. Manageable risk factors associated with lactational incidence elimination, and prevalence of *Staphylococcus aureus* intra mammary infections in dairy cows. *Journal of Dairy Science* **95**: 1283-1300.

Food Standards Agency Scotland. 2007. Milk hygiene on the dairy farm. http//assurance.redtractor.org.uk. (Accessed 10 October 2012).

Frey, Y., Rodriguez, J.P., Thomann, A., Schwendener, S. and Perrenten, V. 2013. Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis. *Journal of Dairy Science* **96**: 1-11.

Gao, J., Ferreri, M., Yu, F., Liu, X., Chen, L., Su, J. and Han, B. 2012. Molecular types and antibiotic resistance of *Staphylococcus aureus* isolates from bovine mastitis in a single herd in China. *The Veterinary Journal* **192**: 550-552.

Gentilini, E., Denamiel, G., Betancor, A., Rebuelto, M., Rodriguez, M. and De Torrest, R.A. 2002. Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina. *Journal of Dairy Science* **85**: 1913-1917.

Gillespie, B.E., Headrick, S.I., Boonyayatra, S. and Oliver, S.P. 2009. Prevalence of coagulase-negative *staphylococcus* species from three dairy research herds. *Veterinary Microbiology* **134**: 65-72.

Grave, K., Greko, C., Nilsson, L., Odensvik, K., Mork., T. and Ronning, M. 1999. The usage of veterinary antibacterial drugs of mastitis in cattle in Norway and Sweden during 1990-1997. *Preventive Veterinary Medicine* **42**: 45-55.

Green, M.J., Green, L.E., Medley, G.F., Schukken, Y.H. and Bradley, A.J. 2002. Influence of dry period bacterial intra mammary infection on clinical mastitis in dairy cows. *Journal of Dairy Science* **85**: 2589-2599.

Grohn, Y.T., Wilson, D.J., Gonzalez, R.N., Hertl, J.A., Schutte, H., Bennett, G. and Schukken, Y.H. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. *Journal of Dairy Science* **87**: 3358-3374.

Haltia, L., Honkanen-Buzalski, T., Spiridonova, I., Olkonen, A. and Myllys, V. 2006. A study of bovine mastitis, milking procedures and management practices on 25 Estonian dairy herds. *Acta Veterinaria Scandinavica* **48**: 22-27.

Hogan, J.S., Smith, K.L., Hoblet, K.H., Schoenberger, P.S., Todhunter, D.A., Hueston, W.D., Pritchard, D.E., Bowman, G.L., Heider, L.E. and Brockett, B.L. 1989. Field survey of clinical mastitis in cow somatic cell count herds. *Journal of Dairy Science* **72**: 1547-1556.

Hogeveen, H., Huijps, K. and Lam, T.J.G.M. 2011. Economic aspects of mastitis: New developments. *New Zealand Veterinary Journal* **59**:16-23.

Hunderra, S., Ademe, Z. and Sintayetu, A. 2005. Dairy cattle mastitis in and around Sebeta, Ethiopia. *International Journal of Applied Research in Veterinary Medicine* **3**: 332-338.

Jones, G.M. 2010. The role of milking equipment in mastitis. www.extension.org (Accessed 10 October 2010).

Jorun, J. 1991. Classification of coagulase negative staphylococci isolated from bovine clinical and subclinical mastitis. *Veterinary Microbiology* **27**: 151-158.

Kemp, M.H., Nolan, A.M., Cripps, P.J. and Fitzpatrick, J.L. 2008. Animal-based measurements of the severity of mastitis in dairy cows. *Journal of Veterinary Record* **63**: 175-179.

Koivula, M., Pitkala, A., Pyorala, S. and Mantysaari, E. 2007. Distribution of bacteria and seasonal and regional effects in a new data base for mastitis pathogens in Finland. *Acta Agricultural Scandinavica*, section A- Animal Science **57**: 89-96.

Lam, T.J.G.M., Schukken, Y.H., van Vliet, J.H., Grommers, F.J., Tielen, M.J.M. and Brand, A. 1997. Effect of natural infection with minor pathogens on susceptibility to natural infection with major pathogens in bovine mammary gland. *American Journal of Veterinary Research* **58**: 17-22.

Layer, F., Ghebremedhin, B., Konig, W. And Konig, B. 2007. Differentiation of *Staphylococcus* spp. by terminal-restriction fragment length polymorphism analysis of glyceraldehydes-3-phosphate dehydrogenase-encoding gene. *Journal of Microbiological Methods* **70**: 542-549.

Lollai, S.A., Ziccheddu, M., Di Mauro, C., Manunta, D., Nudda, A. and Leori, G. 2008. Profile and evolution of antimicrobial resistance of ovine mastitis. *Small Ruminant Research* **74**: 249-254.

Luthje, P. and Schwardz, S. 2006. Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. *Journal of Antimicrobial Chemotherapy* **57**: 966-969.

Makovec, J.A. and Ruegg, P.L. 2003. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *Journal of Dairy Science* **86**: 3466-3472.

Mattews, K.R., Harmon, R.J. and Langlois, B.E. 1992. Prevalence of *Staphylococcus* species during the periparturient period in primiparous and multiparous cows. *Journal of Dairy Science* **75**: 1835-1839.

Mattews, K.R., Harmon, R.J. and Smith, B.A. 1990. Protective effect of *Staphylococcus aureus* infection in the lactating bovine mammary gland. *Journal of Dairy Science* **73**: 3457-3462.

McKenna, M. 2011. More MRSA in milk: A new strain in cows and humans. http://www.wired.com/wiredscience/2011/06/mrsa-milk-new/. (Accessed 22 July 2012).

Moon, J.S., Lee, A.R., Kang, H.M., Lee, E.S., Kim, M.N., Paik, Y.H., Park, Y.H., Joo, Y.S. and Koo, H.C. 2007. Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. *Journal of Dairy Science* **90**: 1176-1185.

Morse, D., De Lorenzo, M.A., Wilcox, C.J., Collier, R.J., Natzke, R.P. and Bray, D.R. 1988. Climatic effects on occurrence of clinical mastitis. *Journal of Dairy Science* **71**: 848-853.

Myllys, V., Honkanen-Buzladki, T., Virtanen, H., Pyorala, S. and Muller, H.P. 1994. Effect of abrasion teat orifice epithelium on development of bovine staphylococcal mastitis. *Journal of Dairy Science* **77**: 446-452.

Myllys, V. 1995. Staphylococci in heifer mastitis before and after parturition. *Journal of Dairy Research* **62**: 51-60.

Nebraska-Lincoln Extension Institute of Agriculture and Natural Resources (NIANR). 2006. Producing milk with low bacteria count. http//elkhorn.unl.edu/epublic/pages. (Accessed 20 September 2012).

Osteras, O., Solverod, L. and Reksen, O. 2006. Milk culture results in a large Norwegian survey- effects of season, parity, days in milk, resistance, and clustering. *Journal of Dairy Science* **89**: 2542-2551.

Olde- Riekerink, R.G.M., Barkema, H.W. and Stryn, H. 2007. The effect of season on somatic cell count and incidence of clinical mastitis. *Journal of Dairy Science* **90**: 1704- 1715

Oliver, S.P., Lewis, M.J., Gillespie, B.E., Dowlen, H.H., Jaenick, E.C. and Roberts, R.K. 2003. Prepartum antibiotic treatment of heifers: milk production, milk quality and economic benefit. *Journal of Dairy Science* **86**: 1187-1193.

Oliver, S.P. 2012. Best management practices to reduced mastitis and improve milk quality. www.extension.org/pages/22418 . (Accessed 10 October 2012).

Onni, T., Sanna, G., Cubeddu, G.P. Marogna, G., Lollai, S., Leori, G. And Tola, S. 2010. Identification of coagulase-negative staphylococci isolated from ovine milk samples by PCR- RFLP of 16S rRNA and gap genes. *Veterinary Microbiology* **144**: 347-352.

Oviedo-Boyso, J., Valdez-Alarcon, J.J., Cajero-Juarez, M., Ochoa- Zarzosa, A., Lopez-meza, J.E., Bravo-Patino, A. and Baizabal-Aguirre, V.M. 2007. Innate Immune response of bovine mammary gland to pathogenic bacteria responsibility for mastitis. *Journal of Infection* **54**: 399-409.

Parker, K.I., Compton, C., Anniss, F.M., Weir, A., Heuer, C. and Mc Dougall, S. 2007. Subclinical and clinical mastitis in heifers following the use of a teat sealant pre-calving. *Journal of Dairy Science* **90**: 207-218.

Peterrson-Wolfe, C.S., Mullarky, I.K. and Jones, G.M. 2010. A review of *Staphylococcus aureus* mastitis: causes, detection and control. Virginia Cooperative Extension USA. 229-404.

Petzer, I.M., Karzis, J., Watermeyer, J.C., van der Schans, T.J. and van Reenen, R. 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. *Journal of the South African Veterinary Association* **80**: 17-22.

Philip, S.R., Penny, M.A. and Collin, D. 2011. Cattle medicine. Manson Publishing Limited, London. Great Britain. United Kingdom, London.

Piessens, V., De Vliegher, S., Verbist, B., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx, M. and Van Coillie, E. 2012. Intra-species diversity and epidemiology varies among coagulase-negative *Staphylococcus* species causing bovine intramammary infections. *Veterinary Microbiology* **155:** 62-71.

Pitkala, A., Haveri, M., Pyorala, S., Myllys, V. and Honkanen-Buzalski, T. 2004. Bovine mastitis in Finland 2001- prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science* **87**: 2433-2441.

Poelarends, J.J., Hogeveen, H., Sampimon, O.C. and Sol, J. 2001. Monitoring subclinical mastitis in Dutch dairy herds. In: *Proceedings of the Second International Symposium on mastitis and milk quality*, Vancouver, British Columbia, pp. 145-149.

Pyorala, S. and Taponen, S. 2009. Coagulase negative staphylococci emerging mastitis pathogens. *Veterinary Microbiology* **134**: 3-8.

Rajala-Schultz, J.P., Smith, K.L., Hogan, J.S. and Love, B.C. 2004. Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. *Veterinary Microbiology* **102**: 33-42.

Reyher, K.K., Dohoo, I.R., Scholl, D.T. and Keefe, G.P. 2012a. Evaluation of minor pathogen intra mammary infection, susceptibility parameters, and somatic cell counts on the development of new intra mammary infections with major mastitis pathogens. *Journal of Dairy Science* **95**: 3766-3780.

Reyher, K.K., Haine, D., Dohoo, I.R. and Revie, C.W. 2012b. Examining the effect of intra mammary infections with minor mastitis pathogens on the acquisition of new intra mammary infections with major mastitis pathogens- A systematic review and meta-analysis. *Journal of Dairy Science* **95**: 6483-6502.

Roberson, J.R., Mixon, J., Rohrbach, B. and Holland, R. 2006. Etiologic agents associated with high SCC dairy herds. In: *Proceedings of the 24*[th] *World Buiatrics Congress*. Nice, France, pp. 19-21.

Rossitto, P.V., Ruiz, L., Kikuchi, Y., Glenn, K., Luiz, K., Watts, J.L. and Cullor, J.S. 2002. Antibiotic susceptibility patterns for environmental streptococci isolated from bovine mastitis in Central California dairies. *Journal of Dairy Science* **85**: 132-138.

Russell, J.B. and Houlihan, A.J. 2006. Ionophore resistance of ruminal bacteria and its potential impact on human health. *Federation of European Microbiological Societies* **27**: 65-74.

Saini, V., Mc Clure, J.T., Scholl, D.T., De Vries, T.J. and Barkema, H.W. 2012. Herd-level association between antimicrobial use and antimicrobial resistance in bovine mastitis *Staphylococcus aureus* isolates on Canadian dairy farms. *Journal of Dairy Science* **95**: 1921-1929.

Sampimon, O.C. 2009. Coagulase-negative staphylococci mastitis in Dutch dairy herds. PhD dissertation, Dutch Animal Health Service, Deventer, The Netherlands.

Sampimon, O.C., Barkema, H.W., Berends, I.M.G.A., Sol, J. and Lam, T.J.G.M. 2009a. Prevalence and herd-level risk factors intra mammary infection with coagulase-negative staphylococci in Dutch dairy herds. *Veterinary Microbiology* **134**: 37-44.

Sampimon, O.C., Zadoks, R, N., De vliegher, S., Supre, K., Haesebrouck, F., Barkema, H.W., Sol, J. and Lam. T.J.G.M. 2009b. Performance of API staph ID 32 and staph-

zym for identification of coagualase-negative staphylococci isolates from bovine milk samples. *Veterinary Microbiology* **136**: 300-305.

Sawant, A.A., Sordillo, L.M. and Jayarao, B.M. 2005. A survey on antibiotic usage in dairy herds in Pennsylvania. *Journal of Dairy Science* **88**: 2991-2999.

Sawant, A.A., Gillespie, B.E. and Oliver, S.P. 2009. Antimicrobial susceptibility of coagulase-negative staphylococcus species isolated from bovine milk. *Veterinary Microbiology* **134**: 73-81.

Shpigel, N.Y., Winkler, M., Ziv, G. and Saran, A. 1998. Clinical, bacteriological and epidemiological aspects of clinical mastitis in Israeli dairy herds. *Preventive Veterinary Medicine* **35**: 1-9.

Simojoki, H., Orro, O., Taponen, S., Taponen, S. and Pyorala, S. 2009. Experimental model of bovine clinical mastitis caused by *Staphylococcus chromogenes*. *Veterinary Microbiology* **134**: 95-99.

Sundrum. 2012. Healthy food from healthy cows. (Accessed 24 January 2013) http://www.cnd.intechopen.com/pdfs/40660/intech_healthy_food_from_healthy_cows.pdfs

Taponen, S., Simojoki, H., Haven, M., Larsen, H.D. and Pyorala, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase- negative staphylococci identified with API or AFLP. *Veterinary Microbiology* **115**: 199-207.

Taponen, S., Koort, J., Bjorkroth, J., Saloniemi, H. and Pyorala, S. 2007. Bovine intra mammary infections caused by coagulase staphylococci may persist throughout lactation according to amplified fragment length polymorphism-based analysis. *Journal of Dairy Science* **90**: 3301-3307.

Taponen, S. and Pyorala, S. 2009. Coagulase negative staphylococci as cause of bovine mastitis- not so different from *Staphylococcus aureus*? *Veterinary Microbiology* **134**: 29-36.

Tenhagen, B.A., Koster, G., Wallmann, J. and Heuwieser, W. 2006. Prevalence of mastitis and their resistance against antimicrobial agents in dairy cows Brandenburg, Germany. *Journal of Dairy Science* **89**: 2542-2551.

Thorberg, B.M. and Brandstrom, B. 2000. Evaluation of two commercial systems and a new identification scheme based on solid substrates for identifying coagulase-negative staphylococci from bovine mastitis. *Journal of Veterinary Medicine* **47**: 683-691.

Thorberg, B.M. 2008. Coagulase- negative staphylococci in bovine subclinical mastitis. PhD dissertation, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Thorberg, B.M., Kuhn, I., Aarestrup, F.M., Brandstrom, B., Jonsson, P. and Nielsson-Than, M.L. 2006. Pheno- and genotyping of *Staphylococcus epidermidis* isolated from bovine milk and human skin. *Veterinary Microbiology* **155**:163-172.

Trinidad, P., Nickerson, S.C. and Adkinson, R.W. 1990. Histopathology of staphylococcal mastitis in unbred dairy heifers. *Journal of Dairy Science* **73**:639-647.

Waage, S., Skei, H.R., Rise, J., Rogdo, T., Sviland, S. and Odegaard, S.A. 2000. Outcome of clinical mastitis in dairy heifers assessed by re-examination of cases one month after treatment. *Journal of Dairy Science* **83**: 70-76.

Waller, K.P., Aspan, A., Nyman, A., Persson, Y. and Andersson, U.G. 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Veterinary Microbiology* **152**: 112-116.

Walther, C., Rossano, A., Thomann, A. and Perreten, V. 2008. Antimicrobial resistance in lactococcus species from bovine milk: Presence of a mature mult- drug transporter

mdt (A) gene in susceptible *Lactococcus garvieae* strains. *Veterinary Microbiology* **131**: 348-357.

Yuen, S.K., Yee, C.F. and Yin, F.H. 2012. Microbiological quality and the impact of hygienic practices on the raw milk obtained from small-scale dairy farmers in Sabah, Malaysia. *International Journal of Agricultural and Food Science* **2**:55-59.

Zadoks, R.N., Allore, H.G., Barkema, H.W., Sampimon, O.C., Wellenberg, G.J., Grohn, Y.T. and Schukken, Y.H. 2001. Cow- and quarter-level risk factors for *Streptococcus uberis* and *Staphylococcus aureus* mastitis. *Journal of Dairy Science* **84**: 2649-2663.

Zadoks, R.N., van Leeuwen, W.B., Kreft, D., Fox, L.K., Barkema, H.W., Schukken, Y.H. and van Belkum, A. 2002. Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulse-field gel electrophoresis, and binary typing. *Journal of Clinical Microbiology* **40**: 3894-3902.

Zadoks, R.N. and Watts, J.L. 2009. Species identification of coagulase-negative staphylococci: genotyping is superior to phenotyping. *Veterinary Microbiology* **134**: 20-28.

Zhang, S. and Maddox, C.W. 2000. Cytotoxic activity of coagulase-negative staphylococci in bovine mastitis. *Journal of Infection and Immunity* **68**: 1102-1108.

CHAPTER 3

Incidence and antimicrobial activity of *Staphylococcus* species in bovine milk from a dairy farm in Eastern Cape, South Africa

Abstract

The aim of the current study was to investigate the incidence of *Staphylococcus* species on a commercial dairy farm and their antimicrobial sensitivity. Several convention biochemical tests and the API® staph kits (bioMerieux, France) were used to identify the bacterial organisms to their species level. The antimicrobial activity was determined according to the Clinical Laboratory Standard Institute (CLSI). A total of 217 milk samples collected over a period of nine months were cultured for bacteria analysis. Out of the 217 milk samples that were examined, thirteen different *Staphylococcus* species were identified from 86 (39.63%) positive isolates, with *Staphylococcus xylosus* (22.09%), *Staphylococcus hominis* (15.11%) and *Staphylococcus haemolyticus* (11.63%) being the most frequently isolated. *Staphylococcus xylosus* (80%), *Staphylococcus sciuri* (60%), *Staphylococcus aureus* (53.33%), *Staphylococcus haemolyticus* (46.67%), *Staphylococcus epidermidis* (46.67%) and *Staphylococcus chromogenes* (40%) showed resistance against fifteen different antimicrobial agents that were used. These results indicate high rates of incidence and multiple drug resistance of *Staphylococcus* species against several antimicrobial drugs, including penicillin and ampicillin, which are intensively used on farms without any antibiotic susceptibility test and consequently call for drastic measures.

Key words: mastitis causing pathogens, *Staphylococcus* species, antibiotic susceptibility, multiple drug resistance.

3.1. Introduction

Mastitis is the most expensive disease in dairy farms (Dufour *et al*., 2012). Increase in the rate of mastitis incidence in a herd affects the welfare of cows (Hogeveen *et al*., 2011). Cows infected with mastitis experience reduced milk yield, which may lead to huge economic losses to the farmer (Halasa *et al*., 2009; Ampe *et al*., 2012). Several hundreds of bacteria have been linked with the cause of mastitis (Smith *et al*., 2001; Batavani *et al*., 2007). The most common bacteria that cause mastitis challenges in dairy farms are *Staphylococcus* species (Taponen *et al*., 2006; Sawant *et al*., 2009), which are comprised of both the coagulase positive staphylococci (CPS) e.g *Staphylococcus aureus* and the coagulase negative staphylococci (CNS) e.g *Staphylococcus epidermidis*, *Staphylococcus sciuri*, *Staphylococcus haemolyticus*, *Staphylococcus hyicus* among others. Coagulase negative staphylococci comprises of a group of species which differ in their ability to cause mastitis infection (Zhang and Maddox, 2000; Waller *et al*., 2011).

Lately, CNS has evolved to be more significant as bovine a mastitis causing organism (Simojoki *et al*., 2011). Isolates of CNS species are habitually identified in bovine milk of intramammary infection (Tenhagen *et al*., 2006). They cause both clinical and subclinical mastitis in cattle (Supre *et al*., 2011). Though, clinical mastitis by CNS species maybe placid in some cases (Taponen *et al*., 2009). Common CNS species isolated from bovine mastitis include *Staphylococcus chromogenes*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Staphylococcus sciuri* and *Staphylococcus haemolyticus* (Taponen *et al*., 2006; Kot *et al*., 2012). Other CNS species e.g *Staphylococcus saprophyticus*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus capitis* e.t.c from bovine mastitis have also been well documented (Sampimon *et al*., 2011).

In several nations of the world CNS species have been indicated to be a preponderant agent causing mastitis infections in cows when compared to other mastitis causing agents (Pitkala *et al*., 2004; Piepers *et al*., 2007; Pyorala *et al*., 2009). However, prevalence of CNS varies from country to country (Taponen *et al*., 2009). Factors like farm area and or environment (Piessens *et al*., 2012), farm management system (Ayano *et al*., 2013) and season of the year may influence the incidence of the bacterial species in a farm.

A survey carried out in South Africa for instance indicated that CNS were a major cause of both clinical and sub-clinical mastitis infection (Petzer *et al*., 2009). Various factors were linked to the CNS spread among cow herds in South Africa these included large herd size, meagre milking parlour, inefficient milking machine maintenance, influx of new stock of cows and high milk yield by cows were the reasons for CNS spread among cow herds in South Africa. Most studies have reported CNS as a group and not at their species level. Conversely, CNS species differ in their ability to cause infection in cows due to their different virulence (Simojoki *et al*., 2011). Distribution of CNS also varies within different regions of a particular country (Koivula *et al*., 2007; Shekhan *et al*., 2011). Influence of time of the year, parity and stage of lactation have also been indicated to determine the distribution of CNS pathogens from different regions within a country (Grohn *et al*., 2004; Koivula *et al*., 2007; Taponen *et al*., 2007). Thus, the need to identify CNS to their species level is important for effective mastitis control programs (Supre *et al*., 2011).

There is increased use of antimicrobials for the treatment of mastitis in many farms (Walther *et al*., 2008), during lactation and in the dry cow stage treatment (Sawant *et al*., 2005) Several *Staphylococcus* species are resistance to antimicrobials (Sampimon *et al*., 2011). According

to the World Health Organisation (WHO, 2000) the rate of increase of bacterial resistance to antibiotics is worrisome.

Dairy farmers in the Eastern Cape Province of South Africa are aware of *Staphylococcus aureus* to be a cause of mastitis, but not the other species. Farmers acknowledged mastitis to be one of the biggest challenges that they face in their dairy farms. This study aimed to investigate the incidence of *Staphylococcus* species and the *in-vitro* antimicrobial sensitivity pattern of these species in milk of cows collected over a nine month period in 2012, from a dairy farm located in the Eastern Cape of South Africa.

3.2. Material and methods

3.2.1. Study site description

The study was conducted in a dairy farm located in Middle drift of Nkonkobe municipality in the Eastern Cape Province of South Africa. The farm was on 250 hectares of land with six hundred milking cows, producing 3000-3500 litres of milk per day. The average annual rainfall of the farm is 591 mm per annum. Rainfall from May – September is less than 25 mm per annum and 50 -100 mm from November – March of every year. The topography of the farm land is flat with gentle slope and an altitude of 420 - 470 m. In July of every year the farm has a mean daily temperature of between 5.0^{o}C and 28.2^{o}C.

3.2.2. Animal description

Three dairy breeds viz Friesian, Jersey and their crosses (Friesian x Jersey) were kept for milk production. Milk samples were collected from each of the breeds for bacterial examination during the nine month period of study. Each cow that was used for milk

sampling was identified based on their colours, information from the farmers and ear-tags. Data including lactation stage and parity of the cows were taken at each sampling day.

3.2.3. Milk sample collection

Composite raw milk samples were collected from selected cows in a 10 ml sterile sampling bottle after pre-milking test (strip cup test and California mastitis test) were carried out on the cows. Milk sampling bottles were thereafter labelled appropriately and immediately kept in a cooler box with ice before they were transported to the laboratory for bacterial culture analysis. Milk samples were collected between March – November, 2012 which accounted for three different sampling periods (hot-wet, cold-dry and the hot-dry) of the year, respectively; and this was done twice for each sampling period.

3.2.4. Isolation and identification of Staphylococcus species

3.2.4.1. Culturing of milk samples in Mannitol salt agar

Milk samples were aseptically streaked in a freshly prepared Mannitol salt agar within 24- 48 hours of collection from the farm and incubated at 37°C for 24-48 hours. Isolates that gave a yellow colour with fermentation on Mannitol salt agar were sub-cultured in nutrient agar.

3.2.4.2. Sub-culturing of milk samples in nutrient agar

Presumed isolates that were positive in Mannitol salt agar were sub-cultured again in a freshly prepared nutrient agar (within 24 hours) and they were then placed in an incubator for 24-48 hours. This is done so as to get a pure colony of the bacteria isolate.

3.2.4.3. Gram's staining test

All presumed cultures of *Staphylococcus* species were subjected to gram's stain. Few colonies of pure cultured isolates were aseptically picked from the nutrient agar and then smeared on a sterile glass slide. The smeared culture was heat fixed to dryness. Crystal violet (0.5%) was added to the smear and left for 1 minute. Water was used to wash off the stain from the slide. Sufficient solution of iodine was again added to the smear and left for I minute. Water was used to wash off the stain. Ethanol (95%) was afterward used to rinse the stain from the slide. Safrani solution was finally added to the smear for 2 minutes before being washed with water. The smear was allowed to dry for a few minutes before they were viewed under a light microscope to determine the colony structure of the bacteria species. The smear of each bacteria species was viewed under the x10, x40 and x100 magnification. Gram's stain smears that were positive gave a grape-like clustered shape with deep blue to purple colour.

3.2.4.4. Oxidase test

Few colonies of isolates from nutrient agar from presumed *Staphylococcus* species were picked aseptically using a sterile loop and then placed on an oxidase test strip paper. All isolates that turned purple on the test strip paper after 10 seconds were suspected to be *Staphylococcus* species.

3.2.4.5. Catalase test

Pure cultures of presumed *Staphylococcus* species from nutrient agar were aseptically transferred onto a clean glass slide. Few drops of 3% H_2O_2 were then added to the cultures. Positive cultures of *Staphylococcus* species gave out bubbles of oxygen within 5 seconds after adding H_2O_2.

3.2.4.6. API Staph kit test for identification of Staphylococcus species

All presumed isolates were finally confirmed to their species level using the API® staph kit (bioMerieux, France). Homogeneous inoculums of pure cultured isolates were prepared with turbidity equivalent to 0.5 McFarland using the API® Staph medium. Pipette was used to fill the micro tubes of the API kit with the inoculated API Staph medium. Mineral oil was added to the arginine dihydrolase (ADH) and urease (URE) tests to ensure anaerobiosis. The incubation box of the API kit was filled with 5mls of distilled water and closed tightly before incubating at 37^0C for 24hrs. After incubation one drop of API Staph reagent was added to the inoculated micro tubes to determine their reactions. The reactions of the inocula were read using the apiweb ™ identification software (Biomerieux., Inc Quebec).

3.2.5. Minimum Inhibitory Concentration (MIC) determination

Isolates that were identified to their species level were subjected to *in-vitro* drug sensitivity tests using the disc diffusion method as postulated by the Clinical Laboratory Standard Institution (CLSI, 2006). Briefly, few colonies of isolates were aseptically inoculated in normal saline to the desired turbidity (0.5 McFarland standards). With the aid of a sterile cotton swab, the inocula were evenly spread on Muller-Hinton agar in a petri dish. The antibiotic discs were then gently pressed on the agar and the petri dishes placed in an incubator for 24 hours at 37° C. The MIC of each isolate was afterward determined by measuring (in millimetres) the zones of inhibition in the Mueller Hinton agar with a metre rule so as to obtain the diameter of inhibition of the different isolates. Among the antibiotics used were Levofloxacin, Ciprofloxacin, Kanamycin, Meropenem, Ofloxacin, Tetracycline, Gentamicin, Erythromycin, Imipenem, Chlorofenicol, Nalidixic acid, Amoxicillin, Penicillin, Ampicillin and Vancomycin.

3.2.6. Statistical analysis

Data were analysed using both Microsoft Excel (2007) and SAS (2003). Descriptive statistics to generate percentage distribution was performed using Microsoft Excel (2007). Chi-square test (p< 0.05) was employed to assess the association between incidence of *Staphylococcus* species and risk factor variables (such as stage of lactation, parity number and period of the year of milk sample collection) using SAS (2003).

3.3. Results

From the total of 217 raw milk samples that were examined for bacterial growth, 13 different *Staphylococcus* species were isolated from 86 (39.63%) positive samples. The distribution of *Staphylococcus* species is presented in Table 3.1. The most frequently isolated *Staphylococcus* species were *Staphylococcus xylosus* (22.09%), *Staphylococcus hominis* (15.11%), *Staphylococcus haemolyticus* (11.63%), *Staphylococcus sciuri* (10.64%) and *Staphylococcus warneri* (9.3%). About 3.38% of the remaining species were due to *Micrococcus spp*. From the 86 positive isolates bacterial species that were identified, the proportion of *Staphylococcus* species across the different sampling periods was 10%, 38% and 38%, respectively with respect to the total milk sampled at the different sampling period (Table 3.2). Among the 13 different *Staphylococcus* species isolated from milk samples, *Staphylococcus xylosus* (10%, 10.52%, and 36.84%), *Staphylococcus epidermidis* (10%, 2.63% and 5.26 %), *Staphylococcus haemolyticus* (40%, 5.26% and 10.65%) and *Staphylococcus sciuri* (10%, 7.89% and 13.15%) were identified at each of the different sampling periods (Table 3.2). There was significant difference (0.0039 and 0.0001) in the incidence of *Staphylococcus* species and some risk factors in the farm which include the period of milk sample collection and the stage of lactation at p < 0.05 (Table 3.3).

Table 3. 1 Proportion of *Staphylococcus* species isolated from 86 positive bovine milk samples.

Staphylococcus species	Number (%)
S. epidermidis	4 (4.65)
S. xylosus	19 (22.09)
S. aureus	3 (3.48)
S. haemolyticus	10 (11.63)
S. hominis	13 (15.11)
S. sciuri	9 (10.46)
S. chromogenes	5 (5.81)
S. warneri	8 (9.3)
S. auricularis	4 (4.65)
S. hyicus	1 (1.16)
S. saprophyticus	2 (2.32)
S. cohnii- cohnii	3 (3.48)
S. cohnii- urealyticus	2 (2.32)
Others	3 (3.48)
Total	86 (100)

Table 3. 2. Distribution of *Staphylococcus* species isolates identified from milk samples at different sampling periods of the year.

Staphylococcus species	N= 76 Cold-dry (%)	N=81 Hot-wet (%)	N=60 Hot-dry (%)
S. epidermidis	1(10)	1 (2.63)	2 (5.26)
S. xylosus	1(10)	4 (10.52)	14 (36.84)
S. aureus	2(20)	-	1 (2.63)
S. haemolyticus	4(40)	2 (5.26)	4 (10.52)
S. hominis	1(10)	12(31.57)	-
S. sciuri	1(10)	3 (7.89)	5 (13.15)
S. chromogenes	-	3 (7.89)	2 (5.26)
S. warneri	-	6 (15.78)	2 (5.26)
S. auricularis	-	4 (10.52)	-
S. hyicus	-	1 (2.63)	-
S. saprophyticus	-	-	2 (5.26)
S. cohnii- cohnii	-	-	3 (6.97)
S. cohnii- urealyticus	-	-	2 (5.26)
Others	-	2 (5.26)	1 (2.63)
Total	10 (100)	38 (100)	38 (100)

N: Total number of milk examined at each sampling period.

The antibiotic sensitivity test results for 78 isolates of CNS species against 15 different antibiotics are represented in Table 3.4. On the whole, the antimicrobial resistance test of the *Staphylococcus* species showed the lowest resistance to Levofloxacin (1.3%), Imipenem (2.6%), Ofloxacin (2.7%), Gentamicin (5.1%), Ciprofloxacin (5.1%), Chlorofenicol (5.1%), Meropenem (6.8%), and Kanamycin (7.7%), whereas *Staphylococcus* species isolates showed the highest resistance to penicillin (83%), Nalidixic acid (79%) and Ampicillin (63%) respectively. Considering individual *Staphylococcus* species, *Staphylococcus cohnii-urealyticus* was 100% resistant to Erythromycin, Nalidixic acid, Imipenem, Vancomycin, Penicillin and Ampicillin, *Staphylococcus saprophyticus* to Vancomycin, Penicillin, Amoxicillin and Amoxicillin and *Staphylococcus cohnii- cohnii* to Nalidixic acid, Penicillin and Ampicillin.

Table 3.3 Chi-square test for potential risk factors associated with the incidence of *Staphylococcus* species.

Risk factor	Group	Number examined	X^2-values	[1]Sig.
Parity	First lactation	25	24.7182	ns
	Second lactation	68		
	Third lactation	57		
	Fourth lactation	67		
Stage of lactation	Early (1-100)	28	51.9845	**
	Mid (101-200)	117		
	Late (201<)	72		
Sampling time	Cold-dry	76	98.2789	***
(Season)	Hot-wet	81		
	Hot-dry	60		

[1]Chi Square values along the same column were significant at ** $P<0.01$; ***$P<0.001$ but not significant at ns $P>0.05$ respectively.

Table 3.4 Distribution of resistant *Staphylococcus* species to different antibiotics.

Staphylococcus spp	N	CIP	LEV	OFX	MEM	E	NAL	IMI	VA	PG	GM	T	K	AM	C	AP
					Number of antimicrobial resistant isolates (%)											
S. haemolyticus	9	0	0	0	0	3(33)	7(78)	0	6(71)	6(71)	0	3(33)	0	2(33)	0	9(100)
S. aureus	3	0	0	0	0	1(33)	2(67)	1(33)	2(67)	2(67)	2(67)	1(33)	0	0	0	3(100)
S. sciuri	7	2(29)	0	0	0	4(57)	7(100)	0	4(57)	5(71)	1(14)	0	3(43)	0	3(43)	6(86)
S. hominis	14	0	0	0	0	0	13(93)	0	2(14)	13(93)	0	4(29)	0	1(7)	0	NA
S. epidermidis	3	0	1(33)	0	0	2(67)	1(33)	0	1(33)	3(100)	0	1(33)	0	2(67)	0	NA
S. xylosus	20	2(10)	0	2(10)	5	14(70)	13(65)	0	16(80)	18(90)	1(5)	2(10)	3(15)	13(66)	0	18(90)
S. auricularis	4	0	0	0	0	0	3(75)	0	0	3(75)	0	2(50)	0	1(25)	0	NA
S. chromogenes	4	0	0	0	0	1(25)	3(75)	0	1(25)	3(75)	0	1(25)	0	1(25)	0	0
S. warneri	6	0	0	0	0	1(17)	6(100)	0	1(17)	5(83)	0	0	0	0	0	6(100)
S. hyicus	1	0	0	0	0	0	1(100)	0	0	0	0	1(100)	0	0	0	NA
S. cohnii- cohnii	3	0	0	0	0	2(67)	3(100)	0	2(67)	3(100)	0	1(33)	0	0	0	3(100)
S. cohnii-urealyticus	2	0	0	0	0	2(100)	2(100)	2(100)	2(100)	2(100)	0	0	0	NA	1(50)	2(100)
S. saprophyticus	2	0	0	0	0	0	1(50)	0	2(100)	2(100)	0	0	0	2(100)	0	2(100)
Total	78	4(5.1)	1(1.3)	2(2.7)	5(6.4)	30(39)	62(79)	2(2.6)	39(50)	65(83)	4(5.1)	16(21)	6(7.7)	22(28)	4(5.1)	49(63)

NA: Not available. N: Number of isolates, CIP: Ciprofloxacin, LEV: Levofloxacin, OFX: Ofloxacin, MEM: Meropenem, E: Erythromycin, NAL: Nalidixic acid, IMI: Imipenem, VA: Vancomycin, PG: Penicillin-G, GM: Gentamycin, T: Tetracyline, K: Kanamycin, AM: Amoxicillin, C:Chlorofenicol,AP:Ampicillin.
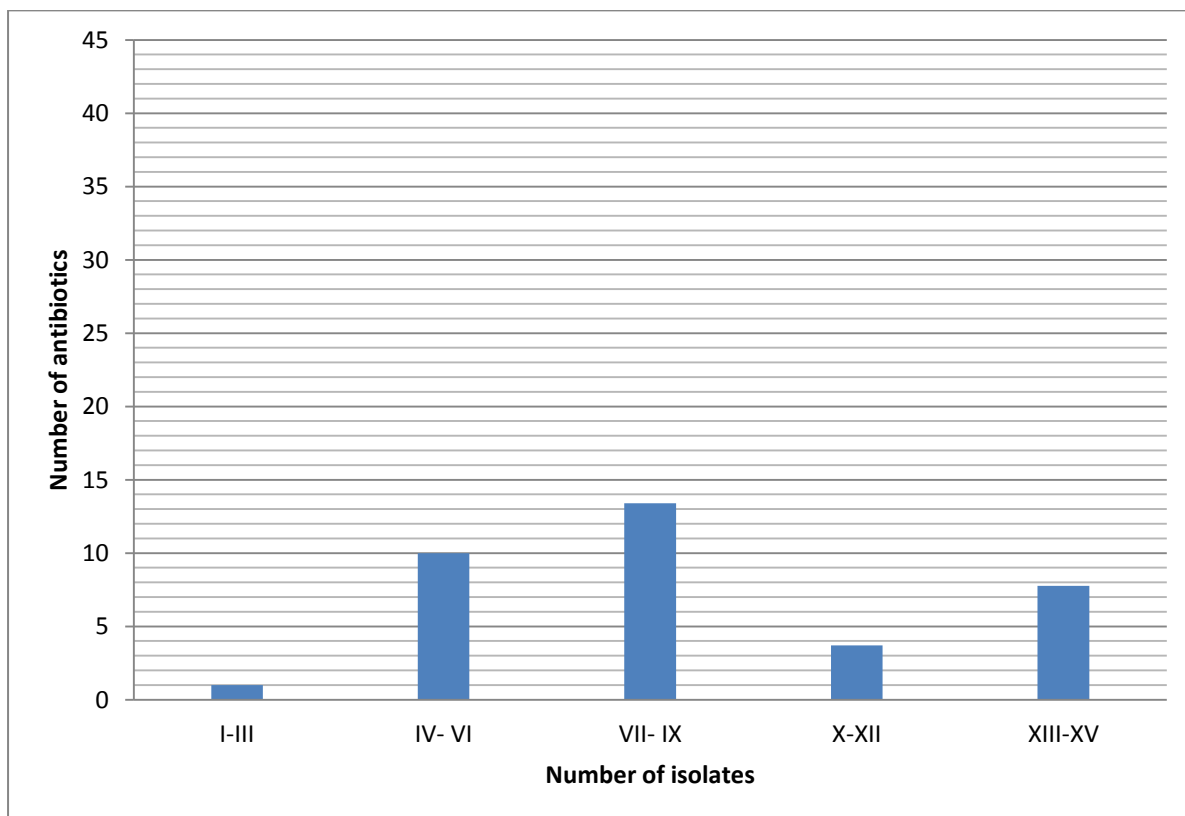
Figure 3. 1 Distribution of multiple drug resistance to 15 antimicrobial drugs among 78 isolates of *Staphylococcus* species from bovine milk.

I-III= Ciprofloxacin, Levofloxacin and Ofloxacin, IV-VI= Meropenem, Erythromycin and Nalidixic, VII-IX= Imipenem, Vancomycin and Penicillin, X-XII= Gentamicin, Tetracycline and Kanamycin, XIII-XV= Amoxicillin, Chlorofenicol and Ampicillin.

3.4. Discussion

From an aggregate of 217 milk samples that were collected throughout the three sampling period of the year, 86 (39.63%) were positive for *Staphylococcus* species. Thirteen different *Staphylococcus* species were identified among the 86 isolates that tested positive. *Staphylococcus xylosus* (22.09%), *Staphylococcus hominis* (15.11%), *Staphylococcus haemolyticus* (11.63%) and *Staphylococcus sciuri* (10.46%) were the most frequently isolated species in this study (Table 3.1). This result is somewhat in line with a study carried out in Canada (Davidson *et al*., 1992). Also, in the study by Kot *et al*. (2012) *Staphylococcus xylosus* was observed to be the most frequently isolated species. Farm management system and the farm surroundings may be a possible reason for such findings (Bendahou *et al*., 2008; Ayano *et al*., 2013). Piessens *et al*. (2012), in their study reported that farm environment may sometimes be a reservoir for *Staphylococcus* species. Some *Staphylococcus* species including *Staphylococcus xylosus*, *Staphylococcus hominis* and *Staphylococcus haemolyticus* are known to be opportunistic in nature and may find their way into the mammary gland of cows (Schukken *et al*., 2009). Conversely, these *Staphylococcus* species have been reported to cause intra mammary infections in cows (Quirk *et al*., 2012).

On account of the period of the year that milk samples were collected, more *Staphylococcus* species (at the species level) were observed in the hot and dry period of the year when compared with the other periods of the year (Table 3.2). The reason for this may be due to the adaptive ability and persistent nature of some *Staphylococcus* species to the mammary glands of cows (Piessens *et al*., 2012). In the current study, a particular *Staphylococcus* species was assumed to persist when it was isolated at least once in each sampling day across the three different sampling periods of the year. Thus, *Staphylococcus epidermidis*, *Staphylococcus xylosus*, *Staphylococcus haemolyticus* and *Staphylococcus sciuri* were assumed to persist in

cow udders. In another study by Taponen *et al*. (2007), *Staphylococcus epidermis* and *Staphylococcus haemolyticus* were also reported to persist in the udder of cows but *Staphylococcus xylosus* was transient. *Staphylococcus* species (especially CNS) may differ in their degree of persistence (Taponen *et al*., 2007). Also, incidences of specific bacteria species vary among the different months of the year (Koivula *et al*., 2007).

In all, *Staphylococcus xylosus* and *Staphylococcus hominis* (22.09% and 15.11%) were the most frequently isolated species that were observed in the current study (Table 3.1). They more often than not occur as harmless organisms in both the human body and some parts of the animal skin (Taponen *et al*., 2008). However, they have been isolated from milk of cows that are lactating (Kenar *et al*., 2012). The least isolated *Staphylococcus* species was *Staphylococcus hyicus* (1.16%). This result is different from the study carried out in Turkey, where *Staphylococcus hyicus* (33.33%) was the most frequently isolated *Staphylococcus* species (Kirkan *et al*., 2005). In another study carried out in Morocco, a similar result in line with the current study was however observed (Bendahou *et al*., 2008).The variation in the frequency of isolation of these species may be as a result of difference in the study areas (Koivula *et al*., 2007). Again, from the current finding *Staphylococcus cohnii-cohnii* was the ninth frequently isolated *Staphylococcus* species. This result was similar to the study carried out by Kirkan *et al*. (2005).

Staphylococcus aureus is a major mastitis causing pathogen in dairy farms. It was observed in this study to be the eighth most frequently (3.48%) isolated *Staphylococcus* species. The distributions of other *Staphylococcus* species include *Staphylococcus warneri* (9.3%), *Staphylococcus auricularis* (4.65%), *Staphylococcus saprophyticus* (2.32%) and *Staphylococcus cohnii-urealyticus* (2.32%) respectively. Few *Staphylococcus* species that

were identified including *Staphylococcus warneri* and *Staphylococcus saprophyticus* may have been as result of contamination from the milking clusters that were used in the farm. These species were also isolated from the milking clusters and not from farmers' hands and may have been transferred from one cow to another from milk droplets in the milking clusters.

Not much work has been done on the incidence of CNS (at the species levels) as regards the time of the year milk samples are collected (Gillespie *et al*., 2009). However, some studies have indicated that the time of the year when milk samples are collected from cows for bacterial culture analysis influences the prevalence of mastitis causing pathogens in milk (Osteras *et al*., 2006; Gillespie *et al*., 2009). According to Koivula *et al*. (2007), *Staphylococcus aureus* were prevalent during the cold and dry period of the year than any other period of the year. The reasons given for their findings were linked with cow's calving periods and farm management style. Shpigel *et al*. (2000) reported that there was increase in the incidence of mastitis caused by coliform bacteria in the wet and cold period of the year, but lower incidence of coliform bacteria was observed in the hot and dry period. In another study in Norway, *Staphylococcus aureus* and *Streptococcus uberis* were prevalent in the hot and dry period of the year but, *Streptococcus dysgalactiae* and CNS (reported as a group) were more prevalent in the cold and dry period (Osteras *et al*., 2006). This may give a rational insight for a probable link in the time of milk sampling and the incidence of *Staphylococcus* species in farms (Gillespie *et al*., 2009).

More *Staphylococcus* species (at the species level) were isolated during the hot and dry period of the year in the current study. The number of species-specific isolates identified were 11, 10 and 6 for the hot-dry, hot-wet and cold-dry period of the year respectively (Table

3.2). The difference in the result may be due to the variation in the climatic conditions of the different sampling periods of the year (Piessens *et al*., 2012; Sampimon *et al*., 2011). The prevalence of mastitis causing pathogens in a farm is linked with multiple complex factors including environment, cow herd, clinical state of the udder of cow, and the farm itself (Thorberg *et al*., 2009; Supre *et al*., 2011; Ayano *et al*., 2013).

Some *Staphylococcus* species cause damage to the mammary cow udder than others depending on their virulent attribute (Zhang *et al*., 2000; Thorberg *et al*., 2009). Their roles in causing damage to mammary tissues of cows (mostly CNS) and to initiate mastitis infection have not been plainly defined (Kirkan *et al*., 2005). However, relationships have been reported to exist between stage of lactation and the incidence of *Staphylococcus* species (Taponen *et* al., 2007; Abera *et al*., 2010). Heifers and primiparous cows are known to be infected with mastitis by *Staphylococcus species* more often when compared with multiparous cows, though the reason for this is not yet clear (Taponen *et al*., 2007). There was no significant difference (p= 0.9845) in the incidence of *Staphylococcus* species and the number of lactations per cow (Table 3.3). A significant association was however observed for the incidence of *Staphylococcus* species in relation to the stage of lactation (p= 0.0039) and the period of year of milk sampling (p= 0.0001). Abera *et al*. (2010) also reported no significant difference in the incidence of *Staphylococcus aureus* and the number of lactation in cows but, reported a significant association between the stage of lactation and the prevalence of *Staphylococcus aureus* in bovine milk. Lack of cleanliness of cow udder during dry periods was suggested by Abera *et al*. (2010) to influence the increase of bacteria on the skin of cow teat leading to possible high infection of bacteria in the udder.

Today, many farms are becoming dependant on antibiotics (Mc Kenna, 2011). The use of antimicrobials in dairy farms plays a significant role in the control of mastitis. Information about the variation that exists among specific *Staphylococcus* species is essential for mastitis therapy and management (Sawant *et al*., 2009). A large proportion of *Staphylococcus* species from this study were resistant to Erythromycin, Nalidixic acid, Vancomycin, Penicillin, Tetracycline, Amoxicillin and Ampicillin (Table 3.4). There is an increasing evolutionary trend of antibiotic resistance in infectious diseases in recent times (McKinzey, 2007). This may be the reason for the observed result in the current study. Hundred percent resistance was observed of several *Staphylococcus* species isolates to Ampicillin in the current study (Table 3.4). This was contrary to the report by Sawant *et al*. (2009) were most of the *Staphylococcus* species were susceptible to Ampicillin.

In the study by Kot *et al*. (2012) high resistance to Penicillin was observed for *Staphylococcus* species. Penicillin and Ampicillin were the mostly used antimicrobials in the farm where this study was conducted. However, from our results Penicillin and Ampicillin may not be a proper choice for the treatment of *Staphylococcus* species (Kot *et al*., 2012) due to the high to these antimicrobials. The highest percentage of *Staphylococcus* species isolates that was resistant to Penicillin was among *Staphylococcus xylosus*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus sciuri* and *Staphylococcus haemolyticus* (Table 3.4). All the isolates of *Staphylococcus epidermidis* gave 100% resistance to Penicillin (Table 3.4). This result is in accordance with the result of Sampimon *et al*. (2011) and Kot *et al*. (2012) whose study showed that Penicillin resistance in *Staphylococcus epidermidis* was very high.

Considering the results for specific species, 100% resistance was observed mostly for *Staphylococcus cohnii-urealyticus* and *Staphylococcus saprophyticus*. Resistance (100%) to

six out of fifteen antimicrobial agents was observed for *Staphylococcus cohnii- urealyticus* while *Staphylococcus saprophyticus* gave resistance (100%) to four out of fifteen antimicrobial agents that were used. The next *Staphylococcus* species with a high resistance (9 out of 15) to multiple antimicrobial agents was *Staphylococcus sciuri* from the current study (Table 3.4).

Staphylococcus aureus gave resistance to 8 (Erythromycin, Nalidixic acid, Imipenem, Vancomycin, Penicillin, Gentamicin, Tetracycline and Ampicillin) out of the 15 antimicrobial (Table 3.4). In Sweden, Persson *et al*. (2011) also observed resistance to Penicillin by *Staphylococcus aureus* in their study but not for Gentamicin and Erythromycin as observed in the current study. *Staphylococcus aureus* was susceptible to Chlorofenicol and Ciprofloxacin in our study and this is in line with the study carried out by Persson *et al*. (2011). All the *Staphylococcus aureus* isolates identified from this study were susceptible to Kanamycin. This is contrary to the report by Persson *et al*. (2011). The variation observed in the trend of *Staphylococcus* species against antimicrobial agents from this study may be as a result of difference in virulence strains of the different species that were identified (Zhang *et al*., 2000). The most susceptible *Staphylococcus* species (11 out of 15) to multiple antimicrobial agents was observed for *Staphylococcus hyicus*.

As regards the number of isolates of *Staphylococcus* species that were resistant to multiple antimicrobial agents, 13 isolates of species were resistant to Imipenem, Vancomycin and Penicillin, while 10 isolates were resistant to Meropenem, Erythromycin and Nalidixic acid (Figure 3.1). Conversely, 4 isolates of *Staphylococcus* species were resistant to Gentamicin, Tetracycline and Kanamycin. This result is contrary to a study that was conducted in Argentina were all *Staphylococcus* species that were tested were susceptible to Gentamicin

(Gentilini *et al*., 2002). The least number of isolates (1) against multiple antimicrobial agents was observed for Ciprofloxacin, Levofloxacin and Ofloxacin (Figure 3.1). The result of huge multi-drug resistance witnessed on the farm may be due to the development of resistance by the *Staphylococcus* species prevalent in the farm of study to antimicrobial agents used for mastitis therapy. Frequent use of the same antimicrobial agents on a farm may lead to transfer of resistant gene among mastitis disease causing organisms (Gentillin *et al*., 2002).

3.5. Conclusions

There was relatively high incidence of *Staphylococcus* species with *Staphylococcus xylosus*, *Staphylococcus hominis* and *Staphylococcus haemolyticus* frequently identified in milk samples from the farm where this study was conducted. Stage of lactation and the period of the year of sample collection influenced the incidence of *Staphylococcus* species in the farm. High rate of multiple drug resistance against antimicrobials agents was also observed on the farm with *Staphylococcus xylosus* and *Staphylococcus sciuri* having the highest resistance rate against antimicrobial agents. The high resistance observed for most *Staphylococcus* species to multiple antimicrobial agents including Penicillin and Ampicillin which are frequently used on the farm suggests that these drugs may not be effective against most of the *Staphylococcus* species for mastitis therapy control and hereby calls for alternative antimicrobial agents to be used for treatment of mastitis disease in cows from the farm.

3.6. References

Abera, M., Demie, B., Aragaw, K., Regassa, F. and Regassa, A. 2010. Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *Journal of Veterinary Medicine and Animal Health* **2:** 29-34.

Ampe, B., Goethals, K., Laevens, H. and Duchateau, L. 2012. Investigating clustering in intervals-censored udder quarter infection times in dairy cows using a gamma frailty model. *Journal of Preventive Veterinary Medicine* **106:** 251-257.

Ayano, A.A., Hiriko, F., Simyalew, A.M. and Yohannes, A. 2013. Prevalence of subclinical mastitis in lactating cows selected in commercial dairy farms of Holeta district. *Journal of Veterinary Medicine and Animal Health* **5:** 67-72.

Batavani, R. V., Asri, S. and Naebzadeh, H. 2007. The effect of subclinical mastitis on milk composition in dairy cows. *Iranian Journal of Veterinary Research* **8:** 205-211.

Bendahou, A., Lebbadi, M., Ennaenei, L., Essadqui, F.Z. and Abid, M. 2008. Characterization of *Staphylococcus* species isolated from raw milk and milk products (Iben and jben) in North Morocco. *Journal of Infection in Developing countries* **2:** 218-225.

Clinical and Laboratory Institute Standards (Former: National Committee for Clinical Laboratory Standards). 2006. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests. Approved Standard. 9th ed. NCCLS document M2-A9, NCCLS, Wayne, PA.

Davidson, T.J., Dohoo, I.R., Donald, A.W., Hariharan, H. and Collins, K. 1992. A cohort study of coagulase negative staphylococcal mastitis in selected dairy herds in Prince Edward Island. *Canadian Journal of Veterinary Research* **56:** 275-280.

Dufour, S., Dohoo, I., Barkema, H.W., Descoteaux, I., De Vries, T.J., Reyher, K.K., Roy, J.P. and Scholl, D.T. 2012. Manageable risk factors associated with lactational incidence elimination, and prevalence of *Staphylococcus aureus* intra mammary infections in dairy cows. *Journal of Dairy Science* **95:** 1283-1300.

Gillespie, B.E., Headrick, S.I., Boonyayatra, S. and Oliver, S.P. 2009. Prevalence and persistence of coagulase- negative *Staphylococcus* species in three dairy research herds. *Veterinary Microbiology* **134:** 65-72.

Gentilini, E., Denamiel, G., Betancor, A., Rebuelto, M., Fermepin, M.R. and De Torres, R.A. 2002. Antimicrobial Susceptibility of Coagulase-Negative Staphylococci Isolated from Bovine Mastitis in Argentina. *Journal of Dairy Science* **85:** 1913-1917.

Grohn, Y.T., Wilson, D.J., Gonzalez, R.N., Hertl, J.A., Schutte, H., Bennett, G. and Schukken, Y.H. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. *Journal of Dairy Science* **87:** 3358-3374.

Halasa, T., Nielen, M., De Roos, A.P.W., de Jong, G., Lam, T.J.G.M., Van werven, T. and Hogeveen, H. 2009. Production loss due to new subclinical mastitis in Dutch dairy cows estimated with a test-day model. *Journal of Dairy Science* **92:** 599-606.

Hogeveen, H., Huijps, K. and Lam, T.J.G.M. 2011. Economics aspects of mastitis: New developments. *New Zealand Veterinary Journal* **59:** 16-23.

Kenar, B., Kuyucuoglu, Y. and Seker, E. 2012. Antibiotic susceptibility of coagulase-negative staphylococci isolated from bovine subclinical mastitis in Turkey. *Pakistan Veterinary Journal* **32:** 390-393.

Kirkan, B., Goksoy, E.O. and Kaya, O. 2005. Identification and Antimicrobial Susceptibility of *Staphylococcus aureus* and Coagulase Negative Staphylococci from Bovine Mastitis in the Aydin Region of Turkey, *Turkey Journal of Veterinary Animal Science* **29:** 791-796.

Koivula, M., Pitkala, A., Pyorala, S. and Mantysaari, E. 2007. Distribution of bacteria and seasonal and regional effects in a new data base for mastitis pathogens in Finland. *Acta Agriculture Scandinavica* section A- Animal Science **56:** 89-96.

Kot, B., Piechota, M., Antos- Bielska, M., Zdunek, E., Wolska, K.M., Binek, T., Olszewska, J., Gulinski, P. and Trafny, E.A. 2012. Antimicrobial resistance and genotypes of staphylococci from bovine milk and the cowshed environment. *Polish Journal of Veterinary Science* **15:** 741-749.

Mc Kenna, M. 2011. More MRSA in milk: A new strain in cows and humans. http://www.wiredscience/2011/06/mrsa-milk-new/. (Accessed 22 July, 2012).

Mc Kinzey, M. 2007. Evolutionary Antibiotic Resistance as Documented in Multiple Strains of *Staphylococcus*. *Eukaryon* **3:** 75-79.

Microsoft Excel. 2007. Microsoft Office Excel. www.microsoft.com/office/excel.

Osteras, O., Solverod, L. and Reksen, O. 2006. Milk Culture Results in a Large Norwegian Survey- Effects of Season, Parity, Days in Milk, Resistance, and Clustering. *Journal of Dairy Science* **89:** 1010-1023.

Persson, Y., Nyman, A. J. and Gronlund-Andersson, U. 2011. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. *ACTA Veterinaria Scandinavica* **53:** 36- 43.

Petzer, I.M., Karzis, J., Watermeyer, J.C., van der Schans, T.J. and van Reenen, R. 2009. Trends in udder health and emerging mastitogenic pathogens in South Africa dairy herds. *Journal of South African Veterinary Association* **80:** 17-22.

Piepers, S., De Meulemeester, L., de Kruif, A., Opsomer, G., Barkema, H.W. and De Vliegher, S. 2007. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. *Journal of Dairy Research* **74:** 478-483.

Piessens, V., De Vliegher, S., Verbist, B., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx, M. and Van Coillie, E. 2012. Intra-species diversity and epidemiology varies among coagulase-negative *Staphylococcus* species causing bovine intramammary infections. *Veterinary Microbiology* **155:** 62-71.

Pitkala, A., Haveri, M., Pyorala, S., Myllys, V. and Honkanen-Buzalski, T. 2004. Bovine mastitis in Finland 2001- prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science* **87:** 2433-2441.

Pyorala, S. and Taponen, S. 2009. Coagulase negative staphylococci emerging mastitis pathogens. *Veterinary Microbiology* **134:** 3-8.

Quirk, T., Fox, L.K., Hancock, D.D., Capper, J., Wenz, J. and Park, J. 2012. Intramammary infections and teat canal colonization with coagulase-negative staphylococci after postmilking teat disinfection: Species- specific responses. *Journal of Dairy Science* **95:** 1906-1912.

Sampimon, O.C., Lam, T.J.G.M., Mevius, D.J., Schukken, Y.H .and Zadoks, R.N. 2011. Antimicrobial susceptibility of coagulase-negative staphylococci isolated from milk samples. *Veterinary Microbiology* **150:** 173-179.

SAS (2003). *SAS User's guide version 9*. USA: Statistical Analysis System Institute Inc., Cary, NC, USA.

Sawant, A.A., Gillespie, B.E. and Oliver, S.P. 2009. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. *Veterinary Microbiology* **134:** 73-81.

Sawant, A.A., Sordillo, L.M. and Jayarao, B.M. 2005. A survey on antibiotic usage in dairy herds in Pennsylvanian. *Journal of Dairy Science* **88:** 2991-2999.

Schukken, Y.H., Gonzalez, R.N., Tikofsky, L.L., Schulte, H.F., Santisteban, C.G., Welcome, F.L., Bennet, G. J., Zurakowski, M.J. and Zadoks, R.N. 2009. CNS mastitis: Nothing to worry about? *Veterinary Microbiology* **134:** 9-14.

Shekhan, M.I., Al- Rodhan, M.A. and AL- Janabi, J.K. 2011. Isolation and identification of *Staphylococcus ssp*. from Bovine mastitis milk and their Sensitivity to some Antibiotics at Al- Qadissiya Province. *AL – Qadisiya Journal of Veterinary Medical Science* **10:** 12-20.

Shpigel, N.Y., Winkler, M., Ziv, G. and Saran, A. 2000. Clinical, bacteriological and epidemiological aspects of clinical mastitis in Israeli dairy herds. *Preventive Veterinary Medicine* **35:** 1-9.

Simojoki, H., Salomaki, T., Taponen, S., Livanainen, A and Pyorala, S. 2011. Innate immune response in experimentally induced bovine intramammary infection with *Staphylococcus simulans* and *S. epidermidis*. *Veterinary Research* **42:** 49-59.

Smith, K.L. and Hogan, J.S. 2001. The world of mastitis. In: Proceedings of the second international symposium mastitis and milk quality, Vancouver, BC, Canada, September 13-15, 1-12.

Supre, K., Haesebrouck, F., Zadoks, R.N., Vaneechoutte, M., Piepers, S. and De Vliegher, S. 2011. Some coagulase-negative *Staphylococcus* species affect udder health more than others. *Journal of Dairy Science* **94:** 2329-2340.

Taponen, S., Koort, J., Bjorkroth, J. and Pyorala, S. 2008. Coagulase-negative staphylococci isolated from bovine extramammary sites and intra-mammary infections in a single dairy herd. *Journal of Dairy Research* **75:** 422-429.

Taponen, S., Koort, J., Bjorkroth, J., Saloniemi, H. and Pyorala, S. 2007. Bovine intramammary infections caused by coagulase staphylococci may persist throughout

lactation according to amplified fragment length polymorphism-based analysis. *Journal of Dairy Science* **90:** 3301- 3307.

Taponen, S. and Pyorala, S. 2009. Coagulase negative staphylococci as cause of bovine mastitis- not so different from *Staphylococcus aureus*? *Veterinary Microbiology* **134:** 29-36.

Taponen, S., Simojoki, H., Haven, M., Larsen, H.D. and Pyorala, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase- negative staphylococci identified with API or AFLP. *Veterinary Microbiology* **115:** 199-207.

Tenhagen, B.A., Koster, G., Wallmann, J. and Heuwieser, W. 2006. Prevalence of mastitis and their resistance against antimicrobial agents in dairy cows Brandenburg, Germany. *Journal of Dairy Science* **89:** 2542-2551.

Thorberg, B.M., Danielsson-Tham, M.L., Emanuelson, U. and Waller, K.P. 2009. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *Journal of Dairy Science* **92:** 4962-4970.

Waller, K.P., Aspan, A., Nyman, A., Persson, Y. and Andersson, U.G. 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Veterinary Microbiology* **152:** 112-116.

Walther, C., Rossano, A., Thomann, A. and Perreten, V. 2008. Antimicrobial resistance in lactococcus species from bovine milk: Presence of a matured mult-drug transporter mdt (A) gene in susceptible lactococcus garvieae strains. *Veterinary Microbiology* **131:** 348-357.

World Health Organization, 2000. Overcoming antimicrobial resistance. World Health Organization report on infectious disease 2000. http//www.who.int/infectious-disease-report/2000/index.html.

Zhang, S. and Maddox, C.W. 2000. Cytotoxic activity of coagulase-negative staphylococci in bovine mastitis. *Journal of Infection and Immunity* **68:** 1102-1108.

CHAPTER 4

In-vitro antibacterial activity of *Usnea barbata* lichen extracted with methanol and ethyl- acetate against selected *Staphylococcus species* from bovine milk

Abstract

This study aimed at evaluating the antimicrobial potential of *Usnea barbata* lichen as a medicinal plant against selected *Staphylococcus* species isolated from raw milk of mastitis cows. *In-vitro* screening of the methanolic and ethyl-acetate extracts of *U. barbata* were evaluated to determine their antimicrobial activity against thirteen different *Staphylococcus* species. The selected organisms were isolated from raw bovine milk by several biochemical tests and identified with an API® staph kit (bioMerieux, France). The antimicrobial activity of the extracts were evaluated using both the agar well diffusion method and the broth micro-dilution technique to determine the mean zone of inhibition and the minimum inhibitory concentration (MIC), respectively. Both the methanolic and ethyl-acetate extracts showed variable antimicrobial activity against the *Staphylococcus* species with mean zones of inhibition ranging from 0 - 34 mm in diameter. Susceptibility by the *Staphylococcus* species tested in the methanol and the ethyl-acetate extract was 92.31% and 53.85%, respectively. The MIC result for the methanol extract ranged from 0.0390 to10 mg/ml, while that of the ethyl-acetate extract ranged from 0.15625 to 5 mg/ml. Results from this study revealed the *in vitro* microbial activity of *U. barbata* extracts which indicate its potential as a medicinal plant to be used in traditional medicine.

Key words: Antimicrobial resistance, mastitis, microbial activity, medicinal plant.

4.1. Introduction

Mastitis, an inflammation of the mammary gland especially in dairy animals is known to be a huge threat due to the resistance of several causative organisms to antimicrobial agents, leading to considerable economic losses in farms (Halasa *et al*., 2009). Mastitis in cows could be clinical or subclinical in nature. Clinical mastitis can be visibly seen from the appearance of raw milk of cows but in subclinical mastitis there is no apparent change in milk (Gianneechini *et al*., 2002). *Staphylococcus* species are among the several pathogenic organisms that cause mastitis and are known to be resistant to a wide range of antimicrobial agents in several parts of the world (Pyorala and Taponen, 2009; Sampimon *et al*., 2011). In South Africa for example, a study conducted by Petzer *et al*. (2009) indicated that *Staphylococcus* species including *Staphylococcus aureus* and coagulase negative staphylococci (CNS) are the most prevalent pathogens (with increasing trend on a yearly bases) that cause mastitis in dairy cows and they have also become important mastitis causing pathogens.

There are different *Staphylococcus* species that may be involved in the cause of mastitis in a dairy farm. There is however sparse or no information on the sensitivity of these bacteria species (isolated from bovine milk) to extracts of *U. barbata*.

Several bacterial resistant organisms are reported to surpass the effect of newly developed antibiotic drugs (Gould, 2009). The rise in the trend of resistance by mastitis causing organisms to antibiotics is posing a serious challenge to the society (Tiwari *et al*., 2013). However, some plants have been discovered to possess active antimicrobial ingredients which can be effective to treat various ailments both in humans and animals (Alviano and Alviano, 2009). Antimicrobials are widely used for treatment of various ailments both in humans and animals. Over the years, the continuous use of antimicrobials in the dairy sector has led to the emergence of resistant strains of several pathogens that are linked to the cause

of mastitis disease in animals (Pitkala *et al*., 2004). Mastitis disease causing organisms develop resistance to antimicrobials through a systematic process which make them to withstand the effect of drugs used against them (Suleiman *et al*., 2010). Even though several efforts have been made to combat the emergence of these organisms, by way of introducing new antimicrobial agents there is yet no lasting solution to the problem at present (Gould, 2009). According to the World Health Organization (WHO, 2011), the global challenge of antimicrobial resistant to drugs is bothersome. Thus, the search for other effective drugs to address the situation is worthwhile (Dhanalakshmi *et al*., 2013).

Many small-scale farmers have resorted to the use of herbal plants as alternatives instead of the conventionally known antimicrobial drugs to treat their animals (Masika *et al*., 2000). This practice has increased greatly in Africa. According to Ndip *et al*. (2007), Africa has the highest record of herbal plants usage as options for treating various diseases both in humans and in animals. Over 80% of Africans use plants as an alternative to treat different kinds of diseases (Madamombe and Afolayan, 2003). Several studies in different countries of the world have evaluated the use of *U. barbata* plant as a remedy to treat diseases in animals and humans because they possess some potential natural antimicrobial agents (Kirmizigul *et al*., 2003; Rankovic *et al*., 2012). *Usnea barbata* lichens are epiphytes that grow on leaves of other trees and plants in a symbiotic relationship. Like other lichens they can also grow on rocks and soils with very low nutrient content (Vrablikova *et al*., 2006). The use of *U. barbata* for the treatment of mastitis by rural dwellers in the Eastern Cape of South Africa was reported by Madamombe and Afolayan (2003).

Presently, we are unaware of any study that has evaluated the antimicrobial activity of *U. barbata* plant on *Staphylococcus* species isolated from raw milk of cows with mastitis. The

current study aimed to evaluate the *in-vitro* antimicrobial activity of methanolic and ethyl-acetate extracts of *U. barbata* lichens on some selected *Staphylococcus* species isolated from milk of cows. Virtually all the *Staphylococcus* species tested in our study have been implicated as potential bacterial organisms involved in mastitis disease of livestock animals (Taponen *et al*., 2006; Sampimon *et al*., 2011; Kot *et al*., 2012). In addition, the increasing trend in the prevalence of these bacterial organisms in our environment causing mastitis disease in cows as earlier indicated by Petzer *et al*. (2009) necessitated the study.

4.2. Materials and Methods

4.2.1. Study site

The study site, milk collection, isolation and identification of *Staphylococcus* species are described in Chapter 3.

4.2.2. Plant sample and extracts preparation

Usnea barbata was harvested from the Hogback forest (32°C 35′S 26°C 57′E) about 30 Km from Alice town, Eastern Cape, South Africa. The *U. barbata* lichen was selected based on the information of their use for the treatment of cattle with mastitis by farmers in that area. Identification of the plant was done at the University of Fort Hare where voucher specimens (Idah 2000/2) have been deposited. The *U. barbata* lichen was air-dried at room temperature (20- 25°C) for 10 days and thereafter ground into powder before it was serially extracted with methanol and ethyl acetate solvent, respectively. Extraction was done using a portion of 400g of the *U. barbata* plant lichen in an extraction bottle before adding the solvents (methanol and ethyl-acetate) and then shaken for 24 hours in a shaker (Edison, N.J., USA). After 24 hours, the mixture was centrifuged at 1500 rpm for 10 minutes and filtered using a Whatman

No. 1 filter paper. Filtrate was concentrated to dryness under reduced pressure at 40° C in a rotary evaporator (Strike 202, Steroglass Italy). Extracts were stored in a tight lid container for further use.

4.2.3. Test for antimicrobial sensitivity

The agar well diffusion technique was used to test for the antimicrobial sensitivity of *Staphylococcus* bacteria to plant extracts. Break point with an inhibition zone of diameter of ≥ 11 was chosen for bacterial susceptibility for the plant extracts and the antibiotic (Nyenje and Ndip, 2011). Few colonies of bacteria isolates from freshly prepared (within 24 hours) nutrient agar were added to sterile normal saline in a test tube with turbidity adjusted to 1 x 10^8 CFU/ml (McFarland standards) to make the required inocula for the experiment (Ndip *et al*., 2007). A sterile cotton swab was thereafter used to inoculate the standardized bacterial suspension in a radial pattern on Mueller Hinton agar plates (Oxoid, Basingstoke, England). The plates were after wards left to dry for about 5-10 minutes. Holes of about 6 mm in diameter were then aseptically punched to make wells (5 holes per plate) in the Mueller Hinton agar with the aid of a sterile cork borer. Each well was thereafter filled with 50 µl of extract at different concentrations (5, 10 and 20 mg/ml). The extracts (methanol and ethyl acetate) were dissolved in 5% dimethyl sulphoxide (DMSO) before adding them into the wells. Each plate was made in triplicate and left for 30 minutes for sufficient diffusion of the extracts into the agar before they were incubated at 37°C for 24 hours. The zones of inhibition were measured to the nearest millimetre after 24 hours. The mean zone of inhibition was calculated for each solvent; 0.01mg/ml of amoxicillin was used as positive control while Dimethyl sulphoxide (5%) was used as negative control.

4.2.4. Determination of minimum inhibitory concentration (MIC) of plant extracts

Minimum inhibitory concentration (MIC) of the plant extracts against the bacterial species was determined using the broth micro-dilution method in 96-well micro-titer plates. Series of dilutions were made for the extracts and for the standard antibiotic (amoxicillin) with concentrations ranging from 0.004875 to 10 mg/ml in 5% DMSO. The DMSO solution was dissolved in sterile distilled water to the desired concentration. Two-fold serial dilution of Mueller Hinton broth from stock solution (10 mg/ml) was prepared in the micro-titer wells and a standardized bacterial suspension (20µl) added into the wells except for the control wells, which contained broth and sterile distilled water, respectively. Plates were then incubated for 24 hours at $37^{o}C$. A drop of rezasurin solution as an indicator was added to the micro titer wells to indicate the growth. Bacterial growth was indicated by a colour change from purple to pink. The least concentration in the wells of the test solution that led to inhibition of growth was taken as the MIC.

4.2.5. Statistical analysis

The SPSS version 17.0 (Illnois USA, 2009) was used for statistical analysis. One way ANOVA test was used to evaluate if there was any significant difference in the diameter of zones of inhibition of the plant extracts and the standard antibiotic (amoxicillin). Statistical significance was considered at $p < 0.05$.

4.3. Results

The zones of inhibition for methanol extract ranged from 10 to 34 mm while that of ethyl-acetate ranged from 0 to 23 mm (Table 4.1). Amoxicillin (0.01 µg/ml) which was used as a positive control gave a zone of inhibition in the range of 17 to 47 mm (Table 4.1). The

DMSO (5%) used as negative control showed no activity. With reference to the break point (inhibition zone diameter ≥ 11), six out of the thirteen bacterial strains were the most resistant organisms to methanol and ethyl-acetate extract viz, *Staphylococcus haemolyticus*, *Staphylococcus capitis*, *Staphylococcus cohnii-urealyticus*, *Staphylococcus cohnii-cohnii*, *Staphylococcus hominis* and *Staphylococcus saprophyticus*. On the other hand, four strains: *Staphylococcus xylosus*, *Staphylococcus sciuri*, *Staphylococcus lentus* and *Staphylococcus epidermidis*, were the most susceptible organisms. There was statistical significance in the mean zone of inhibition of the standard antibiotic (amoxicillin) and the plant extracts (methanol and ethyl-acetate) at $p < 0.05$ (Table 4.1).

Out of the thirteen *Staphylococcus* species that were tested, the susceptibility of the bacterial organisms to the standard antibiotic (amoxicillin) and methanol extract was 100% and 92.31%, respectively while that of the ethyl-acetate extract was 53.85% (Figure 4.1). The MIC results showed that methanol and ethyl-acetate had an antimicrobial activity ranging from 0.0390 to 10mg/ml and 0.15625 to 5mg/ml, respectively while the MIC for amoxicillin on the other hand had a ranged of 0.625 to10 µg/ml (Table 4.2).

Table 4.1 Zone of inhibition (mm) of *U. barbata* extracts and amoxicillin against the test organisms.

	Solvent extracts of *Usnea barbata* plant (mg/ml)						
	Methanol			Ethyl-acetate			Amoxicillin
Species	5	10	20	5	10	20	0.01
S. aureus	14 ±1	NA	NA	0 ± 0	0 ± 0	15 ± 0.58	23 ± 1.5
S. sciuri	15 ±1	17 ± 2	NA	13	14 ± 1.8	15 ± 3	30 ± 0.57
S. xylosus	34 ± 1.7	NA	NA	23 ± 1.2	NA	NA	47 ± 1.5
S. chromogenes	17 ± 1	18 ± 0.57	NA	13 ± 0.7	15 ± 1.7	NA	21 ± 3.1
S. lentus	14 ± 1.7	17 ± 1	19	13 ± 0.57	14 ± 1.7	NA	17 ± 0.57
S. cohnii [a]	10 ± 1.5	NA	NA	12	14 ± 0.9	19 ± 1.5	39 ± 1.5
S. haemolyticus	16 ± 0.6	NA	NA	8 ± 7.5	9 ± 6.3	14 ± 1.7	27 ± 1.5
S. capitis	16 ± 14	NA	NA	0	9 ± 6	10 ± 8	30 ± 6.5
S. epidermidis	26 ± 1.5	NA	NA	16 ± 2	19 ± 1.6	21 ± 0.6	37 ± 2.1
S. warneri	22 ± 3	NA	NA	14 ± 0.5	15 ± 0.4	16 ± 2	40 ± 0.6
S. cohnii [b]	18 ± 1.7	NA	NA	0 ± 0	7 ± 5	9 ± 7	36 ± 1.1
S. hominis	22 ± 1.7	NA	NA	0 ± 0	14 ± 1.4	17 ± 2.5	39 ± 1
S. saprophyticus	14 ± 1	NA	NA	0 ± 0	10 ± 6	12 ± 10	20 ± 2

(*S. cohnii* [a]: *Staphylococcus cohnii – cohnii*, *S. cohnii* [b]: *Staphylococcus cohnii – urealyticus*).

(NA: Not Available). (* values are in mean, ± standard deviation, n= 3).
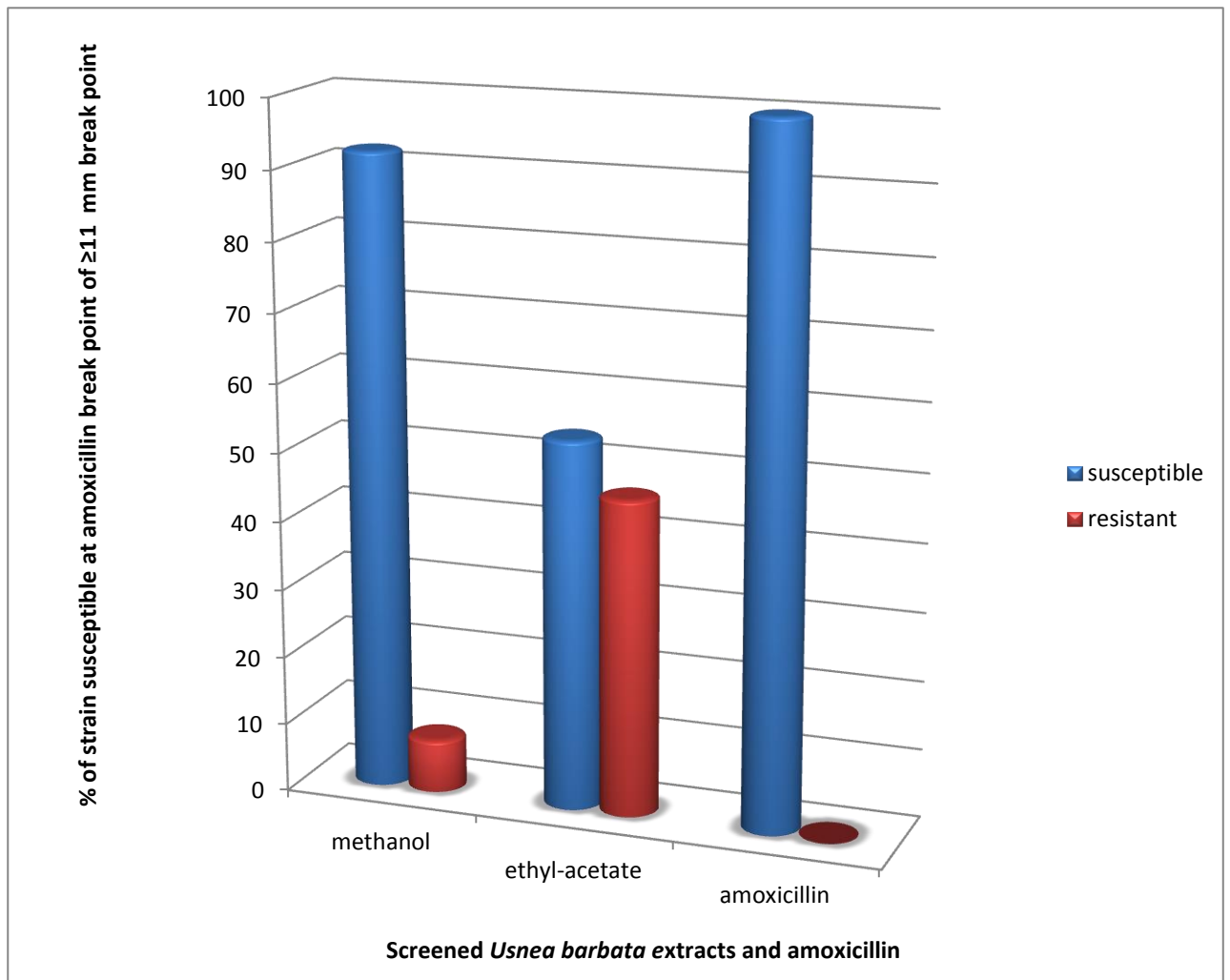
Figure 4.1 Sensitivity of test organisms to amoxicillin, methanol and ethyl-acetate extracts at 5mg/ml.

Table 4.2 Antibacterial activity of *Usnea barbata* extracts and amoxicillin against the test organisms.

	MIC[a] (mg/ml)		
Staphylococcus species	Methanol	Ethyl-acetate	Amoxicillin
S. aureus	1.25	2.5	6.25×10^{-4}
S. sciuri	3.125×10^{-1}	1.5625×10^{-1}	3.125×10^{-4}
S. xylosus	na[b]	na	7.8125×10^{-5}
S. chromogene	10[c]	1.25	6.25×10^{-4}
S. lentus	10	6.25×10^{-1}	5×10^{-3}
S. cohnii- cohnii	3.125×10^{-1}	3.125×10^{-1}	2.5×10^{-3}
S. haemolyticus	6.25×10^{-1}	6.25×10^{-1}	1.25×10^{-3}
S. capitis	6.25×10^{-1}	1.5625×10^{-1}	1.562^{-4}
S. epidermidis	1.5625×10^{-1}	3.125×10^{-1}	1.562^{-4}
S. warneri	3.90×10^{-2}	3.125×10^{-1}	2.5×10^{-3}
S. cohnii-urealyticus	3.125×10^{-1}	3.125×10^{-1}	6.25×10^{-4}
S. hominis	6.25×10^{-1}	2.5	1.0×10^{-2}
S. saprophyticus	10	5	5×10^{-3}

[a] minimum inhibitory concentration, [b] Not active, [c] highest concentration of extract tested.

4.4. Discussion

The screening of the antimicrobial properties of *U. barbata* extracts in the current study indicated a significant activity with a range between 0 to 34 mm against all the *Staphylococcus* species that were tested. Most papers have reported the antimicrobial activity of *U. barbata* on bacteria and fungi isolated from human isolates (Weckesser *et al*., 2006; Kala and Senthilkuma, 2010; Wendakoon *et al*., 2012) but, information regarding the use of *U. barbata* extracts on bacteria isolates from animal origin (raw milk) is sparse. In the current study, the range of mean zone of inhibition for all organisms tested was between 0 to 34 mm. The least mean zone of inhibition was observed for *Staphylococcus cohnii- urealyticus*, *Staphylococcus haemolyticus* and *Staphylococcus capitis* while the highest zone of inhibition was observed for *Staphylococcus xylosus*, *Staphylococcus sciuri*, *Staphylococcus lentus* and *Staphylococcus epidermidis*.   In another study by Wendakoon *et al*. (2012), it was observed that the mean zone of inhibition for *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates (from human origin) was between 11 to 32 mm; with chemical compounds including flavonoids, tannins, lignins and phenolic acid extracted from the lichen mentioned to be responsible for inhibiting the growth of the bacteria. These chemical compounds are known to initiate antimicrobial activities in many plants because they produce biological effect on micro-organisms.

Iwalewa *et al*. (2007) reported that, saponins from plant extracts produce inhibitory effect on inflammatory disease of humans and animals. Rankovic *et al*. (2012) in their study identified usnic acid, norstictic acid, atranorin and chloroatranorin from acetone extract (of *U. barbata* lichen) as the active compounds that may be responsible for antimicrobial activity to bacteria. The differences in the inhibitory activity to the different *Staphylococcus* species in the current study may be due to the structural make up of the bacterial cells. The thickness of bacterial

cell membranes plays a role in the effectiveness (antimicrobial and bactericidal) of antibiotics when they are tested against bacteria *in-vitro* (Fennell *et al*., 2004; Okeleye *et al*., 2013). However, the inhibitory activities of the *U. barbata* lichen against the tested organisms in the current study indicate that they could be effective for treatment of cattle with mastitis disease caused by the selected *Staphylococcus* species.

There is a dearth of information in literatures on the bacterial effect of ethyl-acetate extracts from *U. barbata* lichens. Though, ethyl-acetate has been used as an extraction solvent for other plants including *Peltophorum africanum* and *Combretum molle* (Okeleye *et al*., 2010; Nyenje and Ndip, 2011), but not with *U. barbata* lichen. Extracts of *U. barbata* lichen from other solvents (such as acetone, methanol, carbon dioxide, water) have also been reported to be active against several bacteria organisms including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli* and *Micrococcus viradans* (Madamombe and Afolayan, 2003; Cansaran *et al*., 2006; Weckesser *et al*., 2006; Rankovic *et al*., 2012; Wendakoon *et al*., 2012). In the current study, a good proportion of the bacterial species were susceptible to both the methanol and the ethyl-acetate extracts. All the bacterial organisms that were screened in the standard antibiotic (amoxicillin) were susceptible while the susceptibility of the tested bacterial organisms in methanol and ethyl-acetate extracts gave 92.31% and 53.85% respectively. This may be due to methanol solvent extracting more microbial inhibiting active compounds from the plant lichen because of its high polarity. In the study conducted by Parekh *et al*. (2005), methanol extract from 12 different plants gave a better antimicrobial activity than water (a lesser polar solvent) when screened against 5 different bacteria strain including *Staphylococcus epidermidis*. According to Abu-Shanab *et al*. (2006) and Nyenje and Ndip (2011), organic

solvents effectively extract antimicrobial substances from medicinal plants as compared to other solvents.

Several other studies on the activity of *U. barbata* lichen against bacterial organisms have also been reported. The MIC value observed by Rankovic *et al*. (2012) in their study ranged between 0.125 and 12.5 mg/ml for ten different bacterial organisms including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Bacillus subtilis* in acetone extract which is close to the result in the current study. Madamombe and Afolayan (2003) and Cansaran *et al*. (2006), also reported a significant antimicrobial activity with MIC value as low as 0.1mg/ml for *Staphylococcus aureus* in methanol and acetone extract. However, the MIC value for the methanol extract against *Staphylococcus warneri* in the current study was as low as 0.039 mg/ml though the overall activity of both extracts for all the tested organisms varies between 0.039 - 10 mg/ml. This result suggests that *U. barbata* lichen extracted with methanol and ethyl-acetate solvents possess some potential antimicrobial compound that inhibited the tested organisms and may be broad spectrum. Conversely, the variation in activity of the selected bacterial organisms screened in the *U. babarta* lichen (extract) may not be clearly understood in the current study. Further investigation on the activity of this plant against the tested microbial organisms (using other solvents) may be required in the present search for new antimicrobial drugs.

4.5. Conclusion

The methanol and ethyl-acetate extracts of *U. barbata* exhibited *in-vitro* antimicrobial activities with the methanol extracts being more active. The results of the antimicrobial activity of *U. barbata* in the current study also suggest the rationale behind the traditional use of *U. barbata* lichen for the treatment of mastitis in cattle by local farmers in the Eastern Cape Province of South Africa. It is therefore proposed that further investigation should be

carried out on the plant lichen to determine the natural bioactive compounds present in the plant.

4.6. References

Abu-Shanab, B., Adwan, G., Jarrar, N., Abu-Hijleh, A. and Adwan, K. 2006. Antimicrobial activity of four plant extracts used in Palestine in folkloric medicine against methicillin-resistant *Staphylococcus aureus*. *Turkish Journal of Biology* **30:** 21-23.

Alviano, D.S. and Alviano, C.S. 2009. Plant Extracts: Search for New Alternatives to Treat Microbial Diseases. *Current Pharmaceutical Biotechnology* **10:** 106-121.

Cansaran, D., Kahya, D., Yurdakulol, E. and Atakol, O. 2006. Identification and quantification of Usnic Acid from the Lichen *Usnea* Species of Anatolia and Antimicrobial Activity. *Zeitschrift fur Naturforschung* **61c:** 773-776.

Dhanalakshmi, D., Dhivya, R. and Manimegalai, K. 2013. Antibacterial activity of selected Medicinal plants from South India. *Hygeia: Journal for drugs and medicines* **5:** 63-68.

Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I, Elgorashi, E.E., Grace, O.M. and van Staden, J. 2004. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *Journal of Ethno-Pharmacology* **94:** 205-217.

Gianneechini, R., Concha, C., Rivero, R., Delucci, I. and Moreno Lopez, J. 2002. Occurrence of Clinical and Sub-Clinical Mastitis in Dairy Herds in the West Littoral Region in Uruguay. *Acta Veterinary Scandinavica* **43:** 221-230.

Gould, I.M. 2009. Antibiotic resistance: the perfect storm. *International Journal of Antimicrobial Agents* **34:** S2-S5.

Halasa, T., Nielen, M., De Roos, A.P.W., Van Hoorne, R., de Jong, G., Lam, T.J.G.M., van Werven, T. and Hogeveen, H. 2009. Production loss due to new subclinical

mastitis in Dutch dairy cows estimated with a test-day model. *Journal of Dairy Science* **92:** 599-606.

Iwalewa, E.O., McGaw, L.J., Naidoo, V. and Eloff, J.N. 2007. Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. *African Journal of Biotechnology* **25:** 2868-2885.

Kala, S. and Senthilkumar, S. 2010. Antimicrobial activity of *Acanthephippium bicolour*, Lindley. *Malaysian Journal of Microbiology* **6:** 140-148.

Kirmizigul, S., Koz, O., Anil, H. and Icli, S. 2003. Isolation and Structure Elucidation of Novel Natural Products from Turkish Lichens. *Turkish Journal of Chemistry* **27:** 493-500.

Kot, B., Piechota, M., Antos-Bielska, M., Zdunek, E., Wolska, K.M., Binek, T., Olszewska, J., Gulinski, P., Trafny, E.A. 2012. Antimicrobial resistance and genotypes of staphylococci from bovine milk and the cowshed environment. *Polish Journal of Veterinary Sciences* **15:** 741-749.

Madamombe, I.T. and Afolayan, A.J. 2003. Evaluation of Antimicrobial Activity of Extracts from South Africa *Usnea barbata*. *Pharmaceutical Biology* **41:** 199-202.

Masika, P.J., Van Averbeke, W. and Sonandi, A. 2002. Use of herbal remedies by small-scale farmers to treat livestock diseases in central Eastern Cape Province, South Africa. *Journal of South African Veterinary Association* **71:** 87-91.

Ndip, R.N., Tarkang, A.E.M., Mbullah, S.M., Luma, H.N., Malongue, A., Ndip, L.M., Nyongbela, K., Wirmum, C. and Efange, S.M.N. 2007. *In vitro* anti- *Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. *Journal of Ethinopharmacology* **114:** 452-457.

Nyenje, M. and Ndip, R.N. 2011. *In- vitro* antimicrobial activity of crude acetone extract of the stem bark of *Combretum molle* against selected bacterial pathogens. *Journal of Medicinal Plants Research* **5:** 5315-5320.

Okeleye, B.I., Mkwetshana, N.T. and Ndip, R.N. 2013. Evaluation of the Antimicrobial and Antifungal Potential of *Peltophorum africanum*: Toxicological Effect on Human Chang Liver Cell Line. *The Scientific World Journal*, http://dx.doi.org/10.1155/2013/878735 .

Okeleye, B.I., Samie, A., Bessong, P.O., Mkwetshana, N.F., Green, E., Clarke, A.M. and Ndip, R.N. 2010. Crude ethyl acetate extract of the stem bark of *Peltophorum africanum* (Sond, Fabaceae) possessing *in vitro* inhibitory and bactericidal activity against clinical isolates of *Helicobacter pylori*. *Journal of Medicinal Plants research* **4:** 1432-1440.

Parekh, J., Jadeja, D. and Chanda, S. 2005. Efficacy of Aqueous and Methanol Extracts of Some Medicinal Plants for Potential Antibacterial Activity. *Turkish Journal of Biology* **29:** 203-209.

Petzer, I.M., Karzis, J., Watermeyer, J.C., van der Schans, T.J. and van Reenen, R. 2009. Trends in udder health and emerging mastitogenic pathogens in South Africa dairy herds. *Journal of South Africa Veterinary Association* **80:** 17-22.

Pitkala, A., Haveri, M., Pyorala, S., Myllys, V. and Honkanen-Buzalski, T. 2004. Bovine Mastitis in Finland 2001- Prevalence, Distribution of Bacteria, and Antimicrobial Resistance. *Journal of Dairy Science* **87:** 2433-2441.

Pyorala, S. and Taponen, S. 2009. Coagulase-negative staphylococci- Emerging mastitis pathogens. *Veterinary Microbiology* **134:** 3-8.

Rankovic, B., Kosanic, M., Stanojkovic, T., Vasiljevic, P. and Manojlovic, N. 2012. Biological Activities of *Toninia candida* and *Usnea barbata* Together with Their Norstictic Acid and Usnic Acid Constituents. *International Journal of Molecular Sciences* **13:** 14707- 14722.

Sampimon, O.C., Lam, T.J.G.M., Mevius, D.J., Schukken, Y.H. and Zadoks, R.N. 2011. Antimicrobial susceptibility of coagulase- negative staphylococci isolated from bovine milk samples. *Veterinary Microbiology* **150:** 173-179.

Suleiman, M.M., McGaw, L.J., Naidoo, V. and Eloff, J.N. 2010. Detection of antimicrobial compounds by bio-autography of different extracts of leaves of selected South African tree species. *African Journal Traditional, Complementary and Alternative Medicines* **7:** 64-78.

Taponen, S., Simojoki, H., Haveri, M., Larsen, H.D. and Pyorala, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Veterinary Microbiology* **115:** 199-207.

Tiwari, J.G., Babra, C., Tiwari, H.K., Williams, V., De Wet, S., Gibson, J., Paxman, A., Morgan, E., Costantino, P., Sunagar, R., Isloor, S. and Mukkur, T. 2013. Trends In Therapeutic and Prevention Strategies for Management of Bovine Mastitis: An Overview. *Journal of Vaccines and Vaccination* **4:** 176-187.

Vrablikova, H., McEvoy, M., Solhaug, K.A., Bartak, M., Guaslaa, Y. 2006. Annual variation in photoacclimation and photoprotection of the photobiont in the foliose lichen *Xanthoria parietina. Journal of Photochemistry and Photobiology B: Biology* **83:** 151-162.

Weckesser, S., Engel, K., Simon-Haarhaus, B., Wittmer, A., Pelz, K. and Schempp, C.M. 2006. Screening of plant extracts for antimicrobial activity against bacteria and yeast with dermatological relevance. *Phytomedicine*, doi: 10.1016/j.phymed.2006.12.013.

Wendakoon, C., Calderon., P. and Gagnon, D. 2012. Evaluation of Selected Medicinal Plants Extracted in Different Ethanol Concentrations for Antibacterial Activity against Human Pathogens. *Journal of Medicinally Active Plants* **1:** 60-68.

World Health Organization, 2011. Tackling antibiotic resistance from a food safety perspective in Europe. http//www.euro.who.int/

CHAPTER 5

In vitro study on the antibiogram pattern of _Staphylococcus aureus_ and CNS species isolated from bovine milk in a commercial dairy farm

Abstract

The study aimed to screen milk samples for the presence of _Staphylococcus aureus_ and Coagulase Negative Staphylococci (CNS) and also to evaluate the antimicrobial sensitivity pattern of _Staphylococcus_ species to some selected antibiotics commonly used for the control of mastitis. An aggregate of 250 milk samples were collected from lactating cows in a commercial dairy farm over a period of nine months (March – November, 2012). Identification of bacterial isolates was done using several biochemical techniques and the API$^{®}$ staph kit (bioMerieux, France) was used for confirmation of the isolates to their species level. The antimicrobial susceptibility pattern of individual _Staphylococcus_ species was determined according to the Clinical Laboratory Standard Institute (CLSI). A total of 62 (24.8%) species isolates were identified. On the whole, 11 different bacterial species were identified including _S. xylosus_ (n= 20), _S. saprophyticus_ (n= 10), _S. aureus_ (n= 8), _S. hominis_ (n= 6), _S. warneri_ (n=4), _S. sciuri_ (n= 4), _S. chromogenes_ (n= 3), _S. lentus_ (n= 3), _S. haemolyticus_ (n= 2), _S. capitis_ (n= 1) and _S. cohnii-cohnii_ (n= 1). The most commonly isolated bacteria were _S. xylosus_ (n= 32.25%), _S. saprophyticus_ (n= 16.12%) and _S. aureus_ (12.90%). Most isolates that were identified were resistant to Penicillin (67.7%), Nalidixic acid (66.1%) and Vancomycin (40.5%). The results from the current study indicate that _Staphylococcus_ species are resistance to beta-lactam antibiotics especially Penicillin which is predominantly used in the prevention and treatment of mastitis in the farm where the current study was conducted without prior antibacterial sensitivity test.

Key words: Mastitis, antimicrobial sensitivity pattern, *Staphylococcus* species.

5.1. Introduction

Mastitis is a complex disease generally common in dairy animals. Several causative agents are known to be involved in mastitis infection (Awale *et al*., 2012). The heavy financial burden caused by mastitis disease on farmers and to the dairy sector as a whole is enormous (Sawant *et al*., 2009). Mastitis has become a global issue due to its adverse effects on cow health, milk quality and milk production level of cows (Seegers *et al*., 2003; Awandkar *et al*., 2009; Sharma *et al*., 2011). The disease can be transferred from an infected cow to a health cow causing increase in intra mammary infections if herds are not properly managed (Tremblay *et al*., 2013). Like other major mastitis causing organism, *Staphylococcus aureus* is a well-known bacterial organism that causes mastitis in cows (Heringstad *et al*., 2000). But, unlike *Staphylococcus aureus*, CNS is usually reported to play an insignificant role in the cause of mastitis in cows (Sawant *et al*., 2009).

Technically in mastitis diagnosis, *Staphylococcus* species are grouped into Coagulase Positive Staphylococci and Coagulase Negative Staphylococci based on their ability to coagulate rabbit plasma (Addis *et al*., 2011). *Staphylococcus aureus* is a Coagulase Positive *Staphylococcus* and has been well reported in literature to be a major mastitis causing pathogen (Bradley, 2002; Fergusson *et al*., 2007). But, on the contrary CNS is commonly reported as a minor mastitis causing pathogen (Heringstad *et al*., 2000; Lim *et al*., 2007). However, recent findings in several nations of the world including South Africa have indicated CNS as a significance mastitis causing agent (Wilson *et al*., 1997; Ferguson *et al*., 2007; Petzer *et al*., 2009; Addis *et al*., 2011; Kenar *et al*., 2012; Kateete *et al*., 2013). They are frequently isolated from milk of cows with clinical and subclinical mastitis and have been linked to cause mastitis in dairy cows (Sampimon *et al*., 2009; Oliveira *et al*., 2013).

The result observed from Chapter 3 gave evidence to the fact that several *Staphylococcus* species could be involved in the cause of mastitis in a dairy farm. However, there could be differences in the incidence of *Staphylococcus* species and their antimicrobial sensitivity to conventional antibiotic from different dairy farms due to different management systems adopted by different farms and also due to differences in farm location. Information on the variation of species specific bacterial organisms linked with mastitis and their antimicrobial sensitivity to antibiotics (in different farms) would assist farmers in implementing effective mastitis control and therapy on their farms.

Modern invention of milking practices has reduced the prevalence of several major mastitis causing organisms including *Staphylococcus aureus* and *Streptococcus agalactiae* in dairy farms (Makovec and Ruegg, 2003). Some studies conducted recently in the United States indicated a widespread of environmental micro-organisms including CNS to be the major cause of clinical mastitis in farms (Lago *et al*., 2011; Pinzon-Sanchez and Ruegg, 2011; Schukken *et al*., 2011). Akin to *Staphylococcus aureus*, CNS can also cause persistent intra mammary infections for a long period of time in cows if not checked (Gillespie *et al*., 2009). Mastitis caused by CNS is mostly prevalent in a farm that has successfully managed the spread of mastitis disease caused by major mastitis causing organisms (Gillespie *et al*., 2009). Coagulase negative staphylococci are known to be a peculiar problem in such farms (Myllys and Rautala, 1995).

In most dairy farms today mastitis disease is considered to be a major reason for the use of antibiotics (Roesch *et al*., 2006; Awandkar *et al*., 2009). This is because farmers are involved in the indiscriminate use of antibiotics without carrying out *in vitro* sensitivity tests leading to

repeated failure in mastitis control and therapy of dairy animals (Kaliwal *et al*., 2011). The continual use of antibiotics has led to the evolution of antibiotic resistance genes by *Staphylococcus* species and increases in financial losses due to the treatment of affected animals (Awandkar *et al*., 2009). *Staphylococcus aureus* and CNS are among the bacterial species that are often resistant to several antibiotics used on farms (Tenhagen *et al*., 2006; Pyorala and Taponen, 2009). To achieve a worthwhile result that can be implemented for effective control of mastitis, bacterial identification and their antibiotic resistance pattern is of high necessity (Kaliwal *et al*., 2011). In view of this, the current study was undertaken to identify and also to determine the antibiogram trend of some selected antibiotics to some *Staphylococcus* species isolated from milk samples of cows with subclinical mastitis in a commercial dairy farm located in the Eastern Cape of South Africa.

5.2. Materials and methods

5.2.1. Study site and milk sample collection

The study was conducted in a commercial dairy farm in Eastern Cape, South Africa. Isolates (n= 62) from 250 milk samples were derived from a collection of 11 different *Staphylococcus* species. Milk samples were collected in a 10 ml sterile bottle from lactating cows for nine months from March to November, 2012. After the milk samples were collected they were promptly kept in cooler boxes containing ice before transporting them to the laboratory for microbial analysis.

5.2.2. Bacterial isolation and identification

Milk samples were screened following the procedures recommended by the National Mastitis Council (NMC). In brief, composite foremilk samples collected from individual cows were streaked in Mannitol salt agar that was prepared within 24 hours and incubated for 24-48 hours for bacteria growth. Mannitol salt agar was prepared according to manufacturers' instructions. Presumed isolates of *Staphylococcus* species gave a yellow fermentation in Mannitol salt agar.

5.2.2.1. Sub-culturing of milk samples in nutrient agar
Presumed isolates that gave a positive result in Mannitol salt agar were sub-cultured again in a freshly prepared nutrient agar (within 24 hours) and they were then placed in an incubator for 24 hours. This is done so as to get a pure colony of the bacteria isolate.

5.2.2.2. Gram's staining test

All presumed cultures of *Staphylococcus* species were subjected to gram's stain. Few colonies of pure cultured isolates were aseptically picked from the nutrient agar and then smeared on a sterile glass slide. The smeared culture was heat fixed to dryness. Crystal violet (0.5%) was added to the smear and left for I minute. Water was used to wash off the stain from the slide. Sufficient solution of iodine was again added to the smear and left for I minute. Water was used to wash off the stain. Ethanol (95%) was afterward used to rinse the stain from the slide. Safrani solution was finally added to the smear for 2 minutes before being washed with water. The smear was allowed to dry for a few minutes before they were viewed under a light microscope to determine the colony structure of the bacteria species. The smear of each bacteria species was viewed under x10, x40 and x100 magnification. Gram's stain smears that were positive gave a grape-like clustered shape with deep blue to purple colour.

5.2.2.3. Oxidase test

Few colonies of isolates from nutrient agar from presumed *Staphylococcus* species were picked aseptically using a sterile loop and then placed on an oxidase test strip paper. All isolates that turned purple on the test strip paper after 10 seconds were suspected to be *Staphylococcus* species.

5.2.2.4. Catalase test

Pure cultures of presumed *Staphylococcus* species from nutrient agar were aseptically transferred onto a clean glass slide. Few drops of 3% H_2O_2 were then added to the cultures.

Positive cultures of *Staphylococcus* species gave out bubbles of oxygen within 5 seconds after adding H_2O_2.

5.2.2.5. API® Staph kit test for identification of Staphylococcus species

All presumed isolates were finally confirmed to their species level using the API® staph kit (bioMerieux, France). Homogeneous Inoculums of pure cultured isolates were prepared with turbidity equivalent to 0.5 McFarland using the API® Staph medium. Pipette was used to fill the micro tubes of the API kit with the inoculated API Staph medium. Mineral oil was added to the arginine dehydrolase (ADH) and urease (URE) tests to ensure anaerobiosis. The incubation box of the API kit was filled with 5mls of distilled water and closed tightly before incubating at 37^0C for 24hrs. After incubation one drop of API Staph reagent was added to the inoculated micro tubes to determine their reactions. The reactions of the inocula were read using the apiweb ™ identification software (bioMerieux, Inc Quebec).

5.2.3. Minimum Inhibitory Concentration (MIC) determination

Isolates that were identified to their species level were subjected to *in-vitro* drug sensitivity test using the discs diffusion method as postulated by the Clinical Laboratory Standard Institution (CLSI, 2006). Briefly, a few colonies of isolates were aseptically inoculated in normal saline to the desired turbidity (0.5 McFarland standards). With the aid of a sterile cotton swab, the inocula were evenly spread on Muller-Hinton agar in a petri dish. The antibiotic discs were then gently pressed on the agar and placed in an incubator for 24 hours at 37° C. The MIC of each isolate was afterward determined by measuring (in millimetres) the zones of inhibition in the Mueller Hinton agar with a metre rule so as to obtain the diameter of inhibition of the different isolates. Among the antibiotics used were

Levofloxacin, Ciprofloxacin, Kanamycin, Meropenem, Ofloxacin, Tetracycline, Gentamicin, Erythromycin, Imipenem, Chlorofenicol, Nalidixic acid, Amoxicillin, Penicillin and Vancomycin.

5.3. Results

Results from the current study showed that a total of 62 (24.8%) isolates were identified from an aggregate of 250 milk samples which were examined from subclinical mastitis cows. Table 5.1 shows the prevalence of the 11 different *Staphylococcus* species that were identified. *In vitro* antibiogram studies were conducted for the 62 isolates that were identified. The result revealed Imipenem to be the most effective drug (98.4%) against the bacterial isolates followed by Gentamycin (95.2%), Tetracycline (90.3%) and Meropenem (88.7%), (Table 5.2). The lowest sensitivity was observed for Erythromycin (30.6%) followed by Penicillin (32.3%), Nalidixic acid (33.9%) and Vancomycin (59.7%). Examining individual *Staphylococcus* species (*Staphylococcus aureus* and CNS), *Staphylococcus aureus* was 100% resistant to Penicillin, *Staphylococcus capitis* was 100% resistant to Nalidixic acid and Penicillin, *Staphylococcus cohnii-cohnii* was 100% resistant to Ciprofloxacin, Erythromycin, Nalidixic acid, Penicillin and Vancomycin while *Staphylococcus haemolyticus* gave 100% resistance to Erythromycin and Penicillin.

Table 5. 1 Prevalence of *Staphylococcus aureus* and CNS isolated from bovine milk.

Staphylococcus species	Number of isolated species (%)
S. aureus	8 (12.90)
S. hominis	6 (9.67)
S. warneri	4 (6.45)
S. xylosus	20 (32.25)
S. sciuri	4 (6.45)
S. lentus	3 (4.83)
S. chromogenes	3 (4.83)
S. saprophyticus	10 (16.12)
S. capitis	1 (1.61)
S. cohnii-cohnii	1 (1.61)
S. haemolyticus	2 (3.22)
Total	62 (100)

Table 5. 2 Antimicrobial susceptibility of *S. aureus* and CNS isolated from bovine milk (%).

Staphylococcus Spp	N	CIP	LEV	OFX	MEM	E	NAL	IMI	VA	PG	GM	T	K	AMO	C
S. aureus	8	4(50)	7(87.5)	4(50)	8(100)	3(37.5)	1(12.5)	7(87.5)	6(75)	0	8(100)	8(100)	8(100)	7(87.5)	6(75)
S. hominis	6	6(100)	6(100)	6(100)	6(100)	3(50)	2(33.3)	6(100)	6(100)	3(50)	6(100)	5(83.3)	6(100)	6(100)	6(100)
S. warneri	4	4(100)	4(100)	4(100)	4(100)	2(50)	2(50)	4(100)	4(100)	4(100)	4(100)	4(100)	2(50)	4(100)	4(100)
S. xylosus	20	16(80)	15(75)	16(80)	19(95)	3(15)	6(37.5)	20(100)	11(55)	3(15)	19(95)	17(85)	17(85)	11(55)	17(85)
S. sciuri	4	1(25)	4(100)	4(100)	2(50)	1(25)	2(50)	4(100)	3(75)	1(25)	4(100)	4(100)	2(50)	3(75)	4(100)
S. lentus	3	2(66.7)	2(66.7)	2(66.7)	3(100)	1(33.3)	1(33.3)	3(100)	1(33.3)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)
S. chromogenes	3	3(100)	3(100)	3(100)	3(100)	2(66.7)	2(66.7)	3(100)	1(33.3)	3(100)	3(100)	3(100)	3(100)	3(100)	3(100)
S. saprophyticus	10	8(80)	10(100)	10(100)	7(70)	3(30)	4(40)	10(100)	3(30)	3(30)	8(80)	8(80)	8(80)	7(70)	8(80)
S. capitis	1	1(100)	1(100)	1(100)	1(100)	1(100)	0	1(100)	1(100)	0	1(100)	1(100)	1(100)	1(100)	1(100)
S. cohnii-cohnii	1	0	1(100)	1(100)	1(100)	0	0	1(100)	0	0	1(100)	1(100)	1(100)	1(100)	1(100)
S. haemolyticus	2	2(100)	2(100)	2(100)	1(100)	0	1(50)	2(100)	1(50)	0	2(100)	2(100)	2(100)	2(100)	1(50)
Total (%)	62	47(75.8)	55(88.7)	53(85.5)	55(88.7)	19(30.6)	21(33.87)	61(98.4)	37(59.7)	20(32.3)	59(95.2)	56(90.3)	53(85.5)	48(77.4)	54(87.1)

N: Number of isolates, CIP: Ciprofloxacin, LEV: Levofloxacin, OFX: Ofloxacin, MEM: Meropenem, E: Erythromycin, NAL: Nalidixic, IMI:

Imipenem, VA: Vancomycin, PG: Penicillin-G, GM: Gentamycin, T: Tetracycline, K: Kanamycin, AMO: Amoxicillin, C: Chlorofenicol.

5.4. Discussion

The prevalence of both *Staphylococcus aureus* and CNS in sub-clinical and/or clinical mastitis may pose a serious threat to cows in a dairy herd (Wilson *et al*., 1997; Tremblay *et al*., 2013). Though, CNS is often reported as a group (Addis *et al*., 2011), several different species exist among them (Sawant *et al*., 2009). It is therefore needful to discuss the individual species differently due to the variation that exist in their clinical significance as mastitis pathogens (Piessens *et al*., 2011; Supre *et al*., 2011).

The findings of the current study showed the highest incidence of *Staphylococcus* species to be *Staphylococcus xylosus* (32.25%) followed by *Staphylococcus saprophyticus* (16.12%) and *Staphylococcus aureus* (12.90%). Both *Staphylococcus xylosus* and *Staphylococcus aureus* have been indicated to show more adhesive ability to their host cells (Rohde *et al*., 2007; Tremblay *et al*., 2013), which may be the reason for their high incidence in the farm. Studies have shown that some *Staphylococcus* species possess the ability to adhere and also to invade their host cells (udder) than others in a process known as biofilm formation; this plays an important role to their prevalence in milk of cows (Rohde *et al*., 2007; Kaliwal *et al*., 2011). In addition, *Staphylococcus xylosus* is known to be a free-living opportunistic bacterium that could easily find its way into the udder of cows (Gillespie *et al*., 2009). Clinical mastitis could result from subclinical mastitis due to persistent incidence of CNS in a farm (Gillespie *et al*., 2009). *The* least frequently isolated CNS species from the current study include *Staphylococcus capitis* (1.61%), *Staphylococcus cohnii-cohnii* (1.61%) and *Staphylococcus haemolyticus* (3.22%). This was similar to the study of Kenar *et al*. (2012) that observed the least frequently isolated organisms from milk to be *Staphylococcus capitis* (1.4%), *Staphylococcus cohnii-cohnii* (2.9%) and *Staphylococcus haemolyticus* (4.4%),

respectively. The CNS that were least isolated could be because they are not well adapted to the udder of the cow (Kaliwal *et al*., 2011).

In another study by Gillespie *et al*. (2009), *Staphylococcus haemolyticus* was indicated to be least commonly isolated from milk samples. Also, Matthews *et al*. (1990) in their study reported *Staphylococcus haemolyticus* and *Staphylococcus cohnii-cohnii* to be the least frequently isolated organisms from milk samples. Contrary to our findings, other studies indicated *Staphylococcus chromogenes* and *Staphylococcus hyicus* as the most commonly isolated CNS species from milk samples (Trinidad *et al*., 1990; Taponen *et al*., 2006; Sawant *et al*., 2009). The high prevalence of *Staphylococcus xylosus* and *Staphylococcus aureus* could be linked to a poor level of hygiene in the farm (Harmon, 1993; Sharma *et al*., 2011).

The antibiotic sensitivity tests carried in the current study showed that there was resistance of some *Staphylococcus* species against one or more antibiotics. Imipenem (98.4%), Gentamycin (95.2%), Tetracycline (90.3%), Chlorofenicol (87.1%), Levofloxacin (88.7%), Ofloxacin (85.5%), Kanamycin (85.5%) and Amoxicillin (77.4%) were observed to be very effective on most of the species isolates. In another study by Zahid (2004), it was observed that Gentamycin, Tetracycline, Chlorofenicol, Kanamycin and Amoxicillin were highly effective against most bacterial isolates that cause mastitis. All the isolates of *Staphylococcus aureus* were resistant to Penicillin. Most of the isolates of *Staphylococcus aureus* were also observed to be resistant to Nalidixic acid and Erythromycin but susceptible to Tetracycline, Ofloxacin and Ciprofloxacin. Sharma *et al*. (2011) in their study also observed similar resistance of *Staphylococcus aureus* to Nalidixic acid and Erythromycin but observed a high susceptibility to Tetracycline, Ofloxacin and Ciprofloxacin. Antibiotics such as Gentamycin, Ciprofloxacin and Chlorofenicol among others are new antimicrobial agents introduced into

the market for treating mastitis cows (Sumathi *et al*., 2008). This could be the reason for their effectiveness against the mastitis pathogens in the current study. Mekuria *et al*. (2013) in their study observed high resistance of *Staphylococcus aureus* to Penicillin but resistance to Chlorofenicol, Gentamycin and Vancomycin was negligible. This is in agreement with the current study. According to Sumathi *et al*. (2008), Penicillin resistant bacteria produce certain enzymes (plasmids mediated beta lactamase) that make them resistant against antibiotics.

Erythromycin was observed to be effective against most CNS species isolates except for *Staphylococcus cohnii- cohnii* and *Staphylococcus haemolyticus*. In a study by Sawant *et al*. (2009), Erythromycin was effective against most CNS species. Kaliwal *et al*. (2011) also indicated in their study that antibiotics including Ciprofloxacin, Erythromycin, Gentamycin and Amoxicillin were effective against several CNS species but, Penicillin was not. Their finding is in line with the present study. Mohanty *et al*. (2013) observed that Chlorofenicol, Levofloxacin and Ciprofloxacin were effective against several *Staphylococcus* species apart from Penicillin that was not effective. This is similar to the current findings. On the other hand, Roesch *et al*. (2006) indicated in their study that most CNS species isolated from milk (in an integrated dairy production farm) were resistant to Erythromycin. Variation exists in the effectiveness of antibiotic against individual CNS species which could in turn affect their prevalence in a farm as a result of difference in antimicrobial recommendation by farmers which vary in different areas (Kenar *et al*., 2012).

Though information about how long Penicillin have been consistently used in the farm is not known, arbitrary use of the drug in the farm of the current study could be the reason for the resistance of several species isolates to the antibiotics (Awandkar *et al*., 2009; Sharma *et al*., 2011). Another possible reason could be that antibiotics administered to animals for treatment

of mastitis may be less in terms of dosage than the recommended dose making the drug ineffective as was observed during the few visits to the farm hence causing microbial resistance build up (Malinowski *et al*., 2002). According to (Rajala-Schultz *et al*., 2009), acquisition of new resistant genes and chromosome mutations by previously susceptible strain of *Staphylococcus* species could also be other reason for antibiotic failure of a particular antibiotic.

5.5. Conclusion

The findings from the current study revealed differences in antimicrobial sensitivity patterns among *Staphylococcus* species. Imipenem was observed to be the most effective antibiotic against the isolated bacterial species while Penicillin was the least effective antimicrobial agent. This may be as a result of the frequent use of Penicillin for mastitis treatment in the farm.

5.6. References

Addis, M., Pal, M. and Kyule, M, N. 2011. Isolation and identification of *Staphylococcus* Species from Raw Bovine Milk in Debre Zeit, Ethiopia. *Veterinary Research* **4:** 45-49.

Awale, M.M., Dudhatra, G.B., Kumar, A., Chauhan, B.N., Kamani, D.R., Modi C.M., Patel, H.B. and Mody S.K. 2012. Bovine Mastitis: A Threat to Economy. *Scientific reports*, 1: 295. doi: 10.4172/scienticreports.295.

Awandkar, S.P., Khode, N.V., Sardar, V.M. and Mendhe, M.S. 2009. Prevalence and Current Antibiogram Trend of Mastitis Agents in Udgir and its Vicinity, Maharashtra State, India. *International Journal of Dairy Science* **4:** 117-122.

Bradley, A.J. 2002. Bovine Mastitis: An Evolving Disease. *The Veterinary Journal* **164:** 116-128.

Clinical and Laboratory Institute Standards (Former: National Committee for Clinical Laboratory Standards). 2006. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests. Approved Standard. 9th ed. NCCLS document M2-A9, NCCLS, Wayne, PA.

Ferguson, J.D., Azzaro, G., Gambina, M. and Licitra, G. 2007. Prevalence of Mastitis Pathogens in Ragusa, Sicily, from 2000-2006. *Journal of Dairy Science* **90:** 5798-5813.

Gillespie, B.E., Headrick, S.I., Boonyayatra, S. and Oliver, S.P. 2009. Prevalence and persistence of coagulase-negative *Staphylococcus* species in three dairy research herds. *Veterinary Microbiology* **134:** 65-72.

Harmon, R.J. 1993. Symposium mastitis and genetic evaluation for somatic cell count. *Journal of Dairy Science* **77:** 2103-2112.

Heringstad, B., Klemetsdal, G. and Ruane, J. 2000. Selection for mastitis resistance in dairy cattle: a review with focus on the situation in the Nordic countries. *Livestock Production Science* **64:** 95-106.

Kaliwal, B.B., Sadashiv, S.O., Kurjogi, M.M. and Sanakal, R.D. 2011. Prevalence and Antimicrobial Susceptibility of Coagulase- Negative *Staphylococci* isolated from Bovine Mastitis. *Veterinary World* **4:** 158-161.

Kateete, D.P., Kabugo, U., Baluku, H., Nyakarahuka, L., Kyobe, S., Okee, M., Najjuka, C.F. and Joloba, M.L. 2013. Prevalence and Antimicrobial Susceptibility Pattern of Bacteria from Milkmen and Cows with Clinical Mastitis in and around Kampala, Uganda. *PLoS ONE*, **8**(5): e63413. doi: 10. 1371/journal.pone.0063413.

Kenar, B., Kuyucuoglu, Y. and Seker, E. 2012. Antibiotic Susceptibility of Coagulase- Negative Staphylococci Isolated from Bovine Subclinical Mastitis in Turkey. *Pakistan Veterinary Journal* **32:** 390-393.

Lago, A., Godden, S.M., Bey, R., Ruegg, P.L. and Leslie, K. 2011. The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes. *Journal of Dairy Science* **94:** 4441-4456.

Lim, G.H., Leslie, K.E., Kelton, D.F., Duffield, T.F., Timms, L.L. and Dingwell, R.T. 2007. Adherence and efficacy of an external sealant to prevent new intrammammary infections in the dry period. *Journal of Dairy Science* **90:** 1289-1300.

Makovec, J.A. and Ruegg, P.L. 2003. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *Journal of Dairy Science* **86:** 3466-3472.

Malinowski, E., Klossowska, A., Kaczmarowski, M., Lassa, H. and Kuzma, K. 2002. Antimicrobial susceptibility of Staphylococci isolated from affected with mastitis cows. *Bulletin of the Veterinary Institute of Pulawy* **46:** 289-294.

Matthews, K.R., Oliver, S.P. and King, S.H. 1990. Comparison of Vitek gram-positive identification system with API Staph-Trac system for species identification of staphylococci of bovine origin. *Journal of Clinical Microbiology* **28:** 1649-1651.

Mekuria, A., Asrat, D., Woldeamanuel, Y. and Tefera, G. 2013. Identification and antimicrobial susceptibility of *Staphylococcus aureus* isolated from milk samples of dairy cows and nasal swabs of farm workers in selected dairy farms around Addis Ababa, Ethiopia. *African Journal of Microbiology Research* **7:** 3501-3510.

Mohanty, N.N., Das, P., Pany, S.S., Sarangi, L.M., Ranabijuli, S. and Panda, H.K. 2013. Isolation and antibiogram of *Staphylococcus*, *Streptococcus* and *Escherichia coli* isolates from clinical and subclinical cases of bovine mastitis. *Veterinary World* **6:**739-743.

Myllys, V. and Rautala, H. 1995. Characterization of Clinical Mastitis in Primiparous Heifers. *Journal of Dairy Science* **78:** 538-545.

Oliveira, L., Hulland, C. and Ruegg, P.L. 2013. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. *Journal of Dairy Science* (In Press, Corrected Proof).

Petzer, I.M., Karzis, J., Watermeyer, J.C., van der Schans, T.J. and van Reenen, R. 2009. Trends in udder health and emerging mastitogenic pathogens in South Africa dairy herds. *Journal of the South African Veterinary Association* **80:** 17-22.

Piessens, V., Van Coillie, E., Verbist, B., Supre, K., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx, M. and 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:** 2933-2944.

Pinzon-Sanchez, C. and Ruegg, P.L. 2011. Risk factors associated with short-term post-treatment outcomes of clinical mastitis. *Journal of Dairy Science* **94:** 3397-3410.

Pyorala, S. and Taponen, S. 2009. Coagulase-negative staphylococci: Emerging mastitis pathogens. *Veterinary Microbiology* **134:** 3-8.

Rajala-Schultz, P.J., Torres, A.H., DeGraves, F.J., Gebreyes, W.A. and Patchanee, P. 2009. Antimicrobial resistance and genotypic characterization of coagulase-negative staphylococci over the dry period. *Veterinary Microbiology* **134:** 55-64.

Roesch, M., Perrenten, V., Doherr, M.G., Schaeren, W., Schallibaum, M. and Blum, J.W. 2006. *Journal of Dairy Science* **89:** 989-997.

Rohde, H., Burandt, E.C., Siemssen, N., Frommelt, L., Burdelski, C., Wurster, S., Scherpe, S., Davies, A.P, Harris, L.G., Horstkotte, M.A., Knobloch, J.K., Ragunath, C., Kaplan, J.B. and Mack, D. 2007. Polysaccharide intercellular adhesion or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials* **28:** 1711-1720.

Sampimon, O.C., Barkema, H.W., Berends, I.M.G.A., Sol, J. and Lam, T.J.G.M. 2009. Prevalence and Herd-level risk factors for intramammary infection with coagulase-negative staphylococci in Dutch dairy herds. *Veterinary Microbiology* **134:** 37-44.

Sawant, A.A., Gillespie, B.E. and Oliver, S.P. 2009. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. *Veterinary Microbiology* **134:** 73-81.

Schukken, Y.H., Bennett, G.J., Zurakowski, M.J., Sharkey, H.L., Rauch, B.J., Thomas, M.J., Ceglowski, B., Saltman, R.L., Belomestnykh, N. and Zadoks, R.N. 2011. *Journal of Dairy Science* **94:** 6203-6215.

Seegers, H., Fourichon, C. and Beaudeau, F. 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. *Journal of Veterinary Research* **34:** 475-491.

Sharma, D., Sharma, P.K. and Malik, A. 2011. Prevalence and Antimicrobial Susceptibility of Drug Resistant *Staphylococcus aureus* in Raw milk of Dairy Cattle. *International Research Journal of Microbiology* **2:** 466-470.

Sumathi, B.R., Veeregowda, B.M. and Gomes, A.R. 2008. Prevalence and antibiogram profile of bacterial Isolations from clinical bovine mastitis. *Veterinary World* **1:** 237-238.

Supre, 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:** 2329-2340.

Taponen, S., Simojoki, H., Haveri, M., Larsen, H.D. and Pyorala, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Veterinary Microbiology* **115:** 199-207.

Tenhagen, B.A., Koster, G., Wallmann, J. and Heuwieser, W. 2006. Prevalence of Mastitis Pathogens and Their Resistance Against Antimicrobial Agents in Dairy Cows in Brandenburg, Germany. *Journal of Dairy Science* **89:** 2542-2551.

Tremblay, Y. D.N., Lamarche, D., Chever, P., Haine, D., Messier, S. and Jacques, M. 2013. Characterization of the ability of Coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *Journal of Dairy Science* **96:** 234-246.

Trinidad, P., Nickerson, S.C. and Alley, T.K. 1990. Prevalence of intramammary infection and teat canal colonization in unbred and primigravid dairy heifers. *Journal of Dairy Science* **73:** 107-114.

Wilson, D.J., Gonzalez, R.N. and Das, H.H. 1997.Bovine Mastitis Pathogens in New York and Pennsylvania: Prevalence and Effects on Somatic Cell Count and Milk Production. *Journal of Dairy Science* **80:** 2592-2598.

Zahid, I.A. 2004. Studies on comparative incidence of sub-clinical and clinical mastitis and *in vitro* antibiotic susceptibility of isolates from Holstein-Friesian and Jersey cows and Buffaloes. *Pakistan Veterinary Journal* **24:** 76-81.

Chapter 6: General Discussion, Conclusions and Recommendations

6.1. General discussion

The objective of the study was to determine the incidence of *Staphylococcus* species distribution in bovine milk of cows with subclinical mastitis in commercial dairy farms and to determine their sensitivity to conventional antibiotics and *U. barbata* extracts. The incidence of *Staphylococcus* species and their antibiotic sensitivity pattern was determined in Chapter 3 and Chapter 5. In Chapter 3, association of some possible risk factor variables such as stage of lactation, parity number and time of year milk samples were examined was also tested against the incidence of *Staphylococcus* species in milk. In Chapter 4, the *in-vitro* inhibitory activity of *U. barbata* extracts (methanol and ethyl-acetate) against selected *Staphylococcus* species was determined.

In Chapter 3 and 5, the incidence of *Staphylococcus* species was observed through the sampling period. The most prevalent *Staphylococcus* species that were isolated from milk samples in Chapter 3 include *Staphylococcus xylosus* (22.09%), *Staphylococcus hominis* (15.11%) and *Staphylococcus haemolyticus* (11.63%). While the most prevalent *Staphylococcus* species isolated from the farm in Chapter 5 include *Staphylococcus xylosus* (32.25%), *Staphylococcus saprophyticus* (16.12%). The prevalence of these *Staphylococcus* species maybe a result of the heavy infestation of these bacteria in the surrounding environment of the farm (Bendahou *et al*., 2008; Piessens *et al*., 2012; Ayano *et al*., 2013). Opportunistic *Staphylococcus* species including *Staphylococcus xylosus*, *Staphylococcus hominis* and *Staphylococcus haemolyticus* from the udder or milking clusters often find their way into the mammary gland of cow udders during milking if the hygiene at the farm is poor (Schukken *et al*., 2009).

In Chapter 3, the prevalence of *Staphylococcus* species isolated in milk varied with the time of the year milk was sampled. In the hot-dry season of the year, it was observed that the incidence of *Staphylococcus* species was higher as compared with the other seasons. Climatic variation which may have affected the population of some bacterial species could be the reason for our observation (Piessens *et al*., 2012; Sampimon *et al*., 2011). There was a significant association (p= 0.0039) between the incidence of *Staphylococcus* species and the stage of lactation that was observed in Chapter 3. The level of cleanliness of cows at different stages of lactation may vary, which could have been attributed to our observation (Abera *et al*., 2010).

High resistance of *Staphylococcus* species was observed for Penicillin, Ampicillin and Nalidixic acid in Chapter 3. Resistance to Penicillin and Ampicillin by several *Staphylococcus* species in Chapter 3 could a result of frequent use of the antibiotics in the farm. Penicillin is one of the mostly used antibiotics in the farms where this study was conducted. According to Awandkar *et al*. (2009) and Sharma *et al*. (2011), indiscriminate use of the same antibiotic to treat mastitis disease in cows could lead to the development of a resistant strain against the drug by the causative pathogen.

In Chapter 4, there was significant activity of the *U. barbata* extracts against several *Staphylococcus* species including *Staphylococcus xylosus*, *Staphylococcus sciuri*, *Staphylococcus lentus* and *Staphylococcus epidermidis* with zone of inhibition ranging from 13 to 34 mm. With reference to ≥ 11mm, about half of the total *Staphylococcus* species were susceptible to *U. barbata* extracts. Nyenje and Ndip (2011), proposed a minimum of ≥ 11 mm of zone of inhibition of plant extracts tested against micro bacteria *in*-vitro to be deemed effective. The sensitivity of methanol and ethyl-acetate extracts at 5mg/ml against all tested

organisms gave a susceptibility of 92.31% and 53%, respectively of all test organisms. The inhibitory ability of the lichen extract against the isolated organisms could be due to the presence of bioactive compounds such as flavonoids, tannins, atranorin, lignins, phenolic acid, usnic acid, norstictic acid and chloroatranorin in them (Rankovic *et al*., 2012; Wendakoon *et al*., 2012).

6.2. Conclusion

There was relatively high incidence of *Staphylococcus* species isolated from milk samples of cows with subclinical mastitis from both farms where the study was conducted. Differences existed in the sensitivity of different *Staphylococcus* species to various antibiotics. From the antibiotic susceptibility result observed in the current study most *Staphylococcus* species were resistant to Penicillin and Ampicillin. Methanol and ethyl-acetate extracts of *U. barbata* exhibited *in-vitro* inhibitory activities against selected *Staphylococcus* species which indicate its potential as a medicinal plant.

6.3. Recommendations

The extracts from *U. barbata* lichen showed a potential attribute, *in-vitro*, as antimicrobial agents against *Staphylococcus* species linked with the cause of mastitis disease in cows. It could be recommended that they could be useful for the treatment of animals with mastitis disease.

Areas that require further research:

- The virulence level of individual specific *Staphylococcus* species as mastitis pathogen in cows.

- The active ingredients in the South African ecotype of *U. barbata* lichens responsible for the inhibitory effect of *Staphylococcus* species.

- The effect of the *U. barbata* extracts on mastitis cows *in-vivo*.

- The dosage of *U. barbata* extracts that will be effective against mastitis bacteria.

- The detrimental effect of the *Staphylococcus* species isolated from raw milk on human safety.

6.4 References

Abera, M., Demie, B., Aragaw, K., Regassa, F. and Regassa, A. 2010. Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. *Journal of Veterinary Medicine and Animal Health* **2:** 29-34.

Awandkar, S.P., Khode, N.V., Sardar, V.M. and Mendhe, M.S. 2009. Prevalence and Current Antibiogram Trend of Mastitis Agents in Udgir and its Vicinity, Maharashtra State, India. *International Journal of Dairy Science* **4:** 117-122.

Ayano, A.A., Hiriko, F., Simyalew, A.M. and Yohannes, A. 2013. Prevalence of subclinical mastitis in lactating cows selected in commercial dairy farms of Holeta district. *Journal of Veterinary Medicine and Animal Health* **5:** 67-72.

Bendahou, A., Lebbadi, M., Ennaenei, L., Essadqui, F.Z. and Abid, M. 2008. Characterization of *Staphylococcus* species isolated from raw milk and milk products (Iben and jben) in North Morocco. *Journal of Infection in Developing Countries* **2:** 218-225.

Nyenje, M. and Ndip, R.N. 2011. *In- vitro* antimicrobial activity of crude acetone extract of the stem bark of *Combretum molle* against selected bacterial pathogens. *Journal of Medicinal Plants Research* **5:** 5315-5320.

Piessens, V., De Vliegher, S., Verbist, B., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx, M. and Van Coillie, E. 2012. Intra-species diversity and epidemiology varies among coagulase-negative *Staphylococcus* species causing bovine intramammary infections. *Veterinary Microbiology* **155:** 62-71.

Rankovic, B., Kosanic, M., Stanojkovic, T., Vasiljevic, P. and Manojlovic, N. 2012. Biological Activities of *Toninia candida* and *Usnea barbata* Together with Their

Norstictic Acid and Usnic Acid Constituents. *International Journal of Molecular Sciences* **13:** 14707- 14722.

Sampimon, O.C., Lam, T.J.G.M., Mevius, D.J., Schukken, Y.H .and Zadoks, R.N. 2011. Antimicrobial susceptibility of coagulase-negative staphylococci isolated from milk samples. *Veterinary Microbiology* **150:** 173-179.

Schukken, Y.H., Gonzalez, R.N., Tikofsky, L.L., Schulte, H.F., Santisteban, C.G., Welcome, F.L., Bennet, G. J., Zurakowski, M.J. and Zadoks, R.N. 2009. CNS mastitis: Nothing to worry about? *Veterinary Microbiology* **134:** 9-14.

Sharma, D., Sharma, P.K. and Malik, A. 2011. Prevalence and Antimicrobial Susceptibility of Drug Resistant *Staphylococcus aureus* in Raw milk of Dairy Cattle. *International Research Journal of Microbiology* **2:** 466-470.

Wendakoon, C., Calderon., P. and Gagnon, D. 2012. Evaluation of Selected Medicinal Plants Extracted in Different Ethanol Concentrations for Antibacterial Activity against Human Pathogens. *Journal of Medicinally Active Plants* **1:** 60-68.