

Isolation and characterization of lytic bacteriophages, a potential alternative for bovine mastitis control

By

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A dissertation submitted on requirement of

Masters of Science in Food Science

In the department of Microbial, Biochemical and Food Biotechnology

University of the Free State

Bloemfontein

2018

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DECLARATION

I, Thembakazi Noguda, declare that the dissertation hereby submitted by me for the Magister Scientiae degree at the University of the Free State is my own work and I have not previously submitted in any other University for a degree.

Signature

Date

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people and institutions for their contribution in completion of this study:

- Firstly, to the almighty God for the gift of life and the opportunities he creates.
- My supervisors, Prof Bennie Viljoen and Prof Robbert Bragg for their patience, monitoring, constructive criticism and wise guidance throughout the study.
- National Research Foundation (NRF) for financing the study
- The farmers who allowed us in their farms to collect samples.
- Prof Bennie Viljoen for hosting me in his lab and everyone in his lab.
- The department of Microbial, Biochemical and Food Biotechnology for hosting me.
- Lastly, I would like to say thank you to my parents Mr Siphumle Talbert Noguda and Mrs Nothobile Hilda Noguda for their continuous love, support and wise guidance in this journey of life.
- My siblings, Unathi, Anelisa, Anelisiwe, Alungile, Nceba, Ncedo and Onke for being my family.

SUMMARY

Mastitis is a common disease affecting dairy herds, with high occurrence incidents and tremendous economic losses associated with it. Varieties of infectious agents are associated with mastitis, but bacteria are responsible for most of the cases. Apart from large numbers and heterogeneity between bacterial species associated with mastitis, it is an infectious disease and the process of milk production makes it easy for the disease to spread and difficult to control. Good milking hygiene, antibiotic therapy during lactation and dry off and chemical teat dips are some of the measures used in its control and treatment of mastitis. However, more cases of antibiotic therapy in treatment and control end up in failure and antimicrobial resistance is the reason attributed to this.

The main goal of this study was to investigate the diversity of bacterial species causing mastitis in South African dairy farms, determine their antimicrobial susceptibility profile and isolate lytic bacteriophages for these species as potential alternative for the control of mastitis caused by bacterial pathogens. Milk samples from mastitis and normal cows were analysed using traditional isolation methods on non-selective and selective differential agar plates, identification by standard biochemical tests. The following bacterial species were predominantly found: *Staphylococcus aureus* and members of *Coagulase-negative Staphylococcus* group (*Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus xylois*, *Staphylococcus epidermis*, *Staphylococcus hominis*, *Staphylococcus hycus*, *Staphylococcus capitis*, *Staphylococcus sciuri*), *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Enterococcus* spp., *Enterobacteriaceae* spp., *Proteus* spp., *Citrobacter* spp., *Bacillus* spp., *Bacillus pumilus*, *Acinetobacter* spp., *Lactococcus lactis* and *Pasteurella* spp.

For their antimicrobial susceptibility evaluation, disk-diffusion and broth microdilution methods were used. The highest sensitivity was found on cephalosporins (cefuroxime 94% and cephalexin 65%) and aminoglycosides (streptomycin 82% and kanamycin 82%), tetracycline 65%, bacitracin 59%, novobiocin 59% and ampicillin 53% and resistance to polymyxin B (53%), penicillin (53%), ampicillin (41%), bacitracin (41%), novobiocin (41%). Intermediate resistance was found for neomycin (47%). Also, different teat dip disinfectants with different active ingredients were evaluated. Products tested were, Deosan Teat Form (Chlorhexidine based), Mastocide (Chlorhexidine digluconate based), Deosan Iodel Gel and locally available chemical pre- and post-milking teat dip (Citric acid monohydrate 0.42% ppm m/v and Dodecyl benzene sulphonic acid based) and milking equipment sanitiser Perosan (peracetic

acid based). All teat dips products were found to be effective, with 100% lethal effects at the recommended application rate in inhibiting growth of all mastitis associated causative strains tested. While the local chemical distributor chemical Perosan acid sanitizers was ineffective at the concentration of 0.4% and double these manufacture recommended concentration for both Gram-negative and positive isolates at exposure time of 5 minutes. Deosan Perosan was also ineffective at manufacture recommended working concentration 0.5% and at double this concentration for Gram-negative. The MIC for these strains was predicted to be >0.75% and for Gram-positive strains, their MICs were between 0.75%-0.19%.

For isolation of lytic bacteriophages, 12 lytic phages were successfully isolated from cow manure for the following species *S. aureus*, *Coagulase-negative Staphylococcus*, *Streptococcus* spp., *Corynebacterium* sp., *Acinetobacterium* sp. and *E. coli*. All these phages formed clear round plaques with size between 1-2 mm in diameter and titer between 10^8 - 10^{12} PFU/ml. Their TEM morphology characteristics showed that they belong to Myoviridae family. Spot test and efficiency of plaque formation were used to determine phage host ranges and most showed a broad host range, they were able to lyse other strains from the same species and strains from other species showing their potential for phage therapy application.

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CHAPTER 5 GENERAL DISCUSSION, CONCLUSION AND FUTURE WORK

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Mastitis is an inflammation of mammary gland, a common disease with high occurrence incidence that cannot be eradicated in a dairy herd with heavy economic losses associated with it (Bogni *et al.*, 2011; Larsen 1994; Petrovski *et al.*, 2000). Survey on the prevalence of this disease in most countries showed a rate of approximately 50% (Radistitis *et al.*, 2000) and losses reported were due to less yield, poor quality of milk produced by infected cows and loss of milk discarded before and during antibiotic treatment to name a few (Larsen, 1994; Petrovski *et al.*, 2006). The inflammation occurs as the results of infectious agent or their toxics, physical trauma, or chemical irritation in the udder of a cow (Bogni *et al.*, 2011). However, the most common cause of mastitis in cows are microorganisms, usually bacteria (Sharma *et al.*, 2006) even though viruses and fungi cases have been reported. Clinical signs visible in the udder such as swelling, redness and increase in temperature and watery consistence, clots and flakes in milk are used to diagnose the clinical form, while an increase in Somatic Cell Count (SCC) are considered diagnostic for the subclinical form of this disease.

Currently, more than 200 microbial species are associated with mastitis and regarded as the causative agents (Mallikarjunaswamy and Krishnamurthy, 1997; Watts 1988). However, 96% of all cases are caused by only few species, such as the, contagious pathogens (*Staphylococcus aureus* and *Streptococcus agalactiae*), environmental pathogens (*Escherichia coli*, *Enterobacter spp.*, *Klebsiella spp.*, *Streptococcus spp.*, *Pseudomonas spp.*) and minor pathogens (*Coagulase-negative Staphylococcus (CNS) spp.*, *Corynebacterium spp.*) (Blowey and Edmondson, 2010; Jones and Bradley, 2009; Haltia, 2006; Haveri, 2008; Rodostis *et al.*, 2000; Pereira, 2011). Contagious and minor pathogens mostly cause a subclinical form, while environmental pathogens cause clinical forms of mastitis. The subclinical form of mastitis is the most costly and frequent form, found in dairy herds worldwide and it can persist for long periods without being detected or diagnosed.

Clinical or subclinical cases can be the result of single or mixed bacterial infections. The bacterial species associated with mastitis can differ in different regions or country and different species can be dominant, responsible for most cases in a particular region. Dominance in species in a region is determined by factors that favour their proliferation such as the changes in seasons, environment (housing material,

type of pasture they are exposed too) and antimicrobials used for control and treatment in that region. The dominant species changes as these factors change. For the successful control of mastitis, it is crucial to continuously monitor the diversity of the causative species and their dominance on each farm. This aids farmers and veterinarians in making important decisions concerning changes that need implementation on their mastitis control program, so that they can effectively control this disease.

For decades, treatment and control of this disease has been possible by the use of mastitis control measures, which include good milking hygiene, use of properly functioning milking machines, antibiotic therapy during lactation and at drying off, teat dipping before and after milking and culling of chronic infected cows (Hogeveen *et al.*, 2011). However, there are still some limitations or concern with some of these measures such as high risk rate of milk contamination by pathogens, antibiotics and chemical teat dips which can lead to allergic reactions and transportation of zoonotic pathogens to humans or select for antibiotic resistance strains which can enter the food chain. Another concern for use of chemical based teat dip products is the negative impact they might have on the environment and their continuous use has been reported to cause chapping, lesions, drying, or a caustic reaction in the teat skin.

Research at present on this topic is mostly focus on finding non-antibiotic and non-chemical alternatives such as bacteriophages, vaccines, natural compounds from plants, animals and bacteria, nano particles and cytokines (Gomes and Henriques, 2016). In the field of mastitis vaccine development, some break throughs have been achieved. There are several mastitis vaccines that are commercially available for prevention of new Intramammary Infections (IMI) caused by coliforms, *S. aureus*, and coagulase-negative staphylococci (Tiwari *et al.*, 2013). Nevertheless, even though vaccine development is promising, there are still some drawbacks, such as causing an increase in milk Somatic cell count (SCC), rendering milk of poor quality and farms get penalised and loss of income. In addition, another limitation of vaccine development is the fact that it is impossible to develop one vaccine that is effective against all mastitis agents, due to their numbers and heterogeneity.

Research on bacteriophages is also promising, but studies conducted only focused on mastitis caused by *S.aureus*. In addition, experimental field trials done on phage application were only for phage therapy and no experimentation has been done on other possible applications, such as being used as part of sanitizers in teat dipping. According to literature, phage application as therapeutic agents in humans, animals and plants proved successful whereas in the food industry they were used

as additives to preserve food, sanitizers and disinfect food contact surfaces (Sillankorva *et al.*, 2012). This shows that research should not only focus on phage therapy, but consider other options that can also be productive.

The main aim of this project was to isolate lytic bacteriophages for mastitis associated causative pathogens with an aim of using them as an alternative teat dip. The first objective of this project was to determine species diversity of mastitis associated causative pathogens in South Africa. The efficacy of various antibiotics against isolated bacterial strains were evaluated in an effort to establish the level of resistance to different antibiotics. There is currently a crisis with regard to bacterial antimicrobial resistance and current status of antibiotic resistance to South African strains needs to be evaluated. The second objective was to isolate lytic bacteriophages for these mastitis-causing pathogens, to characterize the phages morphologically, and to determine their host range.

1.2 References

Bergh O, Børsheim K.Y, Bratbak G and Haldal M, (1989). High abundance of viruses found in aquatic environments. *Nature*; 340: 467-468. 30.

Blowey R and Edmondson P (2010). *Mastitis control in Dairy Herds*, 2nd Edition. CAB International, UK.

Bogni C, Odierno L, Raspanti C, Giraud J, Larriestra A, Elina Reinoso, Lasagno M, Ferrari M, Ducrós E, Frigerio C, Bettera S, Pellegrino M, Frola , Dieser S and Vissio C, (2011). War against mastitis: Current concepts on controlling bovine mastitis pathogens. *FORMATEX 2011*: <http://www.formatex.info/microbiology3/book/483-494.pdf>.

Jones G. M and Bailey T. L, (2009). *Understanding the basics of mastitis*. Virginia Cooperative Extension, publication 404-233.

Gomes F and Henriques M, (2016). Control of Bovine Mastitis: Old and Recent Therapeutic Approaches. *Current Microbiology*, 72:377–382. DOI 10.1007/s00284-015-0958-8.

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

Haveri M (2008). *Staphylococcus aureus* in bovine intramammary infection: molecular, clinical and epidemiological characteristics. Academic dissertation, submitted to Department of Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Finland.

Hogeveen H, Pyorala S, Waller K. P, Hogan J. S, Lam T. J .G. M, Oliver S. P, Schukken Y. H, Barkema H .W, and Hillerton J. E, (2011). Current Status and future challenges in mastitis research. National Mastitis Council, Annual Meeting Proceedings (2011).

Larsen J, (1994). The Economic Losses due to Mastitis and the Cost Effectiveness of Mastitis Problem Solving and Monitoring Programs. Udder Health Seminar 22 June, 6.

Mallikarjunaswamy M.C and Krishnamurthy G.V, (1997). Antibiogram of bacterial pathogens isolated from bovine mastitis cases. Indian Veterinary Journal, 74(10): 885-887.

Pereira U.P, Oliveira D.G, Mesquita L.R, Costa G.M and Pereira L.J (2011). Efficacy of *Staphylococcus aureus* vaccines for bovine mastitis: a systematic review. Veterinary Microbiol 148: 117-124.

Petrovski K. R, Trajcev M and Buneski G. A, (2006). Review of the factors affecting the costs of bovine mastitis. Journal of the South African Veterinary Association 77(2): 52–60 (En.).

Radostits O.M, Blood D.C, Gay C.C, Blood D.C and Hinchkliff K.W, (2000). Veterinary Medicine. 9th Edn, ELBS-Bailliere Tindal, London pp: 563-618.

Sharma N, Gautam A, Upadhyay S.R, Hussain K, Soodan J.S and Gupta S.K, (2006). Role of Antioxidants in Udder Health: A Review. Indian Journal Field Veterinary 2(1):73-76.

Sillankorva S. M, Oliveira H and Azeredo J, (2012). Bacteriophages and Their Role in Food Safety- Review. International Journal of Microbiology; Volume 2012, Article ID 863945, pages13. doi:10.1155/2012/863945.

Tiwari J. G, Babra C, Tiwari H.K and 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 & Vaccination. 4 (1): pp. 1-11.

Watts J.L, (1988). Etiological agents of bovine mastitis. *Veterinary Microbiol* 16: 41-66.

CHAPTER 2

LITERATURE REVIEW

2.1 Clinical and subclinical forms of mastitis

There are two forms of mastitis, characterised according to their visibility in milk or udder as clinical and subclinical. Their occurrence depends on the type of pathogen causing infection and the extent of inflammation caused by the immune system of a cow. Clinical signs in the udder or milk such as clots, swelling and increased temperature in an udder and cow being actually ill are symptoms used to identify the clinical form of this disease, and it is further divided into peracute, acute, subacute, chronic and gangrenous mastitis (Bogni *et al.*, 2011).

Subacute- In this form the cow appear normal, with no abnormal changes observed in the udder or milk, only presence of flaky particles in milk during initial ejection.

Acute- In this form changes observed are hot, swollen, red and painful quarter or udder and abnormalities on milk, reduction in quantity, thin and watery sometimes blood stained. The fever of 39⁰C and slightly depression in a cow.

Peracute- In this form, the systemic signs are the same as acute form, but with more intensity. Milk passes with difficult, fever is over 41⁰C, cow has no appetite, shivers and loses weight quickly and lactation often stops. In cases where therapy is delayed, death may occur in a few hours.

Chronic- This form is observed by a inflammatory process that persists over many months or from one lactation period to the next, mostly existing in a subclinical form with periodic flare-ups producing sub acute or acute clinical signs which commonly subside shortly thereafter, reverting to the subclinical form. Systemic signs are mild and hardness of udder or quarter may or may not be present, sometimes fibrosis and yellow coloured or watery milk with flakes. The affected tissue is tough and smaller than normal (due to proliferation of fibrous connective tissue and glandular atrophy). Antibiotic treatments often do not work.

Gangrenous- Affected quarter is blue and cold to the touch. Progressive discolouration from the tip to the top. Necrotic parts drop off. Cow often dies.

Subclinical Mastitis (SCM) - In this form, there are no visible signs in the milk and udder can only be diagnosed by assessing the level of somatic cells in milk. It is

considered to be the most costly form of mastitis as it can persist for long period without being diagnosed, so frequent monitoring of somatic cells is important.

2.2 Mastitis associated causative agents

In the past, a healthy intramammary gland was previously considered to be a sterile organ with milk free from microorganisms, but recently more and more researchers have challenged that notion (Rainard, 2017). The application of molecular methods to the quantification and sequencing of bacterial DNA has yielded results suggesting that there are commensal microbial communities within the mammary gland. More studies reported that the healthy intramammary gland accommodates a wide variety of bacterial species including *S. aureus* and *Streptococcus uberis* (Kuehn *et al.*, 2013; Oikonomou *et al.*, 2014 and 2012). It was further postulated that bacterial species known to exist on the skin or in the intestinal tract of the cow are part of the normal microbiota of the mammary gland. However, at this stage of research the existence of an intramammary gland microbiota have not been clearly stated or analysed in depth and discussed.

In the case of bovine mastitis, a variety of causative agents are associated with it and they include infectious agents (bacteria, *Mycoplasma*, fungi and viruses), chemical irritation and physical injury (bruises and cuts) (Jones and Bailey, 2009). Approximately 70% of bovine mastitis cases are caused by bacteria, 2% by fungi and 28% is of unknown etiology. There are about 200 different microbial species, sub-species and serotypes which have been isolated from bovine mammary gland and identified as the mastitis causative agents (Mallikarjunaswamy and Krishnamurthy, 1997; Watts 1988). Depending on the type of mastitis these bacterial pathogens cause, they are categorized into major and minor pathogens. Major pathogens cause both clinical and subclinical mastitis and minor pathogens cause subclinical mastitis and rarely cause clinical mastitis. The major pathogens are further categorized into contagious and environment pathogens depending on their source of origin and mode of transmission (Hamadani *et al.*, 2013; Hawari and Fazwi, 2008).

Contagious pathogens live and survive in the mammary gland and spread from cow to cow during milking through contaminated milking machines, hands of milkers and towels used for drying teats and such pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma* spp.

Staphylococcus aureus is a Gram-positive cocci, occurring singly, or in pairs and form irregular grape like clusters. They are non-motile, non-spore forming, facultative anaerobic, catalase and coagulase positive and oxidase negative (Schleifer and Bell,

1984). They are found on udder skin, teats, feed and housing material, other animals and humans (Cullor and Tyler, 1996; McDonald, 1977; Roberson *et al.*, 1994). Most infection cases caused by *S. aureus* are chronic subclinical, with some occasional mild cases to moderate clinical mastitis. *S. aureus* is the predominant pathogen found in mastitis cases worldwide and is of substantial, global concern due to a low response rate to antibiotic therapy and high reoccurrence infection rates. Madgwick *et al.* (1989) suggested that this might be due to the ability of this species to form scars in mammary glands where antibiotics cannot reach them. Furthermore, they are engulfed by macrophages, where they survive in those cells (Hebert *et al.*, 2000). *S. aureus* species have become resistant to antibiotic due to β -lactamase production and virulence factors (Fox and Gay 1993; Sandholm *et al.*, 1990). For control of mastitis caused by *S.aureus* strains, post-milking teat dipping and dry cow therapy are effective.

Streptococcus agalactiae are Gram-positive cocci, occurring in chains of less than four cells. They grow well on blood agar plates and exhibit various types of hemolysis (Lehmann and Neumann, 1896). They do not produce the gangrenous form of mastitis but can occur in all other forms. They are highly contagious pathogens that can only survive and multiply on mammary gland, teat lesions and teat ducts and die on the environment (Cullor and Tyler, 1996; McDonald, 1977). They respond well to treatment during lactation and can be eradicated from a herd with good mastitis control practices such as teat dipping and dry-cow treatment (Biggs, 1996; McDonald, 1977; Philpot, 1975).

Mycoplasma spp. are bacteria with no cell wall, only bound by a single plasma membrane. This genus has about 70 species of which five, *Mycoplasma bovis* (most common *Mycoplasma* species isolated from cases of mastitis), *M. bovis genitalium*, *M. californicum*, *M. canadense* and *M. alkalenscens* are known to cause bovine mastitis cases (Kirk and Lauerman, 1994). Their primary source for the isolation is infected udder, respiratory and reproductive tracts. New infections may occur through an introduction of new infected cows to the herd or animal with respiratory infection or encounter with calves with *Mycoplasma* pneumonia or arthritis. *Mycoplasma* can cause both clinical and subclinical mastitis. The infection is recognised by multiple quarters being infected at the same time, increase in incidence of cases that are resistant to therapy, rapid decline in milk production and abnormal milk that is often brown with flaky sediment. Currently, there is no treatment for *Mycoplasma* mastitis; antibiotic therapy is ineffective and only culling and segregation of infected animals prevent the spreading of the disease. Spontaneous recovery occurs even though

these cases are rare and once an animal is infected, it is considered to be infected for life (González, 1996).

The environmental mastitis is caused by the environmental streptococci (*Streptococcus uberis*, *Streptococcus dysgalactiae*), coliforms (*Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp.) and other environmental pathogens (*Citrobacter* spp., *Serratia* spp., *Proteus* spp., *Pseudomonas* spp. and *Bacillus* spp) (Bogni *et al.*, 2011; Schroeder, 2012; Watts, 1988). These pathogens are widely spread in the environment of the cow (fields, soil, bedding material and manure) and their mode of transmission is through inadequate management of the environment such as having soiled bedding, access to manure, mud or pools of stagnant water, poor pre-milking teat preparation, and poor housing system and fly control (Schroeder, 2012). The primary route of infection by *Streptococcus* spp. is organic bedding (straw) and infection rate is much higher during dry period than during lactation. Environment streptococci can cause both clinical and subclinical mastitis, while coliforms and other environment pathogens mostly cause clinical cases with few subclinical cases (Bogni *et al.*, 2011). For treatment, during lactation antibiotic therapy is used for mastitis cases caused by *Streptococcus* spp. and it is very effective. While frequent milking of an infected udder is used to treat mastitis cases caused by coliforms and other environmental pathogens, severe cases have to be treated by systemic and intramammary antibiotic therapy. For control, dry cow therapy, pre- and post-milking teat dipping and non-organic bedding material such as sand are very efficient in controlling environmental streptococci (Bogni *et al.*, 2011; Kulkarni and Kaliwal, 2013). Other environmental pathogens, can be controlled by reducing exposure of the teat ends to these pathogens through keeping the environment clean, cool and dry and also, by increasing the cow's resistance by providing a stress free environment and by feeding a balanced diet rich in Vitamin E and Selenium (Sharma and Maiti, 2005).

Minor pathogens are known to be emerging mastitis pathogens and of less importance, but their importance has increased over the years due to their frequent isolation in mastitis cases (Blignaut, 2016; Katsande *et al.*, 2013; Kasozi *et al.*, 2014; Petzer *et al.*, 2009). They are mostly found as free-living microorganisms in the environment and form parts of the normal microbiota of teat skin and such pathogens include coagulase-negative staphylococci (CNS) and *Corynebacterium* spp (Blignaut, 2016; Petzer *et al.*, 2009). These pathogens cause less udder damage and infectious cases remain subclinical with milk SCC below 500,000 cells/ml (Djabri *et al.*, 2002) and mild cases of clinical mastitis. The increase in SCC has lead to some speculations that minor pathogens, may have a protective effect on new intramammary infections

caused by major pathogens (Matthews *et al.*, 1990; De Vliegher *et al.*, 2004). To prove the hypothesis, different field experimental challenge trials were conducted and the results varied. In two of those studies by Compton *et al.* (2007) and Parker *et al.* (2007), they observed that minor pathogens increased the risk of new Intramammary Infection (IMI) by major pathogens. Zadoks *et al.* (2001) observed no effect, while in other studies by Linde *et al.* (1980) and Matthews *et al.* (1990) they observed protection of the udder from new IMI. CNS has become a predominant pathogen frequently isolated in mastitis cases in most countries especially in African dairy farms (Blignaut, 2016; Katsande *et al.*, 2013; Kasozi *et al.*, 2014; Petzer *et al.*, 2009). There are about 50 different species of CNS, but *S. chromogenes*, *S. simulans* and *S. hyicus* are the most frequently isolated in milk samples from mastitis cows. Most countries do not treat mastitis cases caused by these pathogens during lactation. Frequent milking of infected udders is done and about 70% of the cases have been reported to recover spontaneously (Taponen *et al.*, 2006). Treatment is done at drying off, where antibiotic therapy has been found to respond very well with bacteriological cure rate between 80 to 90% (Pyörälä and Pyörälä, 1998; Taponen *et al.*, 2003; Taponen *et al.*, 2006; Waage *et al.*, 2000). Good milking hygiene, post-milking teat dip and dry cow therapy are very effective in preventing and controlling the minor pathogens (Hogan *et al.*, 1987).

Fungi are other infection causing agents implicated as causative agents in bovine mastitis cases, even though most of these cases are usually not recognised. Due to limited reports on their frequent occurrence as normal diagnosis, scientists only rely on bacteriology examination of milk, with no mycological examination of milk. In fungal infections, yeasts are predominant, followed by filamentous fungi and cases occur as a single case or occasionally outbreaks. The species of importance on bovine mastitis cases are *Aspergillus* spp., *Trichosporon* spp., *Pichia* spp., *Candida* spp., *Saccharomyces* spp. and *Torulopsis* spp., *Cryptococcus neoformans* and *Prototheca zopfii* (Fadlelmula *et al.*, 2009; Hamadani *et al.*, 2013; Khan and Khan, 2006). The source of fungal infections is from mouldy surroundings, contaminated feed or bedding, teat dips and dairy utensils, mud and stagnant water. These fungal species are also normal inhabitants of the skin of the udder and teats, where they exist at low numbers. Most fungal infections seem to occur as secondary infection, occurring immediately after antibiotic therapy. In addition, strict mastitis control programmes render natural udder immunity ineffective and deficiency in vitamin A and Zinc are other factors contributing to occurrence of fungal infection. The symptoms, in infected cows, are not different from that of bacterial infection. All other fungal infection cases appear to recover spontaneously, except those caused by *Cryptococcus neoformans*.

None of these infections responds well to antibiotic therapy, but intramammary infusion of iodine in solution or oil seems to be an effective treatment. For prevention and control, good hygienic practice during milking, sterilization of dairy utensils, using teat dips with iodine and culling of infected cows provide the best method of controlling outbreaks in the absence of effective udder treatment.

Viruses are other infectious agents implicated as causative agents of bovine mastitis. Literature reported that they do not cause mastitis directly; they only cause local dermatitis, including damage of teat and papillaris, which lead to secondary bacterial infections that cause mastitis (Easterday *et al.*, 1959; Francis, 1984; Gibbs, 1984; Scott and Holliman, 1984; Saini *et al.*, 1992; Turner *et al.*, 1976; Wellenberg *et al.*, 2002). Such viruses' include *Bovine herpes mammillitis virus* (BHV2), *Vaccinia*, *Cowpox*, *Pseudocowpox* and *Foot-and-Mouth-Disease* (FMD) viruses. Other viruses such as BHV1, BHV4 and *Bovine Viral Diarrhea virus* (BVDV) were reported to cause immunosuppression effects (Elvander, 1996; Saini *et al.*, 1999).

2.3 Pathogenesis

The mammary gland anatomy has four quarters; each quarter has a teat canal, teat cistern, gland cistern, milk ducts and glandular tissue in its interior (Gruet *et al.*, 2001). The glandular tissue or secretory portion contains millions of microscopic sacs called alveoli, lined with milk-producing epithelial cells and surrounded by muscle cells that contract and squeeze milk from the alveolus during milking. Blood vessels bring nutrients to each alveolus, where epithelial cells convert them into milk. In between milking, milk accumulates in the alveolar spaces, milk ducts and cisterns followed by during milking the accumulated fluid is removed through the teat canal.

Mastitis occurs when infectious agents (bacteria, viruses, fungi and *Mycoplasma*) gain entry into the mammary gland through the teat canal and pass through into the gland cistern and glandular tissue where they multiply (Bogni *et al.*, 2011). Pathogens use two ways to gain entry in the teat canal through adhesion and repulsion (Basdew and Laing, 2015). Contagious pathogens have strong adhesive factors and they are able to attach to the teat canal, multiply and colonise it and grow all the way through into the teat sinus. The environmental pathogens, on the other hand use repulsion due to their lack of adhesive properties. They are forced through the teat canal by reverse flow of milk and this occurs when pressure between teat end and milking unit is imbalanced. In addition, mechanical milking causes sphincter muscle (keeps teat canal closed when not milked) to remain open for 1-2 hrs after milking (Hamadani *et*

al., 2013). It is at this stage that pathogens in the teat and environment may gain entry. Once they reach the glandular tissue, they multiply and compete with alveolus for nutrients and produce toxins, which kill the alveolus.

2.4 Mammary gland defence mechanisms

The mammary gland is however not defenceless and two forms of immune defence mechanisms protect it, namely innate immunity and acquired immunity (Sordillo, 2005). Both work together for protection against invading pathogens. The response of innate immunity stimulates the acquired immunity response and response of the acquired immune response uses many innate immune effector mechanisms to eliminate microorganisms. Its action frequently increases innate immune response antimicrobial activity. The innate immunity predominated in the first stage of infection and is mediated by anatomical, cellular and soluble factors.

In the anatomical factors, the teat canal is the first line of defence against invading pathogens due to the presence of sphincter muscle (Zecconi *et al.*, 2000), which keeps the teat closed when not being milked, preventing milk from escaping, and bacteria from entering into the teat. Once inside the teat canal, the pathogen needs to pass the second line of anatomic defence keratin. A waxy material made from the stratified squamous epithelium that is composed of fatty acids and fibrous proteins (Oviedo-Boyso *et al.*, 2007). The fatty acids are both esterified and non-esterified, representing myristic acid, palmitoleic acid and linolinic acid which are bacteriostatic. The fibrous proteins of keratin in the teat canal bind electrostatically to mastitis pathogens, which alter the bacterial cell wall, rendering it more susceptible to osmotic pressure. Inability to maintain osmotic pressure causes lysis and death of invading pathogens. Accumulation of keratin provides physical obstruction to pathogens and prevents their migration into the gland cistern (Jones and bailey, 2009). In addition, the inside of the teat canal contains specialised cells called Rosette of Furstenberg (ring of lymphocyte cells that detect invading bacteria and initiate an immune response) (Blowey and Edmondson, 2010).

When the anatomical defence fails, pathogens move into the gland cistern and glandular tissue, which contains alveoli that are lined with milk-producing epithelial cells and initiate infection there causing swelling, damage and the killing of these cells. Damaged milk secreting cells, release inflammatory mediators signal which leads to migration of residential and new recruited leukocytes into the site of infection. Leukocytes are cellular factors, cells regulating innate and acquired immune response

and they consists of neutrophils, macrophages, lymphocytes and epithelial cells (Sordillo, 2005).

Macrophages are the dominant cell type found in milk and tissues of healthy, lactating mammary glands, however during early stage of inflammatory process, neutrophils become the predominant cells found in the mammary gland and the main reason observed for an increase in SCC during mastitis (Paape *et al.*, 2003). Macrophages are the first cells to encounter bacteria and function as recogniser and alarm cells initiating immunity in the mammary gland (Sandholm *et al.*, 1990). Both cell types are recruited actively at the site of infection and move from the blood through a weak blood udder barrier into mammary gland where they kill bacteria by phagocytose using two systems oxygen- dependent and oxygen-independent (Paape *et al.*, 2003; Reshi *et al.*, 2015; Sandholm *et al.*, 1990; Sordillo, 2005). The oxygen-dependent system works by production of reactive oxygen species (ROS). The cytoplasmic membrane of phagocytes contains the enzyme oxidase which converts oxygen into superoxide anion (O_2^-) and this can combine with water by way of the enzyme dismutase to form hydrogen peroxide (H_2O_2) and hydroxyl (OH) radicals. In the case of neutrophils, the hydrogen peroxide can then combine with chloride (Cl^-) ions by the action of the enzyme myeloperoxidase (MPO) to form hypochlorous acid (HOCL), and singlet oxygen.

In macrophages, nitric oxide (NO) can combine with hydrogen peroxide to form peroxynitrite radicals. In addition to ROS and NO, macrophages secrete inflammatory cytokines such as TNF-alpha, IL-1, IL-8, and IL-12 to promote an inflammatory response (Sordillo, 2005). These compounds are very microbicidal, as they are powerful oxidizing agents, which oxidize most of the chemical groups found in proteins, enzymes, carbohydrates, DNA, and lipids. Lipid oxidation can break down cytoplasmic membranes. Collectively, these oxidizing free radicals are called reactive oxygen species (ROS). Oxidase also acts as an electron pump that brings protons (H^+) into the phagosome. This lowers the pH within the phagosome so that when lysosomes fuse with the phagosome, the pH is correct for the acid hydrolases, like elastase, to effectively break down cellular proteins. In addition to phagocytes using this oxygen-dependant system to kill microbes intracellularly, neutrophils also routinely release these oxidizing agents, as well as acid hydrolases, for the purpose of killing microbes extracellularly. These agents, however, also wind up killing the neutrophils themselves as well as some surrounding body cells and tissues as mentioned above.

During phagocytosis pathogens are also exposed to several oxygen-independent reactants such as some lysosomes that contain defensins (cationic peptides that alter

cytoplasmic membranes). These include lysozyme (an enzyme that breaks down peptidoglycan), lactoferrin (a protein that deprives bacteria of needed iron), cathepsin G (a protease that causes damage to microbial membranes), elastase (a protease that kills many types of bacteria), cathelicidins (proteins that upon cleavage are directly toxic to a variety of microorganisms) bactericidal permeability inducing protein (BPI) (proteins used by neutrophils to kill certain bacteria by damaging their membranes), collagenase and various other digestive enzymes that exhibit antimicrobial activity by breaking down proteins, RNA, phosphate compounds, lipids, and carbohydrates (Reshi *et al.*, 2015; Sordillo, 2005).

2.5 Diagnoses for mastitis control

Early diagnosis is very important for mastitis control as other forms of mastitis can occur for a long time without being noticed, causing unforeseen losses on the farm. Currently, a number of methods are available for diagnosing mastitis. Methods such as routine visualization of udder and milk using dark surfaces, SCC methods using indirect methods such as California mastitis test (CMT) also known as cow-side testing, or direct methods such as Fossomatic SCC, SCC Scanner, Delaval cell count, Electrical conductivity (EC) test, pH test, Enzymes and culture test (Viguiet *et al.*, 2009). For diagnoses of clinical form of mastitis, visualization is used, where the udder is examined for clinical signs such as swelling, redness, and hardness, increase in temperature and pain in cow when touched before milking and milk is stripped on dark surface looking for abnormal changes in colour, consistency, clots and flakes.

SCC methods are used for diagnoses of subclinical form of mastitis, as there are no visible signs observed in the udder or milk. Somatic cells are white blood cells normal present in milk. During infection, their number increase to help the cow fight the infection (Sordillo, 2005). Delaval cell count and CMT are an on farm method, while Fossomatic SCC, SCC Scanner are laboratory-based methods. CMT indirectly measures somatic cells of individual cow's milk sample based on the principle that when the detergent is added to a sample with a high cell count, they will be lysed releasing nucleic acids which lead to the formation of a gel, by observing the extent of gel formation results can be interpreted as negative or positive. Also individual cow's or in bulk milk somatic cells level are measured, usually on monthly basis by using SCC Scanner and Fossomatic SCC which are more accurate. International Dairy Federation (IDF) gold standard for mastitis diagnosis is identified by SCC $>400 \times 10^3$ cells/ml accompanied by bacterial presence on a herd level (Petzer *et al.*, 2009).

A health cow has SCC below <100,000 cells/ml, this is the limit regarded as normal and values greater than > 200,000 cells/ml are regarded as the sign of subclinical mastitis (Hillerton, 1999; Schukken *et al.*, 2003). In South Africa, raw bulk milk with SCC more than 500,000 cells/ml is not accepted for human consumption, therefore it is not sold (Department of Health, 1997). While in Europe, Canada and United States their raw milk SCC upper limit is 750,000 cells/ml (Larsen, 1994).

2.6 Treatment of mastitis infection

There are three options in which infection can be eliminated from a herd, firstly by spontaneous recovery or secondly by culling chronic infected cows and thirdly by antibiotic therapy (Nickerson, 2001; Hamadani *et al.*, 2013). Antibiotic therapy during lactation and culling are not favourable options for treatment, due to losses incurred of discarded milk and losses of milk in production due to removal of the cow in the herd. The farmer's first option is frequent milking of infected quarters, especially in mild and new cases of mastitis hoping that infection will be eliminated spontaneously. This phenomenon occurs when a cow is cured of infection without medical intervention, however it does not happen frequently and researchers found that 20 to 50% of established infections recover spontaneous (Nickerson, 1996). Currently, there is no proven way of increasing this phenomenon, but some reports say that vaccination and biological agents such as cytokines enhance it (Nickerson, 2001).

Antibiotic therapy is the only remedy proven efficient in eliminating intramammary infections in dairy herds and it is done during lactation and at drying off (Nickerson, 2001). The main goal of therapy is to eliminate the infectious pathogens in the udder, which leads to curing the cow and returning the cow to normal milk production and composition and prevention of mortality. During lactation antibiotic therapy is efficient against some pathogens and ineffective against some and this is due to determining factors such as incorrect diagnosis, inappropriate route of administration and the drug selected, severity of udder pathology, and elimination of predisposing factors (du Preez, 2000). The antimicrobials used are available in two forms as intramammary antibiotics in a tube and systemic antibiotics given by the intramuscular route. The intramammary antibiotic infusion route is the most commonly used, ideal for treating sub-clinical, mild and moderate clinical mastitis cases or when one quarter is infected and when more than one quarter is infected and the cow is ill. The combination of these treatments is therefore used. The intramammary preparations are usually multi-component including more than one antibiotic, while intramuscular products are

usually single active products (Hillerton and Berry, 2005). The most commonly used antimicrobials for treatment are grouped into the following classes, β -lactams and non- β -lactams (CLSI, 2012; du Preez, 2000; Schmidt, 2011).

β -lactams

All these antimicrobial, have the common central, four-member β -lactam ring and their mode of action is inhibition of cell wall synthesis and additional ring structures or substituent groups added to the β -lactam ring to determine whether the agent is classified as a penicillin, cephem, carbapenem, or monobactam. Penicillin is active against non- β -lactimase producing bacteria (penicillin, ampicillin, oxacillin, cloxacillin, cefazolin and cefotaxime, Cefalexin, Cefadroxil, Cefuroxime, Ceftriaxone, Cefotaxime, Ceftazidime, Cefepime) (CLSI, 2012; de Preez, 2000).

Non- β -lactams

Aminoglycosides, work by inhibiting protein synthesis at ribosomal level, where they bind to the 30s ribosome subunit, leading to the misreading of mRNA. This misreading results in the synthesis of abnormal peptides that accumulate intracellularly and eventually lead to cell death. These antibiotics are bactericidal (dihydrostreptomycin, neomycin, gentamicin, kanamycin, spectinomycin) (CLSI, 2012).

Fluoroquinolones inhibit DNA gyrase enzyme, inhibiting DNA synthesis (norfloxacin, Enrofloxacin, ciprofloxacin) (CLSI, 2012).

Tetracyclines, inhibit protein synthesis at the ribosomal level of certain gram-positive and gram-negative bacteria, by irreversibly binding to the 30S ribosomal subunit, inhibiting the elongation step of protein synthesis and such antimicrobials are representatives of oxytetracycline and chlortetracycline (CLSI, 2012).

Macrolides, their mode of action works by inhibiting bacterial protein synthesis at the ribosomal level. They bind at 50S ribosomal subunit during translation, blocking the elongation step and release step of protein synthesis, releasing unfinished or toxic protein and such antimicrobials include erythromycin, tylosin, lincomycin and spiramycin (CLSI, 2012).

Chloramphenicols, their mode of action works by inhibition of polypeptide synthesis, they bind to the bacterial 50S ribosomal subunit, inhibiting the elongation step of protein synthesis (CLSI, 2012).

Folate pathway inhibition, this group include sulphonamides and trimethoprim, their mode of action works by inhibition of the bacterial folate pathway (CLSI, 2012).

Glycopeptides principal mode of action is inhibition of cell wall synthesis at a different site than that of the β -lactams. The activity of this group is directed primarily at aerobic, gram-positive bacteria and include vancomycin in the glycopeptide subclass and teicoplanin in the lipoglycopeptide subclass (CLSI, 2012).

Lipopeptide with subclass polymyxins that include (polymxin B and colistin) their principal target is cell membrane and are bactericidal - appear to act like cationic detergents. They disrupt the integrity of the cell membrane by interacting with phospholipids and increase cell permeability (Gupta *et al.*, 2009; CLSI, 2012).

Table 2.1 and 2.2 show recommended antimicrobial preparation available commercially for mastitis treatment during lactation and dry period and their spectrum of activity.

Table 2.1 Recommended antimicrobial preparations for lactating cows treatment, withdrawal period and activity spectrum (du Preez, 2000; Pieterse and Todorov, 2010).

Trade Name	Milk withdrawal period	Antibiotic active ingredient	Activity spectrum (if sensitive)
Cloxamast LC	3 days	Cloxacillin, ampicillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Curaloc LC	3days	Cloxacillin,ampicillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Dispolac RX4	24 hours, after blue colour has disappeared	Penicillin, dihydrostreptomycin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.), <i>Clostridium perfringens</i> , <i>Bacillus cereus</i> .
Lactaclox	2.5 days	Cloxacillin	<i>S.aureus</i> , streptococci
Lactaciliin	3 days	Ampicillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Lincocin Forte	2.5 days	Lincomycin, neomycin	<i>S.aureus</i> , streptococci
Mastijet Forte	4 days	Oxytetracycline, neomycin, bacitracin, cortisone	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Nafpenzal MC	6 milkings in treatment +3 milkings after treatment	Penicillin, dyhrostreptomycin, nafcillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.) <i>Clostridium perfringens</i> , <i>Bacillus cereus</i> , <i>Arcanobacterium pyogenes</i>

Table 2.2 Recommended antimicrobial preparations for dry cows treatment, withdrawal period and activity spectrum (du Preez, 2000; Pieterse and Todorov, 2010).

Trade name	Milk withdrawal period	Active ingredients	Activity spectrum (if sensitive)
Bocaclox DC	30 days	Cloxacillin, ampicillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Cephudder	21 days	Cephapirin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)

Cepravin DC	4 days	Cephalexin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Curaclox DC	2.5 days	Cloxacillin, ampicillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Dispolac DC	None specified	Penicillin, dihydrostreptomycin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.) <i>Clostridium perfringens</i> , <i>Bacillus cereus</i> , <i>Arcanobacterium pyogenes</i>
Dri Cillin	2.5 days	Cloxacillin, ampicillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Masticillin DC	28 days + 10 milkings after calving	Cloxacillin	<i>S.aureus</i> , streptococci
Masticlox DC	2.5 days	Cloxacillin	<i>S.aureus</i> , streptococci
Masticlox Plus DC	None specified	Cloxacillin, ampicillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Masticlox Plus DC Extra	4 days	Cloxacillin, ampicillin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Nafpenzal DC	3 milkings	Penicillin, dihydrostreptomycin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Neomastitar DC	5 weeks	Penicillin, neomycin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Noroclox DC	2.5 days	Cloxacillin	<i>S.aureus</i> , streptococci
Noroclox DC EXTRA	2.5 days	Cloxacillin	<i>S.aureus</i> , streptococci
Orbenin EXTRA DC	4 days	Cloxacillin, blue trace dye	<i>S.aureus</i> , streptococci
Pendiclox DC	24 hours after blue colour disappears	Cloxacillin, ampicillin, blue tracer dye	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)
Penstrep DC	24 hours after blue colour disappears	Penicillin, dihydrostreptomycin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.), <i>Clostridium perfringens</i> , <i>Bacillus cereus</i> , <i>Arcanobacterium pyogenes</i>
Rilexine 500 DC	4 weeks	Cephalexin, neomycin	<i>S.aureus</i> , streptococci, coliforms (<i>E.coli</i> and <i>Klebsiella</i> spp.)

2.7 Antimicrobial resistance of mastitis associated causative pathogens

Antimicrobials are defined as any substance of natural, semisynthetic or synthetic origin that kills or inhibits the growth of microorganisms but causes little or no damage to the host (VKM, 2016). This term includes all agents that act against all types of microorganisms, bacteria, viruses and fungi. The main classes are “antibiotics”, described as naturally occurring or synthetic organic substance that inhibit or destroy microorganisms within the body and “biocides”, chemical agents with a broad spectrum that inactivate microorganisms (VKM, 2016). Biocides are further divided into “disinfectants”, agents that inhibit or destroy microorganisms on non-living surfaces and “antiseptics” similar to disinfectants but are used on living tissue (McDonnell and Russell, 1999).

Microorganisms are very adaptable and have the ability to become resistant to these antimicrobials. The mechanism of resistance can either be of a natural property of an organism (intrinsic or innate) or acquired by mutation or acquisition of plasmids (self-replicating, extrachromosomal DNA) or transposons (chromosomal or plasmid integrating, transmissible DNA cassettes) (McDonnell and Russell, 1999). Some bacteria produce enzymes that either destroy the antimicrobial agent before it reaches its target site or modify it or alter the target site by mutation so that it no longer binds the antimicrobial agent or some genetically alter specific metabolic pathways so that the antimicrobial agent cannot exert an effect. Some possess an efflux pump that expels the antimicrobial agent from the cell before it can reach its target.

Mastitis is one of the main reasons antibiotics are used in dairy herd. Their frequent use over long periods, treating undiagnosed cases and easy accessibility over the counter by farmers without prescription are considered to be some of contributing factors in major mastitis pathogens such as *S. aureus*, *E. coli*, *coagulate-negative Staphylococcus spp.* (*S. epidermis* and *S. chromogenes*), *Klebsiella pneumonia* and *Pseudomonas aeruginosa* in becoming resistant (El Behiry *et al.*, 2012). Even worse, over the years certain strains of these species have become multidrug-resistant (MDR), which is considered to be a reason for low cure rates and treatment failure, especially in *S. aureus* mastitis cases (du Preez, 2000). However, it is not clear whether this problem is increasing or it has reached its plateau.

Current reports reveal that most *S. aureus* strains are resistant against almost all β -lactam, aminoglycosides and tetracycline antibiotic groups (Beyene, 2016; Ganguly *et al.*, 2016; Kasozi *et al.*, 2014; Schmidt *et al.*, 2015; Tassew *et al.*, 2016; Wang *et al.*, 2015) but not the methicillins. While on other hand, *E. coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* are mostly resistant against ampicillin, tetracycline and fluoroquinolone (Ahmed and Shimamoto, 2011; Ammar *et al.*, 2016; Ibrahim *et al.*, 2015; Mainda *et al.*, 2015; Polotto, 2012). Also of great global concern is an emerging increase in rare occurrence of Methicillin-resistant *S. aureus* (MRSA), Methicillin-resistance *Coagulate-negative Staphylococcus* (MRCNS) and Extended-spectrum β -lactamase (ESBLs) *E. coli*, *Klebsiella pneumonia* strains in bovine mastitis cases (Bandyopadhyay *et al.*, 2015; Igbinosa *et al.*, 2016; Murinda, 2014).

Infectious cases caused by the former pathogens are common and of most importance in human medicine and they have been spreading as acquired hospital pathogens worldwide, but recently community acquired and livestock associated have emerged. Their unusual presence in bovine mastitis cases is of public health concerned as they can be transmitted from food to humans. Methicillin resistance is

caused by *mecA* gene, which encodes the penicillin-binding protein 2a with decreased affinity for β -lactam antibiotics and ESBLs are the plasmid mediated enzymes that confer resistance to 3rd and 4th generation cephalosporins (oxy-imino β -lactam) and monobactam (aztreonam) groups of drugs except carbapenems and cephamycins (Koovapra *et al.*, 2016).

MRSA are resistance to almost all types of β -lactam, aminoglycosides, macrolides, tetracycline, fluoroquinolones chloramphenicol, and lincosamides (Bhattacharyya *et al.*, 2016; Nikaido, 2009). Glycopeptides such as vancomycin have been used as the last resort for their treatment, but also their prolonged use lead to some strains becoming resistant and such rare cases have been reported in dairy herds (Adegoke and Okoh, 2014; Bhattacharyya *et al.*, 2016; Kateete *et al.*, 2013; Pehlivanoglu and Yardimci, 2012). MRSA and MR-CNS in bovine mastitis cases have been recently reported in Uganda (Kateete *et al.*, 2013) and South Africa (Schmidt *et al.*, 2015), while ESBLs *E. coli* and *Klebsiella pneumonia* producing strains have been reported in UK (Timofte *et al.*, 2014), India (Bandyopadhyay *et al.*, 2015) and Egypt (Braun *et al.*, 2016). They are resistant against all β -lactam, including extended-spectrum cephalosporins (cefotaxime, ceftazidime). Globally mastitis cases caused by the aboved mentioned species strains are still rare and their isolation prevalence is still low, but continuous monitoring of their occurrence is still important.

Bacterial resistance to disinfectants is another possibility, which may be responsible for frequent occurrences of mastitis cases, but at present, it is not recognised as a major problem. The mechanism of resistance to disinfectants is similar to that of antibiotics, it can either be of a natural property of an organism (intrinsic or innate) or acquired by mutation or acquisition of plasmids or transposons (McDonnell and Russell, 1999). For disinfectants to be effective, it needs to be taken up by the cell and difference nature and composition in of outer membrane of organisms affects their uptake, as a result certain disinfectants are effective against other organisms and ineffective in others. Experimental evidence has shown that spores, mycobacterium and Gram-negative bacteria are generally more resistant than Gram-positive bacteria (Gnanadhas *et al.*, 2017; McDonnell and Russell, 1999). Mycobacteria cell wall is a highly hydrophobic structure with a mycoylarabinogalactan peptidoglycan, while Gram-negative bacteria cell wall has an outer membrane with porins and Lipopolysaccharides and periplasmic space with peptidoglycan layer and lipoprotein both of these cell wall acts as effective barrier, preventing disinfectant uptake by the cell. On other hand, Gram-positive bacteria cell walls are composed of peptidoglycan and teichoic acid, which neither of these appears to act as an effective

barrier to the entry of disinfectants as a result they are sensitive to most of disinfectants active ingredients (McDonnell and Russell, 1999).

Cationic ions disinfectants, chlorhexidine, Quaternary Ammonium compounds (QACs) are reported to be effective in Gram-negative bacteria and this is due to their ability to damage the cell wall facilitating their uptake (McDonnell and Russell, 1999; VKM, 2016). In addition, some *S. aureus* strains were reported to exist as mucoid strains in nature, with the cells surrounded by a slime layer. Nonmucoid strains are killed more rapidly than mucoid strains by chlorhexidine.

Futhermore, environmental factors like concentration, time of action, hard water, organic load, pH and temperature affect the activity of disinfectant agents (McDonnell and Russell, 1999). Sub-lethal concentrations and inadequate contact time were reported to induce resistance to disinfectants (Gnanadhas *et al.*, 2017; McDonnell and Russell, 1999).

2.8 Why Bovine Mastitis is an Important Disease

There are three diseases of economic importance in the dairy industry, mastitis, infertility and lameness. Mastitis is the number one in these production diseases responsible for most economic losses experienced by the dairy industry, accounting for 38% of the total direct cost (Kossaibati and Esslemont, 1997). The losses occurring were reported to be due to reduction in milk production, discarded milk prior treatment and after, cost of veterinary treatment and drug costs, culling and replacements and premium lost and penalties (Bhikane and Kawitkar, 2000; Blosser, 1979; FAO, 2014;Halasa *et al.*, 2007; Miller *et al.*, 2004; Sudhan and Sharma, 2010).

They estimated that about 70% reductions occur in milk production, both in clinical or subclinical mastitis cases. A reason, being that an infected cow does not return to the same production level within the remainder of lactation and this was found to be due to permanent damage to the milk- secreting cells; however, the extent of damage differs depending on the causing pathogen and cow's immune system (Bogni *et al.*, 2011).

Approximately 9% of economic losses were attributed to milk discarded, during and after antibiotic therapy. Milk with antibiotics residues is unmarketable and there is a withdrawal period until it is free from antibiotics and it is during this time that milk is discarded. In addition, prior treatment of milk may be discarded due to extreme

compositional change. Other losses are due to cost of veterinary services and drugs, which varies between countries.

Involuntary culling and replacements losses accounted for 14%. Age and health issues are one of the reason cows are removed from the herd. Apart from those, mastitis is the main reason most cows are frequently removed from the herd. This is done as the final resort when an animal is chronic and does not respond to treatment. This means that cows are removed from the dairy herd before they reach their optimal economic age leading to losses in farm production.

Other losses reported were significant alterations occurring in the composition of milk especially main milk components such as protein, fat and lactose (Auldism *et al.*, 1995; Le Maréchal *et al.*, 2011; Philpot, 1967). The type of protein present changes, casein major milk protein of high nutritional value decreases while low quality protein whey increases. In addition, blood serum components, chlorine, and sodium increase while important minerals like calcium and potassium decrease. In addition, SCC and bacterial counts increase. These changes subsequently have an effect on properties of milk end products. Apart from that, farmers lose premiums due to good quality milk and receive penalties due to poor qualities milk (Halasa *et al.*, 2007). These losses are estimated by factors that are part of milk payment system such as SCC, bacterial count, protein (%), fat (%), non-fat solids (%) and antibiotic residues.

Another issue associated with mastitis is that of public health significance, due to possible transfer of zoonotic pathogens or their thermostable toxins via milk to humans, especially in unpasteurised dairy products and during pasteurisation failure (Bradley, 2002). In addition, it also increases the risks of antibiotic residues in milk, which can lead to allergic reactions. These residues further increase the risk of emerging antimicrobial resistance strains entering the food chain (White & McDermott, 2001).

It is also associated with other health problems in cows such as reproduction failure and mortality (Sharma *et al.*, 2017). Some of the reasons for reproduction failure have been reported to be associated with reduction in natural estrus, inability to conceive after breeding and pregnancy losses (Barker *et al.*, 1998; Chebel *et al.*, 2004; Hudson *et al.*, 2012; Moore *et al.*, 1991; Santos *et al.*, 2004, Schrick *et al.*, 2001). Pregnancy losses are caused by bacterial toxins released during mastitis, which stimulate the production of prostaglandin F_{2α}, that subsequently causes luteal regression influencing conception and early embryonic survival in affected cattle. Studies showed that the probability of conception decreased by 44% when mastitis

occurred a week before insemination, by 73% when it occurred during the week of insemination, and by 52% when mastitis occurred during the week after insemination. About 17% of cow's adults mortality are caused by mastitis.

2.9 Mastitis Prevalence in African dairy farms

Currently, only about 30% of African countries report cases of bovine mastitis, while 70% of other countries are left behind (motaung *et al.*, 2017). The overall mastitis prevalence reported in some parts of African dairy farms is less than 54% at cow level, while clinical form is less than 14% and subclinical form is between 16-88% (Table 2.3). Previous studies conducted in different parts of Sub-Saharan Africa, mastitis prevalence was more than 50% and subclinical was between 16-80% (FAO.2014). This reveals that over the years, mastitis prevalence has not decrease and the subclinical form has increased. In Asian countries mastitis prevalence is also more than 50% (Sharma *et al.*, 2012). In other countries with developed dairy industry mastitis prevalence is less than 50% (Nickerson, 2009).

Possible explanation for this high rate of mastitis in African countries maybe due to not having enough information about the disease or not being aware of the proven mastitis prevention and control measures. As evidenced, from a recent study conducted in Uganda by Kaneene *et al.* (2016) and Wangalwa *et al.* (2016), in their report high proportion of farmers (77.5%) did not test the milk for mastitis and 92% of farmers were not aware of subclinical form of mastitis and do not use dry antibiotic therapy. In Sudan, 93% of famers do not use teat dipping or dry cow therapy and 70 % do not wash hands before milking, and they do not treat mastitis cases (Salih and Ahmed, 2011).

Table 2.3 Report on mastitis prevalence in African dairy herds.

Country	Sample size	Overall prevalence %	C (%)	SCM (%)	Reference
Ethiopia	384 Cs	52.9	9.4	43.5	G/Michael <i>et al.</i> , (2013)
	340 Qs		5.59	75.5	Duguma <i>et al.</i> , (2014)
	200 Cs		3.0	25.0	Belayneh, <i>et al.</i> ,(2014)
		52.6	14.6	85.4	Teklesilasie <i>et al.</i> , (2014),
	385 Cs	53.25% Cs , 30.32% Qs	9.09	44.16	Tsegaye, <i>et al.</i> ,(2015)
	600 Qs	56			Tassew <i>et al.</i> , (2015)
	384 Cs		5.2	16.1	Amdhun <i>et al.</i> , (2016)

	340, Qs	80.88	5.59	75.3	Mekonnin <i>et al.</i> , (2016)
	319	32.92		33.5-69.8	Mekonnin <i>et al.</i> , (2016)
Kenya	241 Cs		0.9* - 0.5#	56.0* and 65.0#	Gitau <i>et al.</i> ,(2014)
Sudan	400		10.5	72	Ahmed <i>et al.</i> , (2015)
	500				Salih, (2015)
Algeria	560 Qs	28.77			Saidi <i>et al.</i> ,(2013)
Egypt	120		13.3	56.3	Sayed <i>et al.</i> ,(2014)
	200 Cs		5.04	57.7	Ibrahim(2014)
Zimbabwe	584 Cs	21.1	4.8	16.3	Katsande <i>et al.</i> ,(2013)
Zambia	83 B			61.44	Kunda <i>et al.</i> , (2016)
	111	44.4		48.7	Oricksson, (2013)
Uganda	124			87.9	Kasozi <i>et al.</i> ,(2014)
	195			86.2 Cs , 55.4 Q	Abrahmsén <i>et al</i> (2014),
Ruwanda	195			52	Iraguha <i>et al.</i> ,(2015)
B= bulk, C=Clinical , Cs= cows, SCM= Sub-clinical, Q=quarters, *= 1st visit, # = 2nd visit					

2.10 Prevention and control of bovine mastitis

Complete elimination of this disease in the dairy herd is difficult and this is due to its determining factors, host characteristics (cow), nature and number of pathogens causing the disease and the environment (Sudhan and Sharma, 2010). However, its control has been possible for decades now ever since the development of a comprehensive plan of mastitis control by the National Mastitis Council (NMC). This control plan consists of six basic points, proper milking hygiene, use of properly functioning milking machines, post-milking teat dip, antibiotic therapy of clinical mastitis cows during lactation, dry cow therapy and culling of chronic infected cows (Nickerson, 2009). These six basic points were developed to control contagious mastitis which were the major pathogens causing mastitis. However, over years of their application, major pathogens change to environmental pathogens and this lead to the NMC of United States of America (USA) and Canada to expand the six-point plan to a ten-point plan with 73 sub-points (NAAS, 2013). These ten points are (a) establishment of goals for udder health; (b) maintenance of a clean, dry and comfortable environment; (c) proper milking procedures; (d) proper maintenance and use of milking equipment; (e) good record keeping; (f) appropriate management of clinical mastitis during lactation; (g) effective dry cow management;(h) maintenance of bio-security for contagious pathogens and culling of incurable and chronically infected cows; (i) regular monitoring of udder health status; and (j) periodic review of the

mastitis control program. The main objectives of these basic points were to reduce the rate and duration of new infections.

2.10.1 Maintenance of a clean, dry and comfortable environment

The cow's environment such as pasture, milking parlour, housing and bedding serve as primary source of environmental pathogens and the mastitis caused by these pathogens cannot be eradicated from a dairy herd but it can be controlled by reducing the number of bacteria the teat is exposed to. This is done by keeping bedding material such as sand, straw and sawdust clean and dry by changing them frequently and immediately remove all dung and urine. Housing environments should be properly ventilated and free stall barns are recommended with floors and walkways that have concrete and raised stalls with bedding material and steel dividing bars. If cows are allowed to run on pasture, a clean water source should be provided on a concrete area and there should be no access to manure, mud or pools of stagnant water (Sharif and Muhammad, 2009). Poorly designed facilities can contribute to increase incidence of environmental mastitis.

2.10.2 Proper milking hygiene

It involves cleaning milkers' hands, teats and udder with water and sanitizing solution and drying them with individual paper or cloth towels before milking. Also cleaning teat cups and sanitizing them between milking. Mastitis infected cows, undergoing antibiotic therapy are to be milked as a separate group or milked with separate clusters and the milkers should wear gloves. All clusters used for mastitis cows need to be rinsed and sanitized between each cow milking. This prevents transmission of pathogens from infected individuals to healthy cows. It also aids in producing high quality milk with less bacterial loads and reducing incidence of new infections.

2.10.3 Use of properly functioning milking machines

There are few basic functional standards, which all milking machines should meet; first milking systems should meet internationally accepted design and installation standards. Second, they must have stable milking vacuum level of 11 to 12 inches (275 to 300 millimeters or 37 to 41 kilopascals) of mercury at the claw during peak milk flow. Third slipping or squawk teat cup liners during milking must be avoided and lastly vacuum should be closed off to the claw before removing teat cups. Inability to meet

these requirements may lead to machines transferring pathogens from teat to teat and cow to cow.

2.10.4 Good record keeping

It involves the collection of useful data to monitor performance calculation of appropriate indices and decision making based on comparison to target levels. The records are either computerized or manual and useful data recorded involve monitoring of udder health at herd and cow level, using cow side test such as CMT, bulk tank milk SCC and individual cow SCC, teat dip used, antibiotic used for lactation and dry cow therapy, clinical mastitis cases episode occurrence, bacterial culture of bulk tank milk and individual cow milk. For clinical mastitis episode, data recorded involve lactation number, date of calving, type of therapy used and date of milk holding time to be completed. The importance of record keeping aids the farmer and veterans in making important decisions such as the effectiveness of mastitis control program being used and culling purpose decision.

2.10.5 Pre- and Post -milking teat dipping

It involves dipping all cows' teats in a disinfectant before and immediately after milking. Pre- teat dipping is done in conjunction with proper milking hygiene and was developed to control environmental pathogens and was found to reduce IMI caused by them by more than 50%. Apart from environmental pathogens it aids in reducing the spread of microorganisms and incidence of new IMI, and in minimizing the bacterial load in raw milk supply. In addition, the process of preparing teats for milking promotes milk letdown, speeding up the milking process, and helping to ensure that the maximum amount of available milk is harvested without causing damage to the sensitive teat tissues (Nickerson, 2001).

Post-teat dipping is done immediately after milking and it helps in killing microorganisms that survived pre-milking teat dipping. It controls the spread of contagious mastitis and exerts no control over coliform infections. Teat dipping reduces new IMI by more than 50% when used properly with other points. Dips can be applied by hand-held cups with a power dipper or sprayed using spray handgun type with spray nozzle. There is wide variety of teat dips with different active ingredients available commercially and the most widely used are iodine, chlorhexidine, quaternary ammonium compounds, hydrolysed fatty acids, chlorine, and acid anionic compounds. Emollients, such as glycerine, sorbitol, lanolin or propylene glyco are added to the disinfectant preparations to improve teat skin health and so reduce the likely reservoir of mastitis bacteria in teat sores and cracks.

Iodine and iodophors – These compounds are rapidly working and being bactericidal, fungicidal, virucidal, and sporicidal. Aqueous solutions are generally unstable; in solution, at least seven iodine species are present in a complex equilibrium, with molecular iodine (I₂) being primarily responsible for antimicrobial efficacy (Gottardi, 1991). These problems are overcome by the development of iodophors (“iodine carriers” or “iodine-releasing agents”). Iodophors are complexes of iodine and a solubilising agent or carrier, which acts as a reservoir of the active “free” iodine. Although germicidal activity is maintained, iodophors are considered less active against certain fungi and spores. The antimicrobial mode of action of iodine involves attacking key groups of proteins, in particular the free sulfur amino acids cysteine and methionine nucleotides, and fatty acids, which ends in cell death. Less is known about the antiviral action of iodine, but nonlipid viruses and parvoviruses are less sensitive than lipid enveloped viruses. Similarly, to bacteria, it is likely that iodine attacks the surface proteins of enveloped viruses, but they may also destabilize membrane fatty acids by reacting with unsaturated carbon bonds

Chlorhexidine – is a biguanide biocide effective against bacteria, yeast, sporostatic and mycobacteristatic have low activity against many viruses; lipid-enveloped viruses more sensitive than none enveloped viruses (Russell, 1986). Their uptake by bacteria and yeasts causes damage to the outer cell wall but it is not sufficient to induce cell death. The agent then further crosses the cell wall by passive diffusion, and attacks the cytoplasmic membrane or the yeast plasma membrane, damaging it causing leakage of intracellular constituents leading to cell death. High concentrations cause coagulation of intracellular constituents. In viruses, their effect is on viral capsid, perhaps the lipid moieties. When commercialised as teat dip it is available as Chlorhexidine and other unspecified ingredients or in salt form as Chlorhexidine digluconate and Chlorhexidine dicetate (McDonnell and Russell, 1999; Saha *et al.*, 2014).

Quaternary Ammonium compounds – are cationic agents used as both antiseptics and disinfectants, effective against Gram-negative and positive bacteria as well as yeasts, are sporestatic, mycobacteriostatic and not mycobactericidal, have an effect on lipid, enveloped but non-enveloped viruses (McDonnell and Russell, 1999; Russell, 1996; Springthorpe and Satter, 1990). In bacteria, they target cytoplasmic (inner) membrane and the plasma membrane in yeasts. The proposed sequence of events in their mode of action involves (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material;

(iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes. There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell.

Chlorine-based – teat dips products have a broad spectrum of action, are effective against Gram-positive and Gram-negative bacteria, as well as molds, yeasts, less active against spore and mycobacteria, effective against lipid and non-lipid viruses (Bloomfield, 1996; Bloomfield, *et al.*, 1990; Nickeson, 2001). Acidified Sodium Chlorite and Sodium Hypochlorite are two commonly used types of these products. Acidified sodium chlorite forms as the result of combining sodium chlorite with acid such as lactic acid or mandelic acid, forming the active microbicidal components chlorous acid and chlorine dioxide, which are microbicidal. Chlorine-releasing agents are strong oxidizing agents and destroy both structural and enzymatic proteins in prokaryotic cells. At higher concentrations, these agents are sporicidal, the spores lose refractivity, the spore coat separates from the cortex, and lysis occurs.

Acid anionic compounds (dodecyl benzene sulphonic acid, DDBSA) – these compound have rapid bacteriocidal action against most Gram-positive and Gram-negative bacteria except minor pathogens *Corynebacterium bovis* or coagulase negative staphylococci, also have moderate efficacy against fungi and some viruses (Nickerson, 2001; Pankey *et al.*, 1985; 1984). The active chemical ingredients in these disinfectants are anionic surface-active agents and their bactericidal action is due to their ability to disrupt cell membranes, denaturing the cell proteins, inactivating essential enzyme systems and causing leakage, which leads to cell death. Teat dip disinfectant products of these compound are available as conventional or barrier formulations.

2.10.6 Antibiotic therapy of clinical mastitis cows during lactation

It involves detection of the infected quarter and immediate initiation of treatment. Administrating the full series of recommended treatments and maintaining a set of treatment records. Finally, identifying all treated cows, and making sure the milk is free of antibiotic residues before adding to the bulk tank. Only clinical cases are treated, subclinical cases are treated during dry period, however exception is made when milk production is really compromised, the farmer is losing productivity. The main aim of antibiotic therapy during lactation is for elimination of the pathogen, for cow to return to its normal state of milk production and udder and rendering the cow free from pain.

2.10.7 Dry cow therapy

Dry cow therapy was developed with intent of treating contagious mastitis and all cows' quarters were treated with antibiotic immediately at drying off, whether they have mastitis or not. However due to economic costs, selective dry cow therapy is now used where only infected animals and those that are at risks are treated. Selective dry therapy assists in reducing treatment costs and prevents elimination of minor pathogens, which could make cows more susceptible to environment pathogens. This helps control environmental streptococci during the early dry period and it has little or no value in controlling coliforms. The main goal of antibiotic dry therapy is for elimination of existing cases and prevention of new cases.

There are also other non-antibiotic treatments available for use at drying off such as external and internal teat sealants. These teat sealants do not contain antibiotics, so they do not eliminate existing infections, only prevent new IMI. They are usually used in healthy cows during dry period or in combination with antibiotics. More research is still ongoing on internal teat sealants with biological antibacterial active ingredients, such bacteriocin lactecin and nisin; none however has been approved for use (Pyörälä, 2002).

Teat sealants currently available and used is Orbeseal, consists of bismuth subnitrate formulated into an inert viscous malleable paste that does not have any antibiotic or antimicrobial properties (Bradley *et al.*, 2005). It is delivered in a tube and is infused into the teat end after drying off in a manner similar to infusing a dry cow antibiotic. After infusion it fills the fissures and folds of the teat canal thereby creating a seal preventing the entrance of pathogens through the dry period. However, this has some problems also; there are some concerned of sealants residues being found on milk following calving.

2.10.8 Culling

It involves removal of chronic infected cows that does not respond to antibiotic therapy from the dairy herd. Decision to cull depends on varies factors such as the type of pathogen causing clinical mastitis and their response to antibiotic therapy. In addition, how many episode of clinical mastitis did the cow experience. Culling prevents spreading of the disease in a herd.

2.10.9 Regular monitoring of udder health status

SCC and routine visualization of udder and milk are used for regular monitoring of udder health status. Routine visualization of udder (looking for clinical signs) and

milk using dark surfaces (looking for changes in colour and milk consistence and presence of flakes and clots) are done on daily basis before milking. Somatic cells primarily consist of leukocytes (macrophages, lymphocytes, PMN) and epithelial cells (0-7%). SCC from uninfected quarters at the cow and quarter levels are generally below 200,000 cells/ml or even below 100,000 cells/ml during first lactations of cows. During Intramammary infection, SCC gets elevated up to more than 500,000 cells/ml and this is due to an influx of PMN into milk, which has been reported to make 90% of somatic cells. SCC are used as indicator of udder health, they are monitored on individual cow or herd, on daily, weekly or on monthly basis using indirect methods such as the California mastitis test (CMT) or direct methods such as Fossomatic SCC, SCC Scanner, Delaval cell count (Viguiet *et al.*, 2009). These aid in early diagnosis of mastitis cases, leading to early initiation of treatment preventing spreading of the disease and economic losses.

2.10.10 Nutrition

Studies conducted on the role the diet has on cows health has revealed that the deficiency of certain vitamins and trace elements have huge influences on cow's resistance to intramammary infections. Their deficiency have been reported to be related to increase in clinical and subclinical mastitis cases, increase in severity of the cases and increase SCC. These vitamins and trace elements that have been reported to play a crucial role in udder health are vitamins E, A, and b-carotene and the trace minerals selenium, copper, and zinc. Vitamin E is the most important lipid-soluble antioxidant that form integral component of all lipid membranes and has a role in protecting lipid membranes from attack by high tissue concentrations of reactive oxygen species (Rice and Kennedy, 1988). Selenium is an essential micro-nutrient present in tissues throughout the body and is an integral component of the enzyme glutathione peroxidase that functions in the cytosol of the cell (Erskine, 1987). Vitamin E and selenium (Se) are integral components of the antioxidant defence of tissues and cells and their deficiency lead to impaired PMN activity. Dietary supplementation of cows with Se and Vitamin E results in a more rapid PMN influx into milk following intramammary bacterial challenge and increased intracellular killing of ingested bacteria by PMN. They influence phagocytic cell function, and cows feed diets deficient in either component are at greater risk of environmental streptococcal mastitis (Smith *et al.*, 1984). Vitamin E and selenium improved udder health, and the effect of dietary supplementation is most evident at calving and in early lactation (Sharma and Maiti, 2005). Supplemented cows feed with copper were reported to reduce severity of clinical mastitis and cows supplemented with vitamin A and β -

carotene showed a reduction in new IMI (O'Rourke, 2009). Similarly, deficiencies in vitamin A and β -carotene are associated with increased incidence of mastitis. Zinc is reported to be essential trace element required for keratin formation in teat canal of udder. Keratin is a waxy material formed by stratified squamous epithelium cells which contains fatty acids that are bacteriostatic and fibrous proteins, both form a physical obstruction for pathogens preventing their movement to gland cistern. Cows receiving organic zinc (zinc proteinate) supplement were reported to have an increase in keratin formation, significantly low SCC and few new IMI cases (Popovic, 2004; Spain *et al.*, 2005, 1993).

2.11 Alternative therapy for treatment and control of bovine mastitis

Bovine mastitis is one disease that cannot be eradicated from the dairy herd. For many years, antibiotic therapy has been the only efficient remedy available for treatment and control of this disease and ensuring efficient production of high quality milk. Currently, the dairy industry has been experiencing some challenges such as an increase in the number of major mastitis causing pathogens that have become multidrug resistance, rendering treatment ineffective and decline in development of new antibiotics by pharmaceutical companies, lack of response by cows to antibiotics and antibiotic residues in milk have become challenges facing the dairy industry worldwide. Research currently done on treatment and control of disease is focused on finding non-antibiotic alternatives such as bacteriophages, vaccination, natural compounds from plants, animals and bacteria, nano particles and cytokines.

To date, not much has been achieved and the research is still ongoing. However, on vaccine development some break through has been achieved. There are several mastitis vaccines that are commercially available for the prevention of new IMI caused by coliform, *S. aureus*, and coagulase-negative Staphylococci (CNS) (Tiwari *et al.*, 2013). Commercially, there are three coliform mastitis vaccines available; two are identical and sold as J-5 Bacterin and Mastiguard™ and a separate bacterin-toxoid (J Vac®). A 4th gram-negative mastitis vaccine (Endovac-Bovi®) contains re-17 mutant *Salmonella typhimurium* bacterin toxoid. All coliform mastitis vaccine formulations use gram-negative core antigens to produce non-specific immunity directed against endotoxic disease and are reported to be effective in reducing the number of coliforms and severity of mastitis. For *S.aureus* mastitis vaccine such as Somato-Staph® and Lysigin® and Startvac® are available commercially. Startvac® contains inactivated *E. coli* (J5), inactivated *S. aureus* (CP8) SP 140 strain expressing Slime Associated

Antigenic Complex (SAAC) and adjuvant. The vaccine has a label claim for reducing the incidence of subclinical mastitis and the incidence and severity of the clinical signs of clinical mastitis caused by coliforms, *S. aureus*, and coagulase-negative staphylococci (CNS). Somato-Staph® and Lysigin® and are labelled as somatic antigen containing types I, II, III, IV and miscellaneous groups of *S. aureus*. However, due to the number and variety of pathogens, causing the disease development of one vaccine efficient in preventing the disease seems impossible, so more vaccines are still needed.

On the other hand for treatment, bacteriophages and bacteriocins seems to be promising tools as substitute for antibiotic therapy. To date, only Lactococcal bacteriocin, nisin, has been developed for commercial application, used for food preservation and animal probiotics (Pieterse and Todorov, 2010). Researchers have also evaluated nisin potential for treatment and control of bovine mastitis and the results were promising even though they vary (Wu *et al.*, 2007). For treatment so far, no products has been developed, but for prevention and control few products have been developed, (Ambicin® N, Applied Microbiology, Inc., New York, NY) is a nisin - based teat sanitizer and two wipes products , Wipe-Out® Dairy Wipes and Mast Out® were developed by Immucell Corporation (Cotter *et al.*,2005). In addition, there is another bacteriocin, lantibiotic, lacticin 3147, currently researched extensively for its potential as alternative for antibiotic therapy during dry cow treatment and as antimicrobial incorporated in teat sealants (Pieterse and Todorov 2010) and results are promising. For the purpose of this study, we will focus more on bacteriophages.

2.12 Bacteriophages

Phages are viruses specifically infecting bacteria and are harmless to humans, animals and plants; are highly specific to their bacterial host strain or species, occur naturally and they are the most abundant organisms on earth, approximately 10^{30} - 10^{31} particles (Bergh *et al.*,1989 and Burrowes *et al.*, 2011; Sillankorva *et al.*, 2012). Phages reproduce by two types of lifecycles, namely lytic (used by virulent and temperate phages) and lysogenic (used by temperate phage) Figure 2.1(García *et al.*, 2010). The lytic cycle involves attachment and irreversible binding of the phage to bacterial receptors on the cell surface followed by inserting its DNA and taking over of the bacterial cell machinery to produce phage particles and multiply within bacterial cell (host) to finally lyse the host cell and release phage progeny. The lysogenic cycle

phages intergrades its DNA into a host cell chromosomes where it replicate as part of the host genome until induced by external stimuli to enter the lytic cycle.

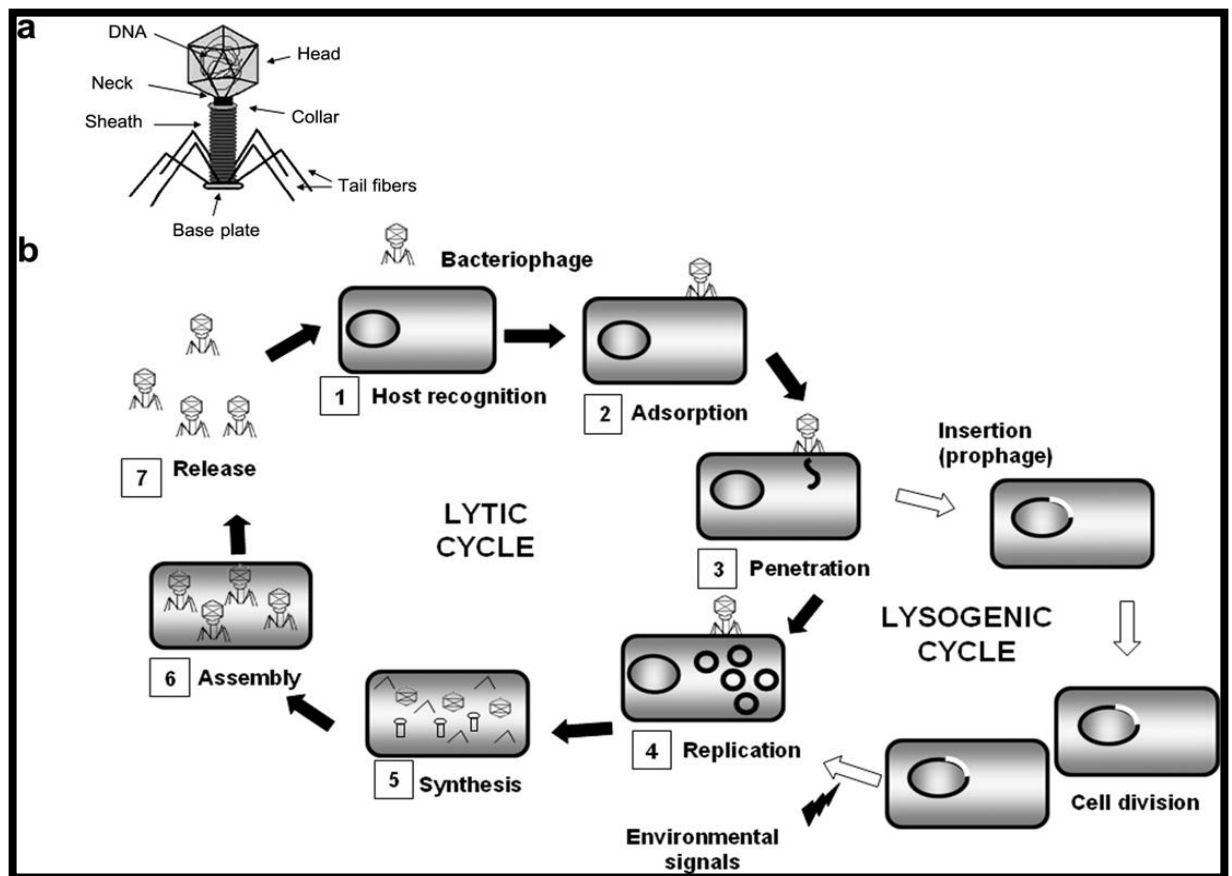


Figure 2.1 Life Cycle of Bacteriophages (García *et al.*, 2010)

Phages are classified according to their morphology, nucleic acid properties and their genome which can either be DNA or RNA, but most phages have double strand DNA (dsDNA), while the rest have single strand RNA (ssRNA), ds RNA or ssDNA (Ackermann, 2006). Morphological phages have few groups: filamentous, isosahedral phage without tails, phages with tails and phages with lipid containing envelop.

To all identified phages, approximately 190 are filamentous and pleomorphic and these are classified into 10 small families (Ackermann, 2004). The pleomorphic phages have three families namely *Plasmavidae*, *Fusselovidae* and *Guttavirus*. All three families have dsDNA, differing in their capsid, the first one is covered by a lipoprotein envelop and the second one have lemon-shaped capsid with short spikes at one end, while the last one has droplet-shaped virus like particles. The filamentous phages, also have dsDNA and their families are *Inoviridae*; long, rigid or flexible filaments or variable length classified by particle length coat structure and DNA content. The second family is *Lipothrixviridae*; they are characterized by combination

of a lipoprotein enveloped and rod-like shape. The last one is the Rudiviridae family representing phages that appear straight rigid rod-like without envelopes.

The other group of phages is the isosahedral shaped capsid and these are divided into four families; *Leviviridae* (ssRNA genome, packed in small capsid), *Corticoviridae* (contains three molecules of dsRNA), *Microviridae* (ssDNA), *Cystoviridae* (dsDNA, with capsid formed by outer layer of proteins with inner lipid bilayer) and *Tectiviridae* (dsDNA, with lipoprotein vesicle that envelops the protein capsid).

The last group are tailed phages classified into the order *Caudavirales* (dsDNA) (Ackermann, 2006). They are widely distributed, representing 96% of all known phages and are separated into three main phylogenetically related families: *Myoviridae* (has contractile tail consisting of sheath and central tubes, makes approximately 25% of tailed phages), *Siphoviridae* (has long, non-contractile tail, makes 61% of all tailed phages and *Podoviridae* (short tails, 14%).

2.12.1 Bacteriophages Application

Numerous ways exist in which phages can be utilised, as diagnoses tool through phage typing, in prevention and treatment of diseases through phage therapy, as biopreservative (added in food as additive) or biosanitiser (used in food contact surfaces and medical tools) and in research. As a therapeutic agent, they have been applied successfully in humans, animals and plants. In human phage therapy, there are two institutions, Bacteriophage Institute, Tbilisi, Georgia and Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland that have studied this phenomena extensively (Sulakvelidze *et al.*, 2001; Weber-Dabrowska *et al.*, 2000). Phages were used to treat patients with infections that were not responding to antibiotic therapy. The diseases treated were purulent meningitis, urinary tract infections, mucopurulent chronic bronchitis, laryngitis, rhinitis, Bronchopneumonia, stomach ulcers caused by *S. aureus*, *E. coli*, *Proteus*, *Klebsiella* and *Pseudomonas* species administered orally three times per day, locally by direct application on wounds or by dropping a phage suspension into the eye, ear or nose. In all those studies phage therapy had outstanding success with between 85 to 100% cure rate, in generally phage therapy success rate was said to be 85%. In Poland, they are currently used as experimental treatment in infection cases where antibiotic therapy had failed.

In the food industry, phage therapy has been used in preventing, reducing, and treating diseases in livestock (Ishani and Tina, 2015; Sillankorva *et al.*, 2012). Also used as biocontrol agents in ready to eat food, added in food as additives to preserve

food, used as biosanitizers for food contact surfaces and equipment. There are commercially available phage preparations in United States and in Europe that are approved for use by Food and Drug Administration (FDA) and United States Department of Agriculture (USDA). ListShield phage (six-phage cocktail) product from Intralytix USA company approved by FDA in 2006 targeting *Listeria monocytogenes* bacteria from ready-to-eat food (RTE) foods and meats. EcoShield from the same company was approved by FDA in 2011, for use on red meat parts and trims intended to be ground for *Escherichia coli* bacteria (Food Contact Notification no. 1018 for ground meat). LISTEX P100 phage-based product from EBI Food Safety, Netherlands, approved for use by USDA is generally recognized as safe to prevent *Listeria* contamination on food products and food processing facilities (Sillankorva *et al.*, 2012).

In agriculture phage therapy has been used to prevent diseases in plants for prevention of bacterial spots in peaches, cabbage and peppers caused by *Xanthomonas pruni* and in tomatoes caused by *Xanthomonas campestris*. In other studies, they were used to control *Ralstonia solanacearum* of tobacco and bacterial blotch of mushrooms caused by *Pseudomonas tolaasii* (Gill and Abedon, 2003).

2.12.2 Bacteriophages for treatment and control of bovine mastitis

The use of bacteriophages for treatment and control of bovine mastitis is the topic that is currently being researched. All the studies conducted were evaluating the effectiveness of phage therapy in treating mastitis cases caused by *S. aureus* during lactation. Swatantra *et al.* (2014), in India, conducted one such study, the experiment consisted of 12 chronic mastitis infected cows and they were treated by single dose intramammary infusion of 5 ml of phage cocktail composed of three phages with a titre of 3×10^{12} PFU /ml of lytic phage. In their results, teats showed gradual reduction in the swelling and ten were completely cured out of twelve within ten days achieving a cure rate of 83%. Another study was conducted by Gill *et al.* (2006), their experimental treatment consisted of 10-ml intramammary infusions of either 1.25×10^{11} PFU/ml of phage K or saline, administered once per day for 5 days. In their results, phages were detectable in milk for up to 36hrs post-infusion but at significantly lower rates, there was an increase in the SCC of treated udders and the cure rate was 16.7%. In another study by Ndlela *et al.* (2016) South Africa; phages were applied to cows as a post-milking udder spray treatment and 87% of phage-treated cows showed a lower bacterial cell count and 96% of the untreated cows showed a higher bacterial cell count compared to treated cows. In another study by O'Flaherty *et al.* (2005), 10 mmol of phosphate buffer was used to deliver a cocktail of the three phages (CS1, DW2 and

K) together with titre 10^8 PFU/ml into the teat sinus, resulted in no significant increase in the SCC. There were several limiting factors observed to be reason for variation in the results obtained in those studies, one such was phage inactivation in the udder caused by milk proteins and fats. Phage therapy provided an alternative, but Intra-mammary infusion of bacteriophages seemed to be not efficient. Use of Bacteriophages in teat dipping, may provide an alternative in preventing new intra-mammary infections in dairy cattle.

The aim of this project was to isolate lytic bacteriophages for mastitis associated causative pathogens with an aim of formulating a phage cocktail that can be used as an alternative teat dip. The first objective of this project was to determine species diversity of mastitis associated causative pathogens in South African dairy farms. The efficacy of various antibiotics against bacterial strains, which were isolated, were also evaluated in effort to establish the level of resistance to different antibiotics. There is currently a crisis with regard to bacterial antimicrobial resistance and current status of antibiotic resistance to South African strains needs to be evaluated. The second objective was to isolate lytic bacteriophages for these mastitis-causing pathogens, to characterize the phages morphologically, and to determine their host range.

2.13 References

Abrahmsén M, Persson Y, Kanyima B.M and Båge R, (2014). Prevalence of subclinical mastitis in dairy farms in urban and peri-urban areas of Kampala, Uganda *Tropical Animal Health Productio*, 46:99–105.

Ackermann H.W, (2006). Classification of bacteriophages. In *The Bacteriophages*, Ed. Calendar R, Oxford University Press, ISBN 0-19-514850-9, New York, USA, pp. 8– 16.

Ackermann H.W, (2004). Bacteriophage classification. In *Bacteriophages. Biology and Applications*. Eds Kutter E, Sulakvelidze A, CRC Press ISBN 978-0-8493-1336-3, Boca Raton, USA, pp. 67–89.

Adegoke A. A and Okoh A.I, (2014). Species diversity and antibiotic resistance properties of *Staphylococcus* of farm animal origin in Nkonkobe Municipality, South Africa. *Folia Microbiologica (Praha)* 59:133–140.

- Ahmed A. G.M, Elfadil A. A. M and Aldeen M.T, 2015. Study on bovine mastitis in smallholder dairy cows in Northern State of Sudan. *International Journal of Veterinary Science*, 4(2): 81-86.
- Ahmed A.M and Shimamoto T, (2011). Molecular characterization of antimicrobial resistance in Gram-negative bacteria isolated from bovine mastitis in Egypt. *Microbiology and Immunology*; 55: 318–327.
- Amdhun K, Tilahun A and Aylate A, (2016). Study on Prevalence of Bovine Mastitis in Tullo District of West Hararghe, Ethiopia. *Advances in Biological Research* 10 (3): 147-153.
- Ammar A, El-Shafii S.S.A, AMO A.E and El-dayim Z. A. A, (2016). Detection of Multidrug Resistance Genes in *Pseudomonas aeruginosa* Isolated from Bovine Mastitic Milk. *Journal of Dairy, Veterinary and Animal Research* 3(2): 00071.
- Auldism M.J, Coats S, Rogers G.L and McDowell G.H, (1995). Changes in the composition of milk from healthy and mastitic dairy cows during the lactation cycle. *Australian Journal of Experimental Agriculture*. 35: p. 427-436.
- Bandyopadhyay S, Samanta I, Bhattacharyya D, Nanda P. K, Kar D, Chowdhury J, Dandapat P, Das A. K, Batul N, Mondal B, Dutta T. K , Das G, Das B. C, Naskar S, Bandyopadhyay U .K, Das S. C and Bandyopadhyay S,(2015). Co-infection of methicillin-resistant *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus* and extended spectrum β -lactamase producing *Escherichia coli* in bovine mastitis – three cases reported from India *Veterinary Quarterly*, Vol. 35(1) 56-61.
- Barker A.R, Schrick F.N, Lewis M.J, Dowlen H.H, and Oliver S.P, (1998). Influence of clinical mastitis during early lactation on reproductive performance of Jersey cows. *Journal of dairy science* 81:1285-1290.
- Basdew I.M and Laing M.D (2015). Investigation of the lytic ability of South African bacteriophages specific for *Staphylococcus aureus*, associated with bovine mastitis, *Biocontrol Science and Technology*, 25:4, 429-443, DOI: 10.1080/09583157.2014.983458.
- Belayneh R, Belihu K and Tesfaye A, (2014). Microbiological study on bacterial causes of bovine mastitis and its antibiotics susceptibility patterns in East Showa Zone, Akaki District, Ethiopia. *Journal of Veterinary Medicine and Animal Health* Vol. 6(4), pp. 116-122, April.

Beyene G.F, (2016). Antimicrobial Susceptibility of *Staphylococcus aureus* in Cow Milk, Afar Ethiopia. International Journal of Modern Chemistry and Applied Science 2016, 3(1)280-283.

Bhattacharyya D, Banerjee J, Bandyopadhyay S, Mondal B, Nanda P.K, Samanta I, Mahanti A, Das A.K, Das G, Dandapat P and Bandyopadhyay S, (2016). First Report on Vancomycin-Resistant *Staphylococcus aureus* in Bovine and Caprine Milk Microbial Drug Resistance Volume 00, Number 00.

Bhikane A.V and Kawitkar S.B, (2000). Hand book for Veterinary Clinician. Venkatesh Books. Udgir, India.

Biggs A, (1996). *Streptococcus agalactiae* - to Blitz or Not to Blitz. In: Proceedings 19th World Buiatrics Congress, 8-12 July 1996, Edinburgh, United Kingdom, Proceedings 1, pp. 296-301.

Blignaut D.J.C, (2016). Prevalence of Mastitogenic pathogens in pasture and total mixed ratio based dairies in 2008 and 2013: Thesis submitted on partial fulfilment of requirement for the degree, master in veterinary medicine in the department of animal production studies, Faculty of Veterinary Sciences, University of Pretoria.

Bloomfield S.F, (1996). Chlorine and iodine formulations, p. 133–158. In J. M. Ascenzi (ed.), Handbook of disinfectants and antiseptics. Marcel Dekker, Inc., New York, N.Y.

Bloomfield S.F, Smith-Burchnell C.A and Dalgleish A.G (1990). Evaluation of hypochlorite-releasing disinfectants against the human immunodeficiency virus (HIV). Journal of Hospital Infection, 15:273–278.

Blosser T.H, (1979). Economic Losses from and the National Research Program on Mastitis in the United States. Journal of Dairy Science Vol. 62:119-127.

Blowey R and Edmondson P, (2010). Mastitis control in Dairy Herds, 2nd Edition. CAB International, UK.

Bradley A, Newton H, Benchaoui H, Tilt N, Cracknell V and Rowan T, (2005). Orbeseal® and Orbenin® for the treatment of intra-mammary infections at drying off and prevention of new infections during the dry period and early lactation in dairy cows. In: Hogeveen, H. (ed.), Mastitis in dairy production Current knowledge and future solutions, Wageningen Academic Publishers, The Netherlands, pp. 339-344.

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

Braun S.D, Ahmed M.F.E, El-Adawy H, Hotzel H, Engelmann I, Weiß D, Monecke S and Ehrlich R, (2016). Surveillance of Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli* in Dairy Cattle Farms in the Nile Delta, Egypt. *Frontiers in Microbiology* 7:1020.

Bogni C, Odierno L, Raspanti C, Giraud J, Larriestra A, Elina Reinoso, Lasagno M, Ferrari M, Ducrós E, Frigerio C, Bettera S, Pellegrino M, Frola, Dieser S and Vissio C, (2011). War against mastitis: Current concepts on controlling bovine mastitis pathogens. *FORMATEX* 2011: <http://www.formatex.info/microbiology3/book/483-494.pdf>.

Chebel R.C, Santos J.E.P, Reynolds J.P, Cerri R.L. A, Juchem S.O and Overton M, (2004). Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. *Animal Reproduction Science*, 84:239–255.

CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition*. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.

Compton C.W, Heuer C, Parker K and McDougall S, (2007). Risk factors for peripartum mastitis in pasture-grazed dairy heifers. *Journal of Dairy Science*, 90, 4171-4180.

Constable P. D, Hinchcliff K. W, Done S.H, Gruenberg W. *Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep*. 11th edition.

Cotter P.D, Hill C, Ross R.P, (2005). Bacteriocins: Developing innate immunity for food. *Nature Reviews Microbiology*, 3: 777-788.

Cullor J.S. and Tyler J.W, (1996). Mammary Gland Health and Disorders. In: *Large Animal Internal Medicine*, ed. Smith Bradford P., 2nd ed., Mosby, St. Louis pp. 1178-1193.

Department of Health, (1997). *The Regulations Relating to Milk and Dairy Products*. R.1555 of 21 November 1997.

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.

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

Duguma A, Tolosa T and Yohannes A, (2014). Prevalence of clinical and sub-clinical mastitis on cross-bred dairy cows at Holleta Agricultural Research Center, Central Ethiopia. *Journal of Veterinary Medicine and Animal Health*, Vol. 6(1), pp. 13-17, January.

Du Preez J.H, (2000). Bovine mastitis therapy and why it fails. *Journal of the South African Veterinary Association*, 71(3): 201–208.

Easterday B.C, Hanson R.P and Simon J, (1959). Experimental viral bovine mastitis. *American Journal of Veterinary Research*, 20, 819–824.

El Behiry A, Schlenker G, Szabo I, Roesler U, (2012). *In vitro* susceptibility of *Staphylococcus aureus* strains isolated from cows with subclinical mastitis to different antimicrobial agents. *Journal of Veterinary Science*, 13(2), 153-16.

Elvander M, (1996). An experimental study of a concurrent primary infection with Bovine Respiratory Syncytical Virus (BRSV) and Bovine Viral Diarrhoea Virus (BVDV) in calves. Ph.D. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Ericksson L, (2013). Prevalence of subclinical mastitis and udder pathogens in small holder dairy farms in Mapepe, Batoka and Choma areas in Zambia. *Online publication of this work: <http://epsilon.slu.se> ISSN 1652-8697 Examensarbete 2013:74.*

Erskine R.J (1993). Nutrition and mastitis. In: *The Veterinary Clinics of North America: Food Animal Practice*. p 551. Andersen KL, Saunders WB (eds). Philadelphia, PA.

Erskine R.J, Eberhart R.J and Hutchinson L.J, (1987). Blood selenium concentrations and glutathione peroxidase activities in dairy herds with high and low somatic cell counts. *Journal of the American Veterinary Medical Association*, 190(11), 1417-21.

Fadlelmula A, Al Dughaym A, Mohamed G, Al Deib M and Al Zubaidy A (2009). Bovine mastitis: epidemiological, clinical and etiological study in a Saudi Arabian large dairy farm. *Bulgarian Journal of Veterinary Medicine* 12: 199-206.

FAO. 2014. Impact of mastitis in small-scale dairy production systems. *Animal Production and Health Working Paper*. No. 13. Rome.

Francis P.G, (1984). Teat skin lesions and mastitis. *British Veterinary Journal* 140, 430–436.

Fox L.K and Gay J.M, (1993) .Contagious Mastitis. *Veterinary Clinics of North America* 9(3), 475-487.

Ganguly S, Wakchaure R, Para P. A, Praveen P. K, Sahoo S, Mahajan T, Qadri K and Kamble S, (2016). MDR Bacteria: A Review of Immense Relevance in Veterinary Microbiology. *Journal of Bio Innovation*, 5(3), pp: 319-324.

García P, Rodríguez L, Rodríguez A and Martínez B, (2010). Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. *Trends in Food Science and Technology*, 21, 373–382. doi:10.1016/j.tifs.2010.04.010.

Gibbs E.J.P, (1984). Viral diseases of the skin of the bovine teat and udder. In: *Proceedings of the Symposium on Large Animal Dermatology*. *Veterinary Clinics of North America*, 6, 187–202.

Gill J and Abedon S.T, 2003. Bacteriophage ecology and plants. *APSnet Feature*. [<http://www.apsnet.org/publications/apsnetfeatures/Pages/BacteriophageEcology.aspx>].

Gitau G. K, Bundi R.M, Vanleeuwen J and Mulei C.M, (2014). Mastitogenic bacteria isolated from dairy cows in Kenya and their antimicrobial sensitivity. *Journal of the South African Veterinary Association* 85(1).

G-Michael L, Deressa B, Begna F and Mekuria A, (2013). Study on prevalence of bovine mastitis in lactating cows and associated risk factors in and around Areka town, Southern of Ethiopia. *African Journal of Microbiology Research* Vol. 7(43), pp. 5051-5056, 25 October.

Gnanadhas D.P, Marathe S. A and Chakravorty D, Biocides - resistance, cross-resistance mechanisms and assessment. Article in *Expert Opinion on Investigational Drugs*. December 2012 DOI: 10.1517/13543784.2013.748035 · Source: PubMed.

Gonzalez R.N, (1996). Prototheca, Yeast, and *Bacillus* as a Cause of Mastitis National Mastitis Council Annual Meeting, Page 82.

Gottardi W, (1991). Iodine and iodine compounds, p. 152–166. In S. S. Block (ed.), Disinfection, sterilization, and preservation. 4th ed. Lea & Febiger, Philadelphia, Pa.

Gruet P, Maincent P, Berthelo X.V. Kaltsatos V, (2001). Bovine mastitis and intramammary drug delivery: review and perspectives. *Advanced Drug Delivery Reviews*, 50, pp. 245–259.

Gupta S, Govil D, Kakar P.N, Prakash O, Arora D, Das S, Govil P and Malhotra A, (2009). Colistin and polymyxin B: A re-emergence. *Indian Journal of Critical Care Medicine*, April-June Vol 13 Issue 2.

Halasa T, Huijps K, Østerås O and Hogeveen H, (2007). Economic effects of bovine mastitis and mastitis management: A review *Veterinary Quarterly*, 29:1, 18-31, DOI: 10.1080/01652176.2007.9695224.

Hamadani H; Khan A.A, Banday M.T, Ashraf I, Handoo N, Bashir A, and Hamadani A, (2013). Bovine Mastitis - A Disease of Serious Concern for Dairy Farmers *International Journal of Livestock Research*, VOL 3 (1) January 13.

Hawari A.D and Fazwi A, (2008). Prevalence and distribution of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Jordan. *American Journal of Animal and Veterinary Sciences*. 3:36-39.

Hebert A, Sayasith K, Senechal S, Dubreuil P, Lagace J,(2000). Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk *FEMS Microbiology Letters* 193, 57-62.

Higgins J.P., Higgins S.E, Guenther K.L, Newberry L.A, Huff W.E and Hargis B.M, (2005). Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry. *Poultry Science Journal*, Vol. 84, pp. 1141-1145.

Hillerton J.E and Berry E.A, (2005). A REVIEW: Treating mastitis in the cow – a tradition or an archaism. *The Society for Applied Microbiology, Journal of Applied Microbiology*, 98, 1250–1255.

Hillerton J.E, (1999). Balancing mastitis and quality. *Proc.British Mastitis Conference*, Stonelergh, UK. Pp: 31-36.

Hogan J.S, White D.G and Pankey J.W, (1987). Effects of teat dipping on intramammary infections by staphylococci other than *Staphylococcus aureus*. Journal of Dairy Science, 70, 873-879.

Hudson C.D, Bradley A.J, Breen J.E, and Green M.J, (2012). Associations between udder health and reproductive performance in United Kingdom dairy cows. Journal of Dairy Science 95:3683-3697.

Ibrahim H.M.M, Ahmed A.M, El-seedy Y.Y and El-Khodery S.A, (2015). Distribution of Multidrug-Resistant Gram Negative Bacteria Causing Clinical Mastitis in Dairy Cows Global Veterinaria 15 (3): 268-277

Ibrahim S.S, El-Said G.W.A, Morgan S.D and El-Garhi M. M, (2014). Prevalence of REVALENCE OF Intramammary infection in some selected commercial dairy farms in Egypt. Journal of the Egyptian Veterinary Medical Association, 74, no 1, 129 – 137.

Igbinsola E. O, Beshiru A, Akporehe L. O and Ogofure A. G, (2016). Detection of Methicillin-Resistant Staphylococci Isolated from Food Producing Animals: A Public Health Implication. Veterinary Science, 3, 14.

Iraguha B, Hamudikuwanda H. and Mushonga B, (2015). Bovine mastitis prevalence and associated risk factors in dairy cows in Nyagatare District, Rwanda. Journal of the South African Veterinary Association 86(1).

Ishani D and Tina S, (2015). Bacteriophages as Twenty-First Century Antibacterial Tools for Food and Medicine. International Journal of Interdisciplinary and Multidisciplinary Studies (IJIMS), Vol 2, No.9,92-97.

IVS. 2015. Index of Veterinary Specialities. Vol 53. Hirt and Carter, Durban, South Africa.

Jones G.M and Bailey T.L, (2009). Understanding the Basics of Mastitis. Virginia Cooperative Extension programs, publication 404-233.

Kaneene J. B, Ssajjakambwe P, Kisaka S, Vudriko P, Miller R and Kabasa J D, (2016). Improving efficiency of the dairy value chain in Uganda; effect of action research-based interventions on milk quality and safety. Livestock Research for Rural Development 28 (1) 2016.

Kasozi k.I, Tingiira J.B and Vudriko P, (2014). High Prevalence of Subclinical Mastitis and Multidrug Resistant *Staphylococcus aureus* are a Threat to Dairy Cattle

Production in Kiboga District (Uganda). Open Journal of Veterinary Medicine, 4, 35-43.

Kateete D.P, Kabugo U, Baluku H, Nyakarahuka L, Kyobe S, Okee M, Najjuka C.F, Joloba M.L, (2013). Prevalence and Antimicrobial Susceptibility Patterns of Bacteria from Milkmen and Cows with Clinical Mastitis in and around Kampala, Uganda. PLoS ONE 8(5).

Katsande S, Matope G, Ndengu M and Pfukenyi D.M, (2013).Prevalence of mastitis in dairy cows from smallholder farms in Zimbabwe. Onderstepoort Journal of Veterinary Research 80(1).

Khan M and Khan A (2006). Basic facts of mastitis in dairy animals: A review. Pakistan veterinary journal 26(4): 204-208.

Kirk J.H and Lauerman L.H (1994). *Mycoplasma* mastitis in dairy cows. The compendium 1994: 541-558.

Koovapra S, Bandyopadhyay S, Das G, Bhattacharyya D, Banerjee J, Mahanti A, Samanta I, Nanda P. K, Kumar A, Mukherjee R, Dimri U and Singh R. K, (2016). Molecular signature of extended spectrum β -lactamase producing *Klebsiella pneumoniae* isolated from bovine milk in eastern and north-eastern India. Infection, Genetics and Evolution 44:395–402.

Kossaibati M.A and Esslemon R.J, (1997). The costs of production diseases in dairy herds in England. Veterinary Journal 154, 41±51.

Kuehn J.S, Gorden P.J, Munro D, Rong R, Dong Q, Plummer P.J, Wang C, Phillips G.J, (2013) .Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. PLoS One8:e61959.

Kulkarni A.G and Kaliwal B.B, (2013). BOVINE MASTITIS: A REVIEW. International Journal of Recent Scientific Research Vol. 4, Issue, 5, pp. 543- 548.

Kunda B, Pandey G.S and Muma J.B, (2016). Somatic Cell Count and Antibiotic Residues in raw milk Produced by Smallholder Dairy Farmers in Lusaka Province of Zambia Asian Journal of Multidisciplinary Studies, 4(5) April.

Larsen J, (1994). The Economic Losses due to Mastitis and the Cost Effectiveness of Mastitis Problem Solving and Monitoring Programs. Udder Health Seminar, 22 June, 6.

Lehmann and Neumann (1896). *Streptococcus agalactiae*, 126AL (nom. cons. Opin. 8 Jud. Comm. 1954, 152). In BERGEY'S MANUAL OF Systematic Bacteriology, Second Edition, Volume Three, The *Firmicutes*. De Vos P, Garrity G.M, Jones D, Krieg N.K, Ludwig W, Rainey F.A, Schleifer K.H and Whitman W.B, pp 684.

Le Maréchal C, Thiéry R, Vautor E and Le Loir Y, (2011). Mastitis impact on technological properties of milk and quality of milk products: a review. Dairy science & technology, 91 (3), pp.247-282.

Linde C, Holmberg O and Astrom G, (1980). The interference between coagulase negative staphylococci and *Corynebacterium bovis* and the common udder pathogens in the lactating cow. Nordisk Veterinary Medicine Journal, 32, 552-558.

Madgwick L, Mayer S. and Keen P, (1989). Penetration of antibiotics into bovine neutrophils and their activity against intracellular *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* 24,709.

Mainda G, Bessell P.R, Muma J.B, McAteer S.P, Chase-Topping M. E, Gibbons J, Stevens M.P, Gally D.L and Barend Bronsvort B.M.D, (2015). Prevalence and patterns of antimicrobial resistance among *Escherichia coli* isolated from Zambian dairy cattle across different production systems Scientific Reports | 5:12439 | DOI: 10.1038/srep12439.

Mallikarjunaswamy M.C and Krishnamurthy G.V, (1997). Antibiogram of bacterial pathogens isolated from bovine mastitis cases. Indian Veterinary Journal, 74(10): 885-887.

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

McDonald J.S, (1977). Streptococcal and Staphylococcal Mastitis. Journal of the American Veterinary Medical Association 170, 10(2), 1157-1159.

McDonnell G and Russell A. D, (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. Clinical Microbiology Reviews 12: 147–179.

Mekonnin E, Eshetu E, Awekew A and Thomas N, (2016). A Study on the Prevalence of Bovine Mastitis and Associated Risk Factors in and the Surrounding areas of Sodo Town, Wolaita Zone, Ethiopia Global Journal of Science Frontier Research, Volume 16 Issue 2 Version 1.0, pp 13-19.

Miller R.H, Norman H.D, Wiggans G.R and Wright J.R, (2004). Relationship of Test-Day Somatic Cell Score with Test-Day and Lactation Milk Yields. *Journal of Dairy Science*, 87:2299-2306.

Moore D. A, Cullor J.S, Bondurant R.H, and Sischo W.M, (1991). Preliminary Field Evidence for the Association of Clinical Mastitis with Altered Interestrus Intervals in Dairy Cattle. *Theriogenology* 36:257-265.

Motaung T.E, Petrovski K.R, Petzer I, Thekiso O and Tsilo T.J, (2017). Importance of bovine mastitis in Africa: Review. *Animal Health Research Reviews*, pp 1-12.

Murinda S, (2014). Methicillin-Resistant *Staphylococcus aureus* (MRSA) Prevalence in Mastitic Dairy Cows from San Bernardino County, CA. Research Proposal. Word. 30 March 2014.

NAAS 2013. Mastitis Management in Dairy Animals. Policy Paper No. 61, National Academy of Agricultural Sciences, New Delhi: 12 p.

Ndlela M, Laing M.D and Basdew I.H, (2016). Biological control of *Staphylococcus aureus*-induced bovine mastitis in dairy cows using bacteriophages. Proc. 6th IDF Mastitis Conf., Nantes, France; 7-9 Sept. 2016.

Nickerson S.C, (2009). A comprehensive plan to control mastitis and milk quality. Animal and Dairy Science Departmental Report. <http://www.ads.uga.edu/ADS2008DepartmentalReports.html>

Nickerson S.C, (2001). Role of drug therapy in mastitis control. Hill farm Research Station,
<http://www.agctr.lsu.edu/wwwac/research/hillfarm/mastitis/drugtherapy.html>

NICKERSON S.C, (1996). Mastitis Control in Replacement Heifers.
<http://www.afns.ualberta.ca/hosted/webs/web96/web96389.htm>

Nikaido H (2009). Multidrug Resistance in Bacteria. *Annual Review of Biochemistry* 78: 119-146.

O'Flaherty S; Ross R.P, Flynn J, Meaney W.J, Fitzgerald G.F and Coffey A, (2005). Isolation and characterization of two anti-staphylococcal bacteriophages specific for pathogenic *Staphylococcus aureus* associated with bovine infections. *Letters in Applied Microbiology*, 41, 482–486.

Oikonomou G, Bicalho M.L, Meira E, Rossi RE, Foditsch C, Machado VS, Teixeira AG, Santisteban C, Schukken Y. H, Bicalho R. C, (2014). Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. PLoS One 9:e85904.

Oikonomou G, Machado V.S, Santisteban C, Schukken Y.H, Bicalho R.C, (2012). Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. PLoS One 7:e47671.

O'Rourke D, (2009). Nutrition and udder health in dairy cows: a review Irish Veterinary Journal Volume 62 Supplement 15-20.

Oviedo-Boyso J, Valdez-Alarco'n J.J, Cajero-Jua'rez M, Ochoa-Zarzosa A, Lo'pez-Meza J. E, Bravo-Patin'o A, Baizabal-Aguirre V.M, (2007). Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. Journal of Infection 54, 399-409.

Paape M.J, Bannerman D.D, Zhao X and Lee J.W, (2003). The bovine neutrophil: structure and function in blood and milk. Veterinary Research 34, 597– 627.

Parker K.I, Compton C, Anniss F.M, Weir A, Heuer C and McDougall S, (2007). Subclinical and clinical mastitis in heifers following the use of a teat sealant precalving. Journal of Dairy Science, 90, 207-218.

Pehlivanoglu F and Yardimci H, (2012). Detection of methicillin and vancomycin resistance in Staphylococcus strains isolated from Bovine milk samples with mastitis. Kafkas University Veteterinary Faculty Dergisi. 18, 849–855.

Pieterse R and Todorov S.D, (2010). Bacteriocin- exploring alternative to antibiotics in mastitis treatment. Brazilian Journal of Microbiology (2010) 41: 542-562.

Pieterse R, (2008).Thesis: Control of bacterial pathogens associated with mastitis in dairy cows with natural antimicrobial peptides produced by lactitic acid bacteria. University of Stellenbosch.

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(1):17-22.

Philpot W.N, (1975). Prevention of Infection - Hygiene. In Proceedings of the International Dairy Federation Annual Bulletin, Document 85,155-164.

Philpot W.N, (1967). Influence of subclinical mastitis on milk production and milk composition, *Journal of Dairy Science*, 50, 978.

Polotto M, Casella T, de Lucca Oliveira M.G, Rúbio F.G and Nogueira M.L, (2012). Detection of *P. aeruginosa* harboring bla CTX-M-2, bla GES-1 and bla GES-5, bla IMP-1 and bla SPM-1 causing infections in Brazilian tertiary-care hospital. *BMC Infect Dis* 12: 176.

Popovic Z, (2004). Performance and udder health status of dairy cows influenced by organically bound zinc and chromium. Ph.D. thesis, University of Belgrade.

Pyörälä S, (2009). Treatment of mastitis during lactation. *Irish Veterinary Journal* Volume 62 Supplement 40-44.

Pyörälä S, (2002). New Strategies to Prevent Mastitis *Reprod, Dom Anim* 37, 211–216.

Pyörälä S.H, Pyörälä E.O, (1998). Efficacy of parenteral administration of three antimicrobial agents in treatment of clinical mastitis in lactating cows: 487 cases (1989-1995). *Journal of American Veterinary Medical Association*, 212, 407-412.

Rainard P, (2017). Mammary microbiota of dairy ruminants: fact or fiction?. *Rainard Veterinary Research*, 48:25. DOI 10.1186/s13567-017-0429-2.

Reshi A.A, Husain I, Bhat S.A, Rehman M.U, Razak R, Bilal S, Mir M. R, (2015). Bovine Mastitis as an Evolving Disease and its Impact on the Dairy Industry. *International Journal Current Research Review* | Vol 7 • Issue 5 • March 2015.

Rice D.A and Kennedy S, (1988). Assessment of vitamin E, selenium and polyunsaturated fatty acid interactions in the aetiology of disease in the bovine. *Proceedings of the Nutrition Society* 47,177-184.

Roberson J.R, Fox L.K, Hannock D.D, Gay J.M and Besser T.E, (1994). Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. *Journal of Dairy Science*, 77, 3354-3364.

Ruegg P. (2000). *Mycoplasma* Mastitis: Can you Control it on Your Farm?

http://milkquality.wisc.edu/wp-content/uploads/2011/09/mastitis-controlprogram_mycoplasma-mastitis.pdf

Russell A.D, (1996). Activity of biocides against mycobacteria. Journal of Applied Bacteriology, Symposium Supplement, 81:87S–101S.

Russell A.D, (1986). Chlorhexidine: Antibacterial Action and Bacterial Resistance. Infection 14, No. 5, 212-215.

Saha K, Butola B.S, Joshi M, (2014). Synthesis and characterization of chlorhexidine acetate drug–montmorillonite intercalates for antibacterial applications. Applied Clay Science 101: 477–483.

Saidi R, Khelef D and Kaidi R, (2013). Subclinical mastitis in cattle in Algeria: Frequency of occurrence and bacteriological isolates. Journal of the South African Veterinary Association 84(1).

Saini M, Sharma B, Singh L.N and Gupta P.K, (1999). Apoptosis in bovine herpesvirus 1 infected bovine peripheral blood mononuclear cells. Indian Journal of Experimental Biology 37, 976–979.

Saini S.S, Sharma J.K and Kwatra M.S, (1992). *Actinomyces pyogenes* mastitis lactating cows following foot-and-mouth disease. Veterinary Record, 131, 152.

Salih R.R.M, (2015). Update on bovine mastitis etiological, clinical and treatment aspects in Khartoum state, Sudan. Online Journal of Animal Feed Research, 5(6): 153-159.

Salih R.R.M and Ahmed A.BO, (2011). Evaluation of Farms Management as A method of Control of Bovine Mastitis in Sudan. <https://www.researchgate.net/publication/272679635>.

Sandholm M, Kaartinen L, and Pyorala S, (1990). Bovine mastitis - why does antibiotic therapy not always work? An overview. Journal of Veterinary Pharmacology and Therapeutics 13(3), 248-60.

Santos J.E.P, Cerri R.L.A, Ballou M. A, Higginbotham G.E and Kirk J.H, (2004). Effect of timing of first clinical mastitis occurrence on lactational and reproductive performance of Holstein dairy cows. Animal Reproduction Science 80:31-45.

Sayed R.H, Salama S.S and Soliman R .T , (2014). Bacteriological Evaluation of Present Situation of Mastitis in Dairy Cows. Global Veterinaria 13 (5): 690-695.

Schleifer K.H and Bell J.A, (1984). Genus I. *Staphylococcus* Rosenbach, 18AL (Nom. Cons. Opin. 17 Jud. Comm. 1958, 153.). In BERGEY'S MANUAL OF Systematic Bacteriology, Second Edition, Volume Three, The *Firmicutes*. De Vos P,

Garrity G.M, Jones D, Krieg N.K, Ludwig W, Rainey F.A, Schleifer K.H and Whitman W.B, pp 392-400.

Schmidt T, Kock M.M and Ehlers M.M, (2015). Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts Journal of Dairy Science, 98:6256–6269.

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

Schroeder J.W, (2012). Mastitis Control Program series, "Bovine Mastitis and Milking Management" AS1129. North Dakota State University Fargo, North Dakota, Extension Service Revised and reprinted July, 1-16.

Schrick F.N, Hockett M.E, Saxton A.M, Lewis M.J, Dowlen H.H, and Oliver S.P, (2001). Influence of subclinical mastitis during early lactation on reproductive parameters. Journal of dairy science 84:1407-1412.

Schukken Y.H, Wilson D.J, Welcome F, Garrison-Tikofsky L and Gonzalez R.N, (2003). Monitoring udder health and milk quality using somatic cell counts. Veterinary Research, 34, 579–596.

Scott F.M.M and Holliman A, (1984). Serum antibodies to bovine mamillitis virus in pregnant heifers. Veterinary Records, 114, 19.

Sharma N, Bato0 A.S, Zul I huma, Sarabpreet Kour1, Misri J and Hussain K, (2017). Impact of Mastitis on Reproductive Performance in Dairy Animals: A Review. Theriogenology Insight: 7(1): 41-49. DOI: 10.5958/2277-3371.2017.00015.8

Sharma N, Rho J.G, Hong Y.H, Kang T.Y, Lee H.K, Yur T.Y and Joeng D.K, (2012). Bovine Mastitis: An Asian Perspective. Asian Journal and Veterinary Advances 7 (6):454-476.

Sharma N and Maiti S.K, (2005). Effect of dietary supplementation of vitamin E and selenium in subclinical mastitis in dairy cows. Indian Journal of Veterinary Medicine, 25(2): 76-79.

Sharif A and Muhammad G (2009). Mastitis Control in Dairy Animals. Pakistan Veterinary Journal, 29(3): 145-148.

Sillankorva S.M, Oliveira H and Azeredo J, (2012). Review Article Bacteriophages and Their Role in Food Safety. International Journal of Microbiology; Volume 2012, Article ID 863945, 13.

Smith K.L, Conrad H.R and Amiet B.A, (1985). Incidence of environmental mastitis as influenced by vitamin E and selenium. Kiel. Milchwirtsch. Forschungsber 37,482.

Smith K.L, Harrison J.H and Hancock D.D, (1984). Effect of vitamin E and selenium supplementation on incidence of clinical mastitis and duration of clinical symptoms. Journal of Dairy Science, 67, 1293.

Sordillo L.M, (2005). Factors affecting mammary gland immunity and mastitis susceptibility. Livestock Production Science 98: 89–99.

Spain J.N, Jones C.A, Rapp C, (2005). The effect of complexed zinc on keratin synthesis in the teat canal and the establishment and severity of experimentally induced *E. coli* mastitis in dairy cows. In: Mastitis in dairy production: Current knowledge and future solutions. Hogeveen H (Ed). P 948.

Spain J.N, Stevens B.J and Hardin D.K, (1993). Effects of Bioplex zinc or zinc oxide on mastitis incidence in lactating dairy cows. Journal of Dairy Science, 76, 265.

Springthorpe, V.S and Satter S.A, (1990). Chemical disinfection of virus contaminated surfaces. Critical Reviews in Environmental Control, 20:169–229.

Sudhan N.A and Sharma N, (2010). Mastitis- An Important Production Disease of Dairy Animals. SMVS'DAIRY YEAR BOOK: pp 72-88.

Sulakvelidze A, Alavidze Z and Glenn Morris JR. J, (2001). Bacteriophage Therapy (minireview). Antimicrobial agents and Chemotherapy. Vol. 45, No. 3, p. 649–659.

Swatantra K. S, Shukla S. K and Hirpurkar S.D, (2014). Therapeutic efficacy of phage lysate in chronic mastitis of farm animals. Pharma Science Monitor 5(3), Jul-Sep 2014, 71-74.

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.

Taponen S, Jantunen A, Pyorala E and Pyorala S, (2003). Efficacy of targeted 5-day combined parenteral and intramammary treatment of clinical mastitis caused

by penicillin-susceptible or penicillin-resistant *Staphylococcus aureus*. Acta veterinaria Scandinavica, 44, 53-62.

Tassew A, Negash M, Demeke A, Feleke A, Tesfaye B and Tesfaye S, (2016). Isolation, identification and drug resistance patterns of methicillin resistant *Staphylococcus aureus* from mastitic cow's milk from selected dairy farms in and around Kombolcha, Ethiopia Journal of Veterinary Medicine and Animal Health, Vol.8 (1), pp.1-10, January.

Teklesilasie T, Kebeta M.M and Fayera T, (2014). Bovine Mastitis: Prevalence and Isolation of Major Pathogens in Dairy Farms of Selected Sites in Addis Ababa, Ethiopia. Applied Journal of Hygiene 3 (3): 31-37.

Timofte D, Maciucă I.E , Evans N.J, Williams H , Wattret A, Fick J.C and Williams N.J,(2014). Detection and Molecular Characterization of *Escherichia coli* CTX-M-15 and *Klebsiella pneumoniae* SHV-12 β -Lactamases from Bovine Mastitis Isolates in the United Kingdom. Antimicrobial Agents and Chemotherapy Vol 58(2) p. 789–794, February.

Tiwari J.G, Babra C, Tiwari H.K and 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 & Vaccination. 4 (1): pp. 1-11.

Tsegaye B, Tekuash R and Abdurhaman Y, (2015). Prevalence and Potential Risk Factors of Bovine Mastitis in Selected Dairy Farms of Dire Dawa Town, Eastern Ethiopia. Applied Journal of Hygiene 4 (1): 06-11.

Turner A.J, Kovessy L and Morgan I.R, (1976). Isolation and characterisation of bovine herpesvirus mammillitis virus and its pathogenicity for cattle. Australian Veterinary Journal, 52, 166–169.

Viguier C, Arora S, Gilmartin N, Welbeck K and O'Kennedy R, (2009). Mastitis detection: current trends and future perspectives (Review). Trends in Biotechnology Vol.27 No.8.

VKM. (2016). Antimicrobial resistance due to the use of biocides and heavy metals: a literature review. Scientific Opinion on the Panel Panel on Microbial Ecology of the Norwegian Scientific Committee for Food Safety, ISBN: 978-82-8259-253-6, Oslo, Norway.

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 reexamination of cases one month after treatment. *Journal of Dairy Science*, 83, 70-76.

Wangalwa R, Tolo C.U, Kagoro G.R and Matofari J.W, (2016). Assessment of on-farm milk handling practices in Mbarara District Southwestern ganda. *African Journal of Dairy Farming and Milk Production*, Vol.3 (2), pp. 141-148.

Wang D, Wang Z, Yan Z, Wu J, Ali T, Li J, Lv Y and Han B,(2015). Bovine mastitis *Staphylococcus aureus*: Antibiotic susceptibility profile, resistance genes and molecular typing of methicillin-resistant and methicillin-sensitive strains in China. *Infection, Genetics and Evolution* 31, 9–16.

Watts J.L, (1988). Etiological agents of bovine mastitis. *Veterinary Microbiology* 16: 41-66.

Weber-Dabrowska B, Mulczyk M and Gorski A, (2000). Bacteriophage Therapy of Bacterial Infections: an Update of Our Institute's Experience (Review). *Archivum Immunologiae et Therapiae Experimentalis*, 48, 547–551.

Wellenberg G.J, van der Poel W.H.M and Van Oirschot J.T, (2002). Viral infections and bovine mastitis: a review *Veterinary Microbiology* 88: 27–45.

White D.G and McDermott P.F, (2001). Emergence and transfer of antibiotic resistance. *Journal of Dairy Science* 84 (E. Suppl.), E151±E5.

Wu J, Hu S and Cao L, (2007).Therapeutic Effect of Nisin Z on Subclinical Mastitis in Lactating Cows. *Antimicrobial agents and chemotherapy*, Sept. Vol. 51, No. 9, p. 3131–3135.

Zadoks R.N, Allore H.G, Barkema H.W, Sampimon O.C, Wellenberg G.J, Gröhn 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.

Zecconi A, Hamann J, Bronzo V, Moroni P, Giovannini G and Piccinini R, (2000). Relationship between teat tissue immune defences and intramammary infections. *Advances in Experimental Medicine and Biology*, 480, 287– 293.

CHAPTER 3

Isolation and characterization of bovine mastitis associated causative pathogens in mastitis and normal milk samples and their antimicrobial susceptibility profiles

Abstract

Bovine mastitis is the inflammation of mammary gland usually caused by bacteria, with more than 200 microbial species associated as causative agents. Its treatment and control have been possible by the use of antibiotics and chemical teat dips, but current reports indicate that more antibiotic therapy cases fail and development of antimicrobial resistance is the major reason. The objectives of this study were to isolate and characterize mastitis associated causative pathogens in clinical and subclinical mastitis milk from different regions in South African dairy farms and to determine their heterogeneity per region, to determine their antimicrobial susceptibility

profile and to compare these profiles per region. For analysis of milk samples, standard traditional microbiology methods using non-selective and selective differential agar plates were used and isolates identified using biochemical tests. The species predominantly found were *S. aureus* and CNS (*S. chromogenes*, *S. haemolyticus*, *S. xylois*, *S. epidermis*, *S. hominis*, *S. hycus*, *S. capitis*, and *S. sciuri*), *Str. dysgalactiae*, *Str. uberis*, *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., *Enterococcus* spp., *Enterobacteriaceae* spp., *Proteus* spp., *Citrobacter* spp., *Bacillus* spp., *B. pumilus*, *Acinetobacter* spp., *Lactococcus lactis* and *Pasteurella* spp. For antimicrobial susceptibility tests, disk-diffusion and broth microdilution methods were applied. The highest sensitivity found was on cephalosporins (cefuroxime 94% and cephalixin 65%) and Aminoglycosides (streptomycin 82% and kanamycin 82%), tetracycline 65%, bacitracin 59%, novobiocin 59% and ampicillin 53% and resistance to polymyxin B (53%), penicillin (53%), ampicillin (41%), bacitracin (41%), novobiocin (41%) and intermediate resistance on neomycin (47%). For disinfectants, teat dip products evaluated were Deosan Teat Form (Chlorhexidine based), Mastocide (Chlorhexidine digluconate based), Deosan Iodel Gel and different chemical pre- and post-milking teat dips commercially available (Citric acid monohydrate 0.42% ppm m/v and Dodecyl benzene sulphonic acid based) and milk equipment acid sanitizer Perosan. All teat dips were effective in inhibiting growth of all mastitis associated causative strains tested, with 100% lethal effect. While some manufacturers chemical Perosan acid sanitizers was ineffective at the concentration of 0.4% and double these manufacture recommended concentration for both Gram-negative and positive isolates at exposure time of 5 minutes. Deosan Perosan was also ineffective at manufacture recommended working concentration 0.5% and at double this concentration for Gram-negative. The MIC for these strains was predicted to be >0.75% whereas for Gram-positive strains, their MICs were between 0.75%-0.19%.

3.1 Introduction

Mastitis is defined as inflammation in the mammary gland regardless of the cause, however almost all the cases in dairy cows are caused by microorganisms, usually bacteria that have entered the teat canal and mammary tissue (Jones and Bailey, 2009; Sharma *et al.*, 2006). Physical, chemical and bacteriological changes observed in milk and pathological changes in the udder are characteristics used to diagnose this disease (Radostits *et al.*, 2000).

There are over 200 microbial species identified as causative agents, but about 96% of the cases are usually caused by a few bacterial species such as Coagulase-negative Staphylococci (CNS), *Staphylococcus aureus*, *Streptococcus agalactiae*,

Streptococcus dysgalactiae, *Streptococcus uberis*, *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus* spp., *Corynebacterium* spp. (Mallikarjunaswamy and Krishnamurthy, 1997; Watts 1988).

Antibiotic therapy is used for treatment and control of this disease and it is done during lactation and at drying period. The main goal of antibiotic therapy is to eliminate the infectious pathogens in the udder, which leads to curing the cow and returning the cow to normal milk production and composition and prevention of mortality (Sudhan and Sharma, 2010). However, during lactation, most antibiotic therapy cases ends up in failure (Du Preez, 2000; Sandholm *et al.*, 1990). Determining factors such as incorrect diagnosis, inappropriate route of administration and the drug selected, severity of udder pathology, and elimination of predisposing factors are some of the reasons said to be responsible for this (Du Preez, 2000). Also development of antimicrobial resistance by bacteria, caused by frequent use of antibiotics in undiagnosed cases, overuse in animal feed and their easy accessibility over the counter without prescription (Barkema, 2008; Williams, 2000).

Apart from the use of antibiotics, disinfectants are also used to control this disease, during milking as pre- and post- milking teat dips and as part of good milking hygiene as sanitizers for milking equipment such as milking units and clusters, parlour surfaces and milk tanks and milkers hands. Teat dipping involves dipping all cows' teats in a disinfectant before and immediately after milking. Pre- teat dipping is done in conjunction with proper milking hygiene and was developed to control environmental pathogens, where it was found to reduce intramammary infections (IMI) by more than 50% (Philpot and Nickerson, 1992). Post-teat dipping was developed to control the spread of contagious mastitis pathogens and it was reported to have no effect over coliform infections. Nickerson (2001) reported post-teat dipping to reduce new IMI by more than 50% when used properly with other points.

Wide varieties of approved teat dips products are available commercially and the active ingredients include iodine, chlorhexidine, quaternary ammonium compounds, hydrolysed fatty acids, hypochlorite, and organic acid (lactic acid and acetic acid). The emollients, such as glycerine, sorbitol, lanolin or propylene glyco are added to the disinfectant preparations to improve teat skin health and so reduce the likely reservoir of mastitis bacteria in teat sores and cracks (Nickerson, 2001). Their antimicrobial mode of action are either chemical or biologically such as oxidation-reduction mechanisms, denaturation or precipitation of cytoplasmic proteins, inhibition of enzyme activity, and disruption of cell membranes. They all have broad-spectrum

antimicrobial activity against Gram-positive and negative bacteria, fungi and some viruses (Anon, 1965; Nickerson, 2001, Russell, 1986; Sykes, 1965).

The objectives of this study were to isolate and characterize mastitis associated causative pathogens in milk samples from mastitis and normal cows. Furthermore, to determine their heterogeneity per region, determine their antimicrobial susceptibility profile and to compare these profiles per region.

3.2 Materials and Methods

3.2.1 Sampling area and sample types

Samples used for the study were collected in two rounds, in first round two dairy farms in Bloemfontein were visited and 30 samples in total were collected, 10 from clinical mastitis cows and 20 from subclinical mastitis cows. During, the second round, five dairy farms were visited, three from Free State region (Farm A, B and E), one from KwaZulu-Natal region (Farm C) and one from Eastern Cape region (Farm D). In total 717 samples were collected, sample size varied between 34 and 349. In Farm A (FS), number of samples collected were 349, in Farm B (FS) 221, in Farm C (KZN) 62, Farm D (EC) 51 and in Farm E (FS) samples collected were 34.

3.2.2. Isolation and Identification of the isolates

All the samples collected were analysed using traditional microbiology methods, following protocols proposed by NMC, (1999). In brief, 10 μ l of sample was spread on 5% cattle Blood agar and MacConkey agar plates and incubated at 37°C for 48hrs. Identification of the isolates was done by using Bergey's manual of Systematic Bacteriology, second edition, (2009) and (2005). Characterization was primarily based on colony morphology (size, shape, pigmentation, appearance, elevation) on plates and Gram and spore staining and followed by biochemical tests such as catalase, coagulase (slide), oxidase, urease, indole, citrate, motility and sugar fermentation test (lactose, glucose, maltose and mannitol) to verified identity of the isolates and to confirm the genus and species of the isolates.

The species diversity correlation of the farms from different regions was determined using Principal Coordinate Analysis (PCoA) (Gower, 1966).

3.2.3 Antimicrobial susceptibility tests of mastitis associated pathogens

3.2.3.1 Antibiotics susceptibility test by disk-diffusion method

Antibiotic susceptibility tests of the isolates were done using the disk-diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2012). Eleven different antibiotics obtained from Oxoid were applied as agents. These included ampicillin (10 µg), penicillin (10 U), cephalixin (30µg), cefuroxime (30µg), kanamycin (30 µg), novobiocin (30µg), neomycin (30µg), bacitracin (10U), polymyxin B (50µg), tetracycline (30 µg) and streptomycin (10 µg). Fresh cultures of the isolates were prepared by streaking them onto Tryptic soya agar (TSA), and incubated overnight (12hrs) at 37°C. The liquid suspension of the isolates were prepared by inoculating well isolated single colony into 4ml Tryptic soya broth (TSB) and incubated for 3hrs at 37°C, then 100 µL of isolate suspension was spread plated on sterile Mueller Hinton agar (MHA) (Biolab, Merck). Antibiotic discs were placed on Mueller Hinton agar plates using sterile forceps, pressed down to ensure contact with the surface and the plates incubated aerobically at 37 °C for 18h – 24h. Inhibition zone diameters were measured using the ruler and values obtained from the National Committee on Clinical Laboratory Standards were used to interpret the results obtained CLSI, (2015). Isolates were classified as resistant, intermediate resistant or susceptible to a particular antibiotic.

3.2.3.2. Determination of Minimum Inhibitory Concentrations (MICs) of disinfectants by Broth Micro-dilution

Disinfectants used: Seven agents were tested, from two local manufacturing companies. A locally produced chemical Perasan as an acid based sanitiser used before and after milking to disinfect milking equipment with recommended working concentration of 2-4 ml/L, (0.2-0.4%) at 5min exposure time.

Deosan Perasan – is a clear colourless peracetic acid based oxidising disinfectant, which is free rinsing and low foaming with broad-spectrum efficacy kill against bacteria and fungi. Perasan is used at ambient temperature to disinfect milking equipment before and after milking at concentrations of 0.15 to 0.5% (1.5 to 5ml per litre of water) for 5min.

Deosan Teat foam – is a ready to use liquid product for teat dipping and for udder, washing is diluted to 6ml/ litre of water. It is non-iodine teat disinfectants with fast acting broad spectrum, effective against a wide range of both Gram-positive and

Gram-negative bacteria. A clear green coloured liquid contains chlorhexidine with glycerine and eucalyptus oil as active ingredients. Suitable for both pre- and post-milking teat dipping at 30 seconds contact time for pre-milking teat dip.

Desosan Mastocide – is highly viscous deep blue or purple colour, post-milking barrier teat dip that contains chlorhexidine digluconate, and glycerol, allantoin and Eucalyptus oil for conditioning and moisturising minimising cracks and crevices where bacteria can hide and breed.

Deosan Iodel Gel – is a dark brown thickened barrier iodophor post teat dip containing 0.4% w/w available iodine with glycerine emollient.

Teat treat – is ready to use dip with Citric acid monohydrate 0.42% ppm m/v and Dodecyl benzene sulphonic acid 2% m/v, Emollient 11% m/v. Pre-milking teat dip light yellow liquid with contact time of 30 seconds and post-milking teat dip is ready to use red coloured liquid with contact time of 30 minutes contact time.

Preparation of test bacterial suspension

Test bacteria suspension were prepared by culturing strains aerobically on TSA at 37°C for 24h, followed by re-suspension of 3-4 single colonies into TSB incubated at 37°C for 18hrs.

Broth microdilution was used for determination of agents MIC using 96-well micro plates. Serial 2-fold dilutions of the agents were performed starting with double the manufacturer's recommended working concentration for Perosan 0.75% until 0.1%. The starting concentration for teat dip products was 100% undiluted as they were provided in ready to use form and they were diluted until 0.1%. The MICs were done simulating the products intended working conditions and this was done by using the diluent prepared by adding 300ppm hard water, 1% skimmed milk in 100ml of sterile distilled water as recommended by SANAS (SANS 51276:2011, Edition 2; EN 1276:2009, Edition 2). The dilutions of the agents were done by adding 100 µl of the diluent in all wells of plates, followed by addition of 100-µl agent in the first column of wells plates and serial diluted 2-fold until the 10th well. The 11th well of plate was used for negative control (contained growth broth) and 12th well was used for positive control (contained growth broth and test bacteria). 10 µl of test bacteria suspension was added in product dilutions for specified contact time, there after transferred to a corresponding well into a new microplate with 90 µl TSB and also in the positive control well. This was done in triplicate for each isolate and the plates were incubated aerobically at 37°C for 18-24h and then examined for growth by detection of visible

turbidity in each well. The lowest concentration of the tested agent to inhibit visible bacterial growth was recorded as the MIC of that agent.

3.3 Results and Discussion

3.3.1 Isolation and prevalence of pathogens in milk samples

From the first 30 samples collected in two Bloemfontein farms, 26 samples were positive for bacterial growth, 10 from clinical mastitis cow and 16 from the subclinical mastitis cows and 4 were negative. From the second samples collected in different regions, farm A (Free State) samples analysed were 349 and samples with no bacteria isolated were 185(53.01%), those positive for bacteria isolation were 164 (47%), samples containing one or two isolates were 161(46.13%) and with multiple mixed bacteria were 3(0.86%). Farm B (Free State), samples analysed were 221, those negative for bacterial isolates were 6(2.71%), those positive for bacteria isolates were 215(97.3%) and mixed bacterial isolates 11(5.00%). Farm C (KwaZulu-Natal), samples analysed were 62 and all were positive for bacterial growth 62(100%), samples containing one or two isolates were 61(98.4%) and mixed bacterial isolation 1(1.61%). Farm D (Eastern Cape), samples analysed were 51, all were positive for bacterial growth, and those, which contained mixed bacterial isolates were 4(7.84%). Farm E (Free State), samples analysed were 34, all were positive for bacterial growth 34(100%) and they either contained one or two isolates.

The diversity and prevalence of mastitis causative species

The specie and species found from first 30 samples analysed collected from two dairy farms in Bloemfontein are shown in Table 3.1, they consisted of major pathogens (contagious and environment pathogens), minor and other pathogens. These were the species found *Corynebacterium* spp. (19.72%), CNS (18.31%), *S. aureus* (8.45%), *Streptococcus* spp. (15.49%), *E. coli* (4.23%), *Pseudomonas* sp. (1.41%), *Proteus* sp. (1.41%), *Enterobacter* sp. (1.41%), *Vibrio* sp. (1.41%), *Bacillus* spp. (4.23%), *Mycobacterium* sp. (1.41%) and other pathogens *Acenetobacter* spp. (21,13%) and *N. meningitides* (1.41%). The predominant species were *Acenetobacter* spp. (21,13%), *Corynebacterium* spp. (19.72%), CNS spp. (18.31%), *Steptococcus* spp. (15.49%), and *S. aureus* (8.45%).

The species found in 717 samples from other five farms, three from Free State region, one from KwaZulu-Natal region and one Eastern Cape are shown in Table 3.2; they also consisted of major pathogens, minor and other pathogens. The range of

species found on each farm was between 14 and 19, except one farm where only three bacterial species were found. The species isolated were similar in four of these farms, except in only one. Mostly detected were *Staphylococcus* spp. *S. aureus* and CNS (*S. chromogenes*, *S. haemolyticus*, *S. xylois*, *S. epidermis*, *S. hominis*, *S. hycus*, *S. capitis*, *S. sciuri*), *Str. dysgalactiae*, *Str. uberis*, *E. coli*, *klebsiella* spp., *Pseudomonas* spp., *Enterococcus* spp., *Enterobacteriaceae* spp., *Proteus* spp., *Citrobacter* spp., *Bacillus* spp., *B. Pumilus*, *Lactococcus lactis* and *Pasteurella* spp.

From Farm A (FS), four pathogens were predominant, namely CNS especially *S. chromogenes* (19.21%), followed by *S. aureus* (12.58%), *Bacillus* spp. (12.58%) and *Klebsiella* spp. (9.93%). Other pathogens present were *S. haemolyticus*, *S. xylois*, *S. epidermis*, *S. hominis*, *S. hycus*, *S. capitis*, *S. sciuri*, *E. coli*, *Str. uberis*, *Enterococcus* spp., *Enterobacteriaceae* spp., *Citrobacter* spp., *B. pumilus*, *Acinetobacter* spp. and *Lactococcus lactis*.

On farm B (FS), CNS pathogens *S. chromogenes* (57.07%) and *S. haemolyticus* (14.13%) were the predominant isolates followed by *E. coli* (10.33%), *Str. dysgalactiae* (3.26%) and *Str. uberis* (2.72%). Other isolates which were present but not in significant numbers were *S. aureus*, *S. hycus*, *S. epidermis*, *S. xylois*, *S. hominis*, *Klebsiella* spp. and *Bacillus* spp., *Pasteurella* spp. Farm C, predominant pathogens were *S. aureus* (51.39%) and *Str. agalactiae* (27.78%) followed by *Bacillus* spp. (20.83%).

On Farm D (EC), *Pseudomonas* spp. (25.71%), *Str. dysgalactiae* (17.14%) were predominant isolates followed by *Enterococcus* spp. (12.86%), *Citrobacter* spp. (11.43%) and *Proteus* spp. (8.57%). Other pathogens found were *Streptococcus* spp., CNS (*S. haemolyticus*, *S. epidermis* and *S. simulans*), *Klebsiella* spp., *E. coli*, *Bacillus* spp and *Lactococcus lactis*. Farm E, predominant isolates were *S. chromogenes* (34.00%) followed by *S. hycus* (12.00%), *S. aureus*, *E. coli*, *Enterococcus* spp., *S. haemolyticus* and *S. epidermis*. Other isolate present were *Bacillus* spp., *Pseudomonas* spp., *Klebsiella* spp., *Enterobacter cloacae*, *Str. dysgalactiae* and *Acinetobacter* spp.

On each farm between one to five species were predominant. However the most dominant strains isolated from most of the farms were CNS spp. especially *S. chromogenes*. This was followed by *S. aureus*, *Bacillus* spp. and *Streptococcus* spp., *E. coli*, *Pseudomonas* spp. and *B. pumilis*, which were dominant in particular farms. The species dominant on each farm were different, in one farm either minor, environmental and contagious pathogens were dominant as seen on farm A and E or

contagious with environmental pathogens on farm C or minor with environmental pathogens farm B or only environmental pathogens on farm D (Table 3.2).

In previous studies conducted in the country, similar results were reported. One such study was by Schmidt *et al.* (2015) who reported 78.4% for *S. chromogenes* prevalence in the study diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. Petzer *et al.* (2009), found from a study conducted between the year 1996 and 2007, that the predominant isolates frequently isolated in 7 provinces in South African dairy herds were CNS spp. (61%), *S.aureus* (25%) and *Streptococcus* (6%). Also in recent study by Blignaut (2016) conducted between 2008 and 2013 on prevalence of mastitogenic pathogens in pasture and total mixed ratio based dairies, they reported CNS spp. in total mixed ratio being predominant by (29.6%) and (26.9%) in pasture on 2008 and in 2013, total mixed ratio they were predominant by (20.2%) and (22.7%). Based on our results and the above mentioned previous studies conducted in the country, for the past 10 years pathogens responsible for mastitis in South African dairy herds have not changed with the minor pathogens CNS spp., still being the dominant species causing mastitis followed by *S.aureus* and *Streptococcus* spp.

Over the past few years, minor pathogens CNS spp. have become the predominant species usually found in mastitis cases as seen in numerous studies conducted worldwide. In Finland a study conducted by Koivula *et al.* (2007), from 77 051 mastitis routine samples submitted to laboratories during 2004-2006, CNS spp. were the most frequently isolated bacteria in clinical (18%) and subclinical mastitis (24%). Similar findings were obtained in the USA and Canada (Dingwell *et al.*, 2004; Lim *et al.*, 2007; Roberson *et al.*, 2006). Nigo *et al.* (2013) in Sudan also reported *Staphylococcus* spp. (61.1%), *Streptococcus* spp. (15%), *Corynebacterium* spp. (6%) and *Bacillus* spp (2%) to be the dominant species. Katsande *et al.* (2013) in Zimbabwe also reported species dominant were CNS spp. (27.6%), *E. coli* (25.2%), *S. aureus* (16.3%), *Klebsiella* spp. (15.5%) and *Streptococcus* spp. (1.6%).

Minor pathogens in the past were considered as pathogens of less importance. However now, their importance has increased. CNS species are opportunistic pathogens with some species being highly pathogenic, and their source in South African dairy farms maybe caused by poor milking hygiene, lack of maintenance in their milking machines, increase in herd size and introduction of many new cows into herds without knowing their health history. Also, another point that needs to be considered is that mastitis control plan currently used worldwide was developed to control mastitis cases caused by contagious and environmental not minor pathogens,

so this sudden increase in mastitis cases caused by these pathogens may be that some changes need to be implicated on mastitis control plan to fix this problem.

There were also other rare mastitis associated species isolated and these were species of *Lactococcus lactis*, *Pasteurella* spp and *Acinetobacter* spp. *Pasteurella* spp. that are mostly found in the upper respiratory tract of mammals and birds. They are spread from the respiratory tract of cows to the udder via the blood or lymph system and what is presently unknown is how they are spread between mammary glands (Swartz and Petersson-Wolfe, 2016). In Korea, they were isolated in bulk milk tanks (Jayarao and Wang, 1999), while recently in Ethiopia, they were found in mastitis cases (Teklesilasie *et al.*, 2014).

For *Lactococcus lactis*, their source is reported to be the cows' environment and it spread from cow to cow during milking. Mastitis cases caused by *Lactococcus lactis* are characterized by herd outbreak with poor treatment response and extremely high SCC. Its identification is reported to be difficult, conventional culture methods and biochemical techniques usually fail as they require specialised diagnostic tests. They are usually misdiagnosed as *Streptococcus* or *Enterococcus*, which means *Lactococcus* mastitis cases may probably be more common than reported (Smith *et al.*, 2016). One *Lactococcus* mastitis case has been reported in Uganda (Kateete *et al.*, 2013).

Acinetobacter spp. are opportunistic pathogens, ubiquitous in nature present on skin of warm-blooded animals, milking machines and from the dairy farm environment (Al Atrouni *et al.*, 2016). Cases of *Acinetobacter* spp. isolation have been reported in bulk milk tank and milk samples from mastitis quarters in Korea (Gurung *et al.*, 2013; Nam *et al.*, 2010) and United States (Jayarao and Wang, 1999). All the above-mentioned species are reported to be highly resistant to antibiotics; as a result, the only mastitis control measure that seems to work is segregation or culling of infected cows. However, a lot is still unknown about mastitis cases caused by them and this warrants further investigation.

Neisseria meningitidis was also one of unusual species isolated in mastitis case from this study. In general, it forms normal microbiota of mucosa respiratory track and cerebra spinal fluid, but it becomes a problem when found in blood, and brain lining were it causes meningitis (Tonjum *et al.*, 1994). Its source maybe the blood that was present in some milk samples, due to distraction of mammary gland.

From all mastitis pathogens, *S. aureus* species are of great importance due to the nature of mastitis cases they cause, mostly are chronic subclinical with low antibiotic

therapy cure rate during lactation and drying off and with high reoccurrence infection rate (Madgwick *et al.*, 1989). Also on evidence based from this study and previous studies conducted in the country and other African countries, *S. aureus* species are always the first or second dominant species in mastitis cases.

Table 3.1 Prevalence of bacterial isolates in raw milk samples from subclinical (SCC> 500x 10³ cell/ml) and clinical mastitis cows analysed for mastitis pathogens in two dairy farms in Bloemfontein.

Organisms	Pathogen Category (Major or minor and other pathogens)	Clinical	Sub-clinical	Total NO (%)
<i>Staphylococcus aureus</i>	major	6	-	6 (8.45)
Coagulase-negative <i>Staphylococcus</i> spp.	minor	7	6	13 (18.31)
<i>Streptococcus</i> spp.	major	5	6	11 (15.49)
<i>Acinetobacter</i> spp.	major	8	7	15 (21.13)
<i>Neisseria meningitidis</i>	other	1	-	1 (1.41)
<i>Escherichia coli</i>	major	-	3	3 (4.23)
<i>Pseudomonas</i> sp.	major	-	1	1 (1.41)
<i>Proteus</i> sp.	major	-	1	1 (1.41)
<i>Enterobacter</i> sp.	major	1	-	1 (1.41)
<i>Vibrio</i> sp.	major	1	-	1 (1.41)
<i>Bacillus</i> spp.	major	-	3	3 (4.23)
<i>Corynebacterium</i> spp.	minor	2	12	14(19.72)
<i>Mycobacterium</i> sp.	major	-	1	1 (1.41)
Total		31	40	71(100)

Table 3.2 Prevalence of mastitis associated pathogens in raw milk samples from five dairy farms in different regions.

Organisms	Pathogen category (major or minor pathogen)	Farm A (FS)	Farm B (FS)	Farm C (KZN)	Farm D (EC)	Farm E (FS)
<i>S. chromogenes</i>	minor	29	105	-	-	17
<i>S. haemolyticus</i>	minor	6	26	-	3	3
<i>S. xylois</i>	minor	6	1	-	-	-
<i>S. epidermis</i>	minor	5	2	-	2	3
<i>S. hominis</i>	minor	2	1	-	-	-
<i>S. hycus</i>	minor	1	4	-	-	6
<i>S. capitis</i>	minor	1	-	-	-	-
<i>S. sciuri</i>	minor	1	-	-	-	-
<i>S. aureus</i>	major	19	1	37	-	3
<i>S. simulans</i>	minor	-	-	-	1	1
<i>Klebsiella</i> spp.	major	15	1	-	2	1
<i>E. coli</i>	major	8	19	-	1	4

<i>Str. uberis</i>	major	2	5	-	3	-
<i>Str. dysgalactiae</i>	major	-	6	20	12	1
<i>Pseudomonas</i> spp.	major	-	2	-	18	1
<i>Enterococcus</i> spp.	major	1	-	-	9	4
<i>Enterobacteriaceae</i> spp.	major	1	-	-	-	-
<i>Interobacter cloacea</i>	major	-	-	-	-	1
<i>Proteus</i> spp.	major	-	-	-	6	-
<i>Citrobacter</i> spp.	major	1	-	-	8	-
<i>Bacillus</i> spp.	major	19	5	15	2	2
<i>B. pumilus</i>	major	13	2	-	1	2
<i>Acinetobacter</i> spp.	major	12	2	-	-	1
<i>Lactococcus lactis</i>	major	9	-	-	2	-
<i>Pasteurella</i> spp.	major	-	2	-	-	-
Total		151	184	72	70	50

Species diversity correlation in the farms from different regions

Fig. 1 shows the results of mastitis associated species diversity correlation in different farms. Three farms A, B and E are from Free State region and they showed close correlation in the diversity of mastitis-associated species found there. This close correlation in species diversity maybe due to similarity in the environment these cows are exposed to such as field pasture, housing, bedding material, water and region temperature or similarity in the type of teat dip they use and antibiotic products or cleaning reagents used for milking equipment. The above factors may be responsible for selecting the occurrence of certain species in this region. Farm C, from KwaZulu-Natal region, species diversity was similar with the above three, consisting with only three different species, which were dominant. Farm D, from Eastern Cape region was different from all other farms, environment pathogens were predominant and most of the species found, were only present there.

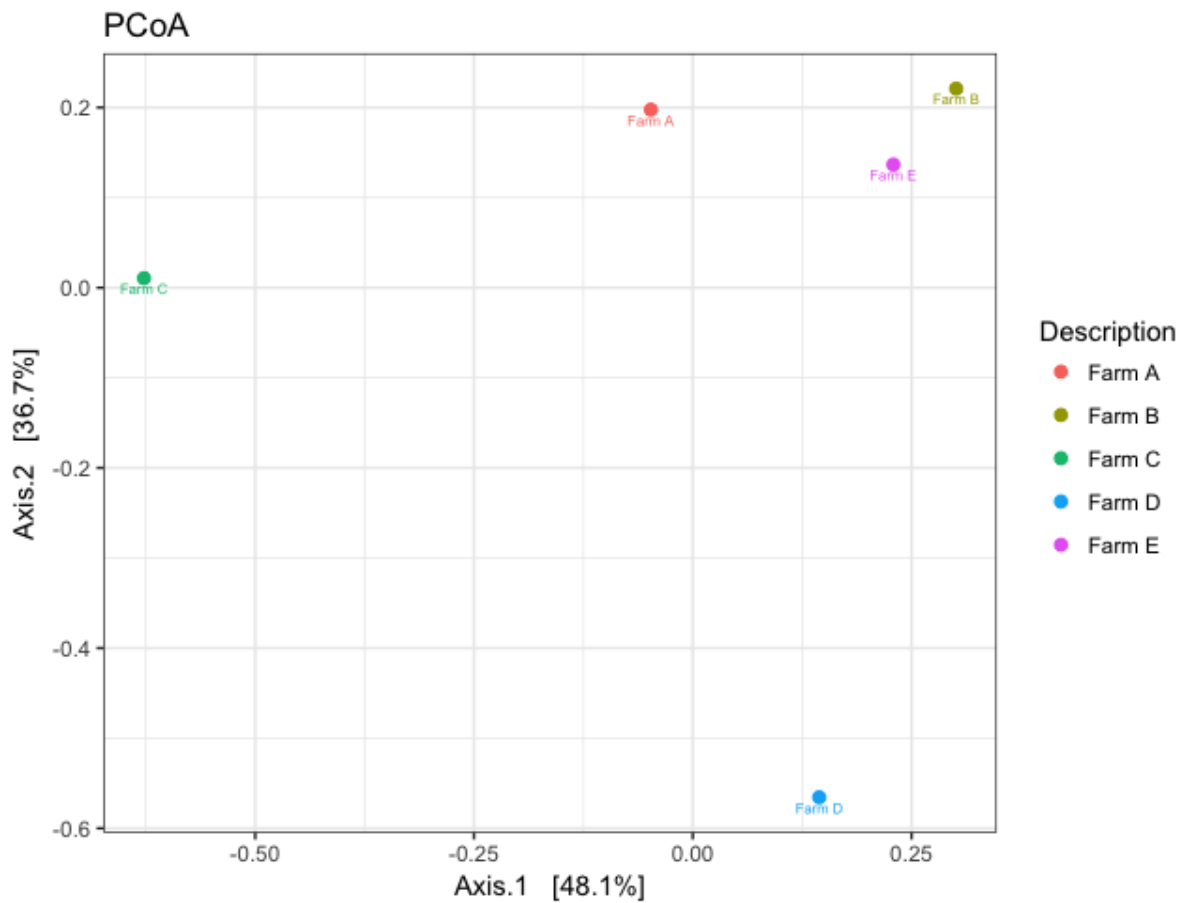


Fig. 1 Correlation of mastitis associated species diversity in different farms

3.3.3 Antimicrobial susceptibility test of mastitis associated pathogens

3.3.3.1 Antibigram profile

For antibiogram profile of the isolates, strains tested were *E. coli* (6), *S. aureus* (3), CNS spp. (2), *Streptococcus* spp. (5) and *Klebsiella* spp. (1) and results are shown in Table 3.3. *E. coli* strains were found to be resistant to novobiocin (100%), penicillin (100%), bacitracin (100%) and ampicillin (67%), tetracycline (33%), cephalexin (17%) and intermediate resistant to ampicillin (17%), Neomycin (83%), Tetracycline (33%), Cephalexin (67%), and Cefuroxime (17%). They were sensitive to kanamycin (100%), neomycin (17%), ampicillin (17%), polymyxin B (100%), tetracycline (33%), streptomycin (83%), cephalexin (17%) and cefuroxime (83%).

S. aureus strains were all resistant to polymyxin B (100%) and sensitive to all other antimicrobials tested and CNS spp. were all resistant to ampicillin (100%), penicillin (100%) and polymyxin B (100%).

Streptococcus spp. were resistant to polymyxin B (80%), kanamycin (40%), neomycin (40%) and streptomycin (40%) and intermediate resistant to kanamycin (20%), neomycin (40%), tetracycline (20%). They were found to be sensitive to

ampicillin (100%), novobiocin (100%), penicillin (100%), bacitracin (100%), cephalexin (100%) and cefuroxime (100%), tetracycline (80%), streptomycin (60%), kanamycin (40%) polymyxin B (20%), and neomycin (20%).

Klebsiella sp. was resistant to ampicillin (100%), novobiocin (100%), penicillin (100%), bacitracin (100%) and tetracycline (100%) and intermediate resistant to neomycin (100%). They were sensitive to kanamycin (100%), polymyxin B (100%), streptomycin (100%), cephalexin (100%) and cefuroxime (100%).

Similar results were observed in Ethiopia by Belayneh and co-workers (2014) who reported high resistance of *S. aureus*, CNS spp. and *Streptococcus* spp. to penicillin (75%, 75% and 70%), polymyxin (80%, 75%, 90%), and amoxicillin (75% , 50%) and *E. coli* resistant to penicillin was (100%) and bacitracidin (100%). Also in the study in Uganda by Kateete *et al.* (2013) they reported that Coliforms resistance to ampicillin was (71%) while it was moderate for cephalothin (33%), cefuroxime (25%). However, our studies results were in contrast to Schmidt (2011) results, in the study conducted in South Africa, who reported resistance of penicillin (47.8%) and ampicillin (65.6%) for *S.aureus*. Similar results were obtained in other studies conducted in China, they reported (77.3%) resistance to penicillin and ampicillin, while in Denmark and Brazil reported figures of 75%, 55.1% (Aarestrup and Jensen 1998; Li *et al.* 2009; Rabello, 2005).

Based on the antibiogram profiles of our strains tested, highest sensitivity was found to cephalosporins (cefuroxime 94% and cephalexin 65%) and Aminoglycosides (streptomycin 82% and kanamycin 82%), tetracycline 65%, bacitracin 59%, novobiocin 59% and ampicillin 53% and resistance to polymyxin B (53%), penicillin (53%), ampicillin (41%), bacitracin (41%), novobiocin (41%) and intermediate resistance on neomycin (47%) (Table3.3). Resistance to beta-lactams class of antimicrobials is not surprising considering the fact that about 97% of the intramammary preparations available in South Africa used in dairy industry for treatment and control either contains penicillin or ampicillin (IVS, 2015; Pieterse and Todorov, 2010). The resistance to penicillin and ampicillin may be due to the production of β -lactamase, an enzyme that inactivates penicillin and closely related antibiotics.

Various studies have suggested that resistance to polymyxins B may be due to alterations of the outer membrane of bacteria cell loss of Lipopolysaccharides or reduction of specific outer membrane proteins or reduction in cell envelope mg^{2+} and Ca^{2+} content (Yu *et al.*, 2015). Most strains were found to be multidrug resistant and

some still acquiring resistance. This results show how important is frequent monitoring of antibiotics susceptibility to mastitis associates causative strains and how important it is to diagnose pathogens before treatment is implicated, this can reduce the number of pathogens that become multidrug resistance.

Table 3.3 Antibiogram profile of mastitis associated causative pathogens (n = 17).

Antimicrobial (concentration)	<i>E. coli</i> n= 6			<i>S. aureus</i> n = 3			<i>Coagulase-negative Staphylococcus</i> n= 2			<i>Streptococcus</i> spp. n=5			<i>Klebsiella</i> spp. n= 1			Total number (%)		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Ampicillin (10µg)	4	1	1	-	-	3	2	-	-	-	-	5	1	-	-	7 (41%)	1 (6%)	9 (53%)
Kanamycin (30µg)	-	-	6	-	-	3	-	-	2	2	1	2	-	-	1	2 (12%)	1 (6%)	14 (82%)
Novobiocin (30µg)	6	-	-	-	-	3	-	-	2	-	-	5	1	-	-	7(41%)	-	10 (59%)
Penicillin (10U)	6	-	-	-	-	3	2	-	-	-	-	5	1	-	-	9 (53%)	-	8 (47%)
Neomycin (30µg)	-	5	1	-	-	3	-	-	2	2	2	1	-	1	-	2 (12%)	8(47%)	7 (41%)
Bacitracin (10U)	6	-	-	-	-	3	-	-	2	-	-	5	1	-	-	7 (41%)	-	10 (59%)
Polymyxin B (50µg)	-	-	6	3	-	-	2	-	-	4	-	1	-	-	1	9 (53%)	-	8 (47%)
Tetracycline (30µg)	2	2	2	-	-	3	-	-	2	-	1	4	1	-	-	3 (18%)	3 (18%)	11 (65%)
Streptomycin (10µg)	1	-	5	-	-	3	-	-	2	2	-	3	-	-	1	3(18%)	-	14(82%)
Cephalexin (30µg)	1	4	1	-	-	3	-	-	2	-	-	5	-	-	1	1 (12%)	2 (18%)	11 (65%)
Cefuroxime (30µg)	-	1	5	-	-	3	-	-	2	-	-	5	-	-	1	-	1 (6%)	16(94%)

R = Resistant, I= Intermediate resistant, S = Sensitive

3.3.3.2 Minimum Inhibitory Concentrations (MIC) of disinfectants by Broth Microdilution

The teat dip disinfectants were provided in “ready to use form”, 100% manufactures recommended working concentration. The active ingredients in these disinfectants were; Deosan Teat form (Chlorhexidine), Deosan Mastocide (Chlorhexidine digluconate), Deosan Iodel Gel (iodine). Eco-sure chemical pre- and post-milking teat dips (Citric acid monohydrate 0.42% ppm m/v and Dodecyl benzene sulphonic acid).

All the disinfectants tested were effective in inhibiting growth of all our mastitis associated causative strains tested. They showed 100% lethal effect and their MICs results are shown in Table 3.4. The MICs were different for each teat dip tested. Based on the MICs values the most effective product was Deosan Teat Form (Chlorhexidine based) which is used as pre-and post-milking teat dip with MICs results lower than 2% at 30 second exposure time. This was followed by Mastocide (Chlorhexidine digluconate based), a post-milking teat dip, with MICs lower than 2% at 30 minutes exposure time. For other teat dips, Eco-Sure chemical pre-milking teat dip (Citric acid monohydrate and Dodecyl benzene sulphonic acid based) MICs were between 25%-1%, with the majority of strains showing MICs values of 3%. For other post-milking teat dips Deosan Iodel Gel and Eco-Sure chemical post-milking teat dip (citric acid monohydrate and Dodecyl benzene sulphonic acid based) MICs values were between 50%-12.5%, with majority of strains showing MICs values of 12.5%. Based on this study, Chlorhexidine based teat dips were the most efficient in inhibiting bacterial growth, followed by Citric acid monohydrate and Dodecyl benzene sulphonic acid and lastly was Iodine based.

Different active ingredients of disinfectant exhibit different antimicrobial activity spectrum against different microbes. Gram-negative bacteria are found to be more resistant than Gram-positive bacteria. Also, the activity of disinfectant agent is affected by external factors such as organic load, concentration used, contact time, hard water conditions, pH and temperature (McDonnell and Russell, 1999). Sub-lethal concentrations and inadequate contact time are reported to induce resistance to disinfectants as results; more studies are conducted, evaluating the effectiveness of these teat dip active ingredients. In this study, the teat dips evaluated showed no resistance, while in other studies resistance has been observed even though it was not that high. One such study was conducted by Mohammed (2014), assessing the antimicrobial resistance patterns and *in vitro* efficacy of three disinfectants as teat dip against Iodine (0.5%) resistant mastitogenic pathogens. The disinfectant tested was iodine glycerin (0.8%) using broth macro-dilution method with 45 strains of CNS, *S.aureus* and *E.coli*, 15 in each species. The results were Iodine glycerine had lethal effect of 90%, 92% and 79% at 30 sec for CNS, *S.aureus* and *E.coli* and 100%, 100% and 85% after 60-second exposure.

Terolli *et al.* (2015) also conducted a study *in vitro* evaluation of Chlorhexidine based disinfectants used post-dipping against mastitis pathogens, using excised teat method. The products evaluated were Alpha Blue (Chlorhexidine digluconate 20%, used as solution with 5000-ppm active principles digluconate 20% used as solution

4500-ppm active substance) and Deosan (chlorhexidine digluconate 20%, used as solution 4500 ppm active substance). These were their results, for Alpha Blue for for *Streptococcus agalactiae*, *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* these were the figure obtained 93.4%; 98.4%; 97.6%; 96.0% and for Deosan these were the values obtained 97.3%;96.1% 95.4%; 94.0%.

In another study by Santos *et al.* (2016), *In vitro* efficacy of teat disinfectant against *S. aureus* strains isolated from bovine mastitis. The study evaluated the *in vitro* efficacy of chlorhexidine (2.0%) and iodine (0.6%) against *S. aureus* isolated from mastitic milk and the results were Chlorhexidine (84%, 90%, 94% and 96%) and Iodine (46%, 58%, 66% and 78%) at 15, 30, 60 and 300 sec.

Barnum *et al.* (1982) evaluated the effectiveness of a teat dip with dodecyl benzene sulfonic acid (1.94%) for the prevention of intramammary infections in cows using experimentally challenge, with *Streptococcus agalactiae* and *Staphylococcus aureus*. The infection rates with *Streptococcus agalactiae* and *Staphylococcus aureus* were 62.5% and 75% in undipped quarters, 12.5% and 21.5% in dipped quarters with a reduction rate of 80% and 71% respectively.

However, for citric acid, there are no studies available for its use as a teat dip. The studies which are available are for its application as an acidulant commonly used in the food and beverage industry as a chelator that is a lipophobic, thus dissociated acid inhibiting growth of microorganisms by chelating divalent metal ions from the medium (Brul and Coote, 1999; Stratford, 1999). Unlike other acidulants that works as lipophilic, undissociated acid inhibits microbial growth at low pH by causing intracellular acidification (Stratford, 1999).

For milking equipment sanitisers, two Perosan products were evaluated, a chemical Perasan with active ingredient not specified and Deosan Perosan (Peracetic acid based). These products were also different in manufactures recommended working concentrations. The first chemical Perasan was found to be ineffective at manufactures recommended working concentration 0.4% and at double the recommended working concentration for both gram negative and positive isolates at 5 minutes contact time. Deosan perosan was also found to be ineffective at manufactures recommended working concentration 0.5% and at double the recommended working concentration for gram-negative isolates at 5 minutes contact time. The MIC for gram-negative strains was reported to be >0.75% as this was the highest concentration tested. On other hand, it was effective for Gram-positive isolates with their MICs between 0.75%-0.19%. The possible explanation for this may be due

to development of bacterial resistance by Gram-negative isolates to the manufactures recommended concentration, which use to be effective and now they require much higher concentration. Also, the differences in outer membranes of gram-negative and positive bacteria may be another reason for the difference in these products MICs. Gram-negative bacteria have thicker outer membrane, which may act as permeability barrier leading to reduction in uptake of disinfectant. While Gram-positive, have thin outer membrane making it easy for disinfectant to enter the cell cytoplasm membrane causing their destruction (McDonnell and Russell, 1999).

Table 3.4 Minimum Inhibitory Concentrations (MICs) of agents that resulted in complete inhibition of test organisms.

MIC (%) of agents							
Exposure time	5min		30 sec		30 min		
Organisms	Perosan	Deosan Perosan	Teat Foam	Eco-sure pre-dip	Mastocide	Iodel Gel	Eco-sure post-dip
<i>S.aureus</i> 1	> 0.6%	0.38%	1.56%	3.13%	1.56%	50%	12.5%
4	> 0.6%	0.75%	1.56%	1.56%	0.78%	25%	12.5%
9	> 0.6%	0.38%	1.56%	0.78%	0.78%	25%	12.5%
CNS 12	> 0.6%	0.38%	0.78 %	3.13%	0.78%	12.5%	25%
CNS17	> 0.6%	0.38%	0.78%	3.13%	0.78%	12.5%	25 %
<i>S. epidermis</i>	> 0.6%	0.19%	0.78%	3.13%	0.78%	12.5%	12.5%
<i>Streptococcus</i> sp.2	> 0.6%	0.19%	0.39%	12.5%	0.78%	12.5%	25%
9	> 0.6%	0.19%	0.39%	1.56%	0.78%	12.5%	12.5%
11	> 0.6%	0.19%	0.39%	12.5%	0.78%	12.5%	12.5%
<i>E.coli</i>	> 0.6%	> 0.75%	0.78%	3.13%	0.78%	50%	12.5%
<i>Enterobacter</i> sp.	> 0.6%	0.38%	1.56%	25%	1.56%	12.5%	12.5%
<i>Pseudomonas</i> sp.	> 0.6%	> 0.75%	0.78%	3.13%	1.56%	12.5%	12.5%
<i>Proteus</i> sp.	> 0.6%	> 0.75%	1.56%	12.5%	1.56%	12.5%	50%
<i>Vibrio</i> sp.	> 0.6%	> 0.75%	1.56%	3.13%	1.56%	25%	0.39%
<i>Bacillus</i> sp.	> 0.6%	> 0.75%	0.78%	3.13%	0.78%	12.5%	25%
<i>Corynebacterium</i> sp.	> 0.6%	> 0.75%	1.56%	3.13%	0.78%	50%	50%

3.4 Conclusions

The diversity of bacteria species found on most farms was similar and the dominant species were *S. chromogenes*, *S. aureus*, *Str. dysgalactiae*, *E. coli*, *Klebsiella* spp. and *Bacillus* spp. Based on this study as well as other studies results CNS spp. are the dominant pathogens causing mastitis in South African dairy farms, followed by *S.aureus*, *Streptococcus* spp. Other rare mastitis associated species isolated were *Lactococcus lactis*, *Pasteurella* spp and *Acinetobacter* spp. Most strains were found to be sensitive to cephalosporins, aminoglycosides, bacitracin, novobiocin

and tetracycline and resistant to penicillins and polymyxin B. Teat dip disinfectants were found to be effective in inhibiting growth of test isolates even at lower concentrations, while milking equipment sanitizer was found to be ineffective at tested concentrations for gram-negative bacteria. Due to the nature of this disease its complete elimination in dairy herd is very difficult. To successfully control this disease the farmer is dependent on the effectiveness of the currently available antimicrobial. The frequent monitoring of the efficacy of these antimicrobials that is very important. Also, to reduce the likelihood of the pathogens which cause mastitis to develop resistance to these antimicrobials, it is importance that causative pathogen be isolated and antimicrobial efficacy tested before antibiotic treatment is implicated and effective disinfectants at effective concentration must be used.

3.5 References

Aarestrup F.M and Jensen N.E, (1998). Development of penicillin resistance among *Staphylococcus aureus* isolated from bovine mastitis in Denmark and other countries. *Microbial Drug Resistance* 4: 247–256.

Anon, (1965). Report of the Public Health Laboratory Service Committee on the Testing and Evaluation of Disinfectants. *British Medical Journal*, 408–413.

Al Atrouni A, Joly-Guillou M-L, Hamze M and Kempf M (2016). Reservoirs of Non-*baumannii* *Acinetobacter* Species. *Frontier Microbiology*, 7:49. doi: 10.3389/fmicb.2016.00049.

Barnum D.A, Johnson R.E and Brooks B.W, (1982). An Evaluation of a Teat Dip with Dodecyl Benzene Sulfonic Acid in Preventing Bovine Mammary Gland Infection from Experimental Exposure to *Streptococcus agalactiae* and *Staphylococcus aureus*. *Canadian Veterinary Journal*, 23: 50-54.

Barkema H.W, (2008). New Approaches to Mastitis Prevention. *WCDS Advances in Dairy Technology* Volume 20: 341-350.

Belayneh R, Belihu K and Tesfaye A, (2014). Microbiological study on bacterial causes of bovine mastitis and its antibiotics susceptibility patterns in East Showa Zone, Akaki District, Ethiopia. *Journal of Veterinary Medicine and Animal Health* Vol. 6(4), pp. 116-122, April.

Blignaut D.J.C, (2016). Prevalence of Mastitogenic pathogens in pasture and total mixed ratio based dairies in 2008 and 2013: Thesis. Thesis submitted on partial fulfilment of requirement for the degree, master in veterinary medicine in the department of animal production studies, Faculty of Veterinary Sciences, University of Pretoria.

Brul S and Coote P, (1999). Preservative agents in foods—mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, 50:1–17.

CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CLSI document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.

CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.

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, Conclin J (2004). Association of cow and quarter-level factors at drying-off with new intramammary infections during the dry period. *Preventive Veterinary Medicine* 63:75-89.

Du Preez J.H, (2000). Bovine mastitis therapy and why it fails. *Journal of the South African Veterinary Association*, 71(3): 201–208.

Gower J.C, (1966). Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika*, 53:325-338.

Gurung M, Nam H. M, Tamang M.D, Chae M. H, Jang G.C, Jung S.C and Lim S. K, (2013). Prevalence and antimicrobial susceptibility of *Acinetobacter* from raw bulk tank milk in Korea. *Journal of Dairy Science*, 96:1997–2002. <http://dx.doi.org/10.3168/jds.2012-5965>.

IVS. 2015. Index of Veterinary Specialities. Vol 53. Hirt and Carter, Durban, South Africa.

Jayarao B.M and Wang L, (1999). A study on the prevalence of gram-negative bacteria in bulk-tank milk. *Journal of Dairy Science*, 82:2620–2624.

Katsande S, Matope G, Ndengu M. and Pfukenyi D.M. (2013). 'Prevalence of mastitis in dairy cows from smallholder farms in Zimbabwe. *Onderstepoort Journal of Veterinary Research* 80(1), Art. #523, 7 pages. <http://dx.doi.org/10.4102/ojvr.v80i1.523>.

Kateete D.P, Kabugo U, Baluku H, Nyakarahuka L, Kyobe S, Okee M, Najjuka C.F, Joloba M.L, (2013). Prevalence and Antimicrobial Susceptibility Patterns of Bacteria from Milkmen and Cows with Clinical Mastitis in and around Kampala, Uganda. *PLoS ONE* 8(5).

Kelsey J.C and Maurer I.M, (1972) .The Use of Chemical Disinfectants in Hospitals. Public Health Laboratory Service Monography Series No. 2. London: HMSO.

Kitis M, (2004). Disinfection of wastewater with peracetic acid: a review. *Environment International* 30:47– 55.

Koivula M, Pitkala A, Pyorala S, Mantysaari E, (2007). Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland. *Acta Agriculturae Scandinavica, Animal Sciences*, In press.

Li J.P, Zhou H.J, Yuan L, He T, Hu S.H, (2009). Prevalence, genetic diversity, and antimicrobial susceptibility profiles of *Staphylococcus aureus* isolated from bovine mastitis in Zhejiang Province, China. *Journal of Zhejiang University* 10:753–760.

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

Mallikarjunaswamy M.C and Krishnamurthy G.V, (1997). Antibiogram of bacterial pathogens isolated from bovine mastitis cases. *Indian Veterinary Journal*, 74(10): 885-887.

McDonnell G and Russell A. D, (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews*, 12:147–179.

Mehmet K, (2004). Disinfection of wastewater with peracetic acid: a review. *Environment International* 30: 47– 55.

Mohammed A.N, (2014). Assessing the Antimicrobial Resistance Patterns and in Vitro Efficacy of three Disinfectants as teat dip against Iodine (0.5%) Resistant Mastitogenic Pathogens *International Journal of Advanced Research* (2014), Volume 2, Issue 12, 946-953.

Nam H.M, Lim S. K, Kim J. M, Joo Y. S, Jang K. C and Jung S. C, (2010). In vitro activities of antimicrobials against six important species of gram-negative bacteria isolated from raw milk samples in Korea. *Foodborne Pathogens and Disease*, 7:221–224.

National Mastitis Council (1999). *Laboratory and field handbook on bovine mastitis*, 2nd edn., National Mastitis Control Council, Madison.

Nickerson SC. (2001). Choosing the Best Teat Dip for Mastitis Control and Milk Quality. National Mastitis Council. Retrieved November 18, 2014 from <http://www.nmcoline.org/articles/teatdip.htm>.

Nigo K.L.S, Elfadil A.A.M and Bakhiet M.R (2013). Epidemiological and Bacteriological Investigation of Bovine Mastitis in Khartoum State, Sudan. *Sudan Journal of Science and Technology* Vol. 14.

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(1):17-22.

Pieterse R and Todorov S.D, (2010). Bacteriocin- exploring alternative to antibiotics in mastitis treatment. *Brazilian Journal of Microbiology*, 41: 542-562.

Rabello R.F, Souza C.R.V.M, Duarte R.S, Lopes R.M.M, Teixeira L.M, Castro A. C.D (2005). Characterization of *Staphylococcus aureus* isolates recovered from bovine mastitis in Rio de Janeiro, Brazil. *Journal of Dairy Science* 88: 3211–3219.

Radostits O.M, Gay C.C, Blood D.C and Hinchcliff K.W, (2000). Mastitis. In: *Veterinary Medicine*, 9th ed., W.B. Saunders Company Ltd., London, pp. 603-687.

Robert A, Seegers H and Bareille N, (2006). Incidence of intramammary infections during the dry period without or with antibiotic treatment in dairy cows - a quantitative analysis of published data. *Veterinary Research*, 37:25-4.

Russell A.D, (1995). Mechanisms of microbial resistance to disinfectant and antiseptic agents. In *Chemical Germicides in Health Care* (ed. Rutala, W.A.), pp. 256–269. Morin Heights: Polyscience.

Russell A.D, (1986). Chlorhexidine: Antibacterial Action and Bacterial Resistance. *Infection* 14, Nr. 5, 212-215.

Saha K, Butola B.S, Joshi M, (2014). Synthesis and characterization of chlorhexidine acetate drug–montmorillonite intercalates for antibacterial applications. *Applied Clay Science* 101: 477–483.

Santos R.P, Souza F.N, Vasconcelos C.G.C, Cortez A, Rosa D.L.S.O, Jardim A.B, Cunha A.F, Lana A.M.Q, Heinemann M.B and Cerqueira M.M.O.P, (2016). In vitro efficacy of teat antiseptics against *Staphylococcus aureus* strains isolated from bovine mastitis. *Semina: Ciências Agrárias, Londrina*, v. 37, n. 4, p. 1997-2002, Jul. /ago. DOI: 10.5433/1679-0359.2016v37n4p1997.

Schmidt T, Kock M.M and Ehlers M.M, (2015). Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts. *Journal of Dairy Science* 98:6256–6269.

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

Shaker L.A, Russell A.D and Furr J.R, (1986). Aspects of the action of chlorhexidine on bacterial spores. *International Journal of Pharmaceutics*, 34, 51–56.

Sandholm M, Kaartinen L and Pyörälä S (1990). Bovine mastitis--why does antibiotic therapy not always work? An overview. *Journal of Veterinary Pharmacology Therapeutics* 13: 248-260.

Smith J.S, Moroni P, and Nydam D, (2016). *Lactococcus*: an emerging mastitis pathogen. Dairy business and Holstein world- PRO-DAIRY, October, pp.36-38. www.TheFutureOfDairy.com.

Stratford M, 1999. Weak acid and “weak-acid preservative” inhibition of yeasts. In: Tuijelaars, A.C.J., Samson, R.A., Rombouts, F.M., Notermans, S. (Eds.), *Food microbiology and food safety in the next millennium*. Foundation Food Micro 099. Veldhoven, the Netherlands, pp. 315–319.

Sudhan N.A and Sharma N. (2010). Mastitis- An Important Production Disease of Dairy Animals. *Farm Management and disease, SMVS‘DAIRY YEAR BOOK*, pp.72-88.

Swartz T and Petersson-Wolfe C.S, (2016). Produced by Communications and Marketing, College of Agriculture and Life Sciences, Virginia Tech, 2016. VT/0316/DASC-67P. www.ext.vt.edu.

Sykes G, (1965). *Disinfection and Sterilization*, 2nd edn. London: E. & F.N. Spon.

Teklesilasie T, Kebeta M.M and Fayera T, (2014). Bovine Mastitis: Prevalence and Isolation of Major Pathogens in Dairy Farms of Selected Sites in Addis Ababa, Ethiopia. *Applied Journal of Hygiene* 3 (3): 31-37.

Terolli L, Shoshi N, Cabeli V, Koleci X and Qirinxhi E, (2015). In vitro evaluation of Chlorhexidine based disinfectants used post-dipping against mastitis pathogens. Sixth International Scientific Agricultural Symposium “Agrosym 2015”1565- 1569. 10.7251/AGSY15051565T.

Tonjum T, Caugant D. A, Namork E and Koomey J.M, (1994). Characterization of the phosphoglucose isomerase of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Proceedings of the 9th International Pathogenic Neisseria Conference*, Winchester, U.K, p. 208.

Watts J.L, (1988). Etiological agents of bovine mastitis. *Veterinary Microbiology* 16: 41-66.

Williams R, (2000). The impact of antimicrobial resistance. *Acta Veterinaria Scandinavica, Supplementum*, vol. 93, 2000, p. 17-20.

Yu Z, Qin W, Lin J, Fang S and Qiu J, (2015). Antibacterial Mechanisms of Polymyxin and Bacterial Resistance. *Hindawi Publishing Corporation BioMed Research International* Volume 2015, Article ID 679109, 11 pages
[.http://dx.doi.org/10.1155/2015/679109](http://dx.doi.org/10.1155/2015/679109).

CHAPTER 4

Isolation and characterization of lytic bacteriophages for bovine mastitis causative pathogens in raw milk and cow manure samples

Abstract

Bovine mastitis is an infectious disease mostly caused by bacteria and the main reason for huge economic losses experienced by the dairy industry. For decades, treatment and control have been possible through the use of antibiotics and chemical teat dips. However, over the years, major mastitis associated pathogens have become multidrug resistance, treating the treatment and control of this disease. In addition, the use of antibiotics and chemical teat dips put milk in high risk of antibiotics and chemical contamination, which render it unfit for human consumption. To combat these challenges, more innovative tools of biological origin are still needed and Bacteriophages provide a promising alternative. For therapeutic application of phages, certain properties are a necessity such as being lytic, have a broad host range, short latency period and high burst size. The objective of this study was to isolate lytic bacteriophages for mastitis associated causative pathogens, to characterize them morphologically and to determine their host range. A total of 12 lytic phages were successfully isolated in cow manure and hosts used were *S. aureus*, *Coagulase-negative Staphylococcus*, *Streptococcus* spp., *E. coli* and *Acinetobacter* sp. Their morphology characteristics showed that they belong to *Myoviridae* family. Based on the spot test and efficiency of plaque formation, most phages showed a broad host range, they were able to lyse other strains from the same species and strains from other species.

4.1 Introduction

Bovine mastitis is an inflammation of mammary gland, usually caused by infectious agents such as bacteria, fungi and viruses or physical and chemical irritancy, although bacteria cause most cases (Bogni *et al.*, 2011; Sharma *et al.*, 2006). Mastitis is the common disease affecting the dairy industry, with great economic losses associated with it, losses such as less milk yields, poor quality of milk, which subsequently affect the quality of milk end products and discarded milk before, during and after antibiotic treatment (Halasa *et al.*, 2007; Sudhan and Sharma, 2010). For characterisation of this disease, physical, chemical and bacteriological changes in milk and pathological changes in the udder are used (Radostis *et al.*, 2000). Complete

irradication of this disease from the dairy herd is difficult, for decades its control and prevention have been possible by comprehensive planning of mastitis control proposed by National Mastitis Council (NMC) (Nickerson, 2009). This plan consists of six basic points, which comprise of proper milking hygiene, use of properly functioning milking machines, post-milking teat dipping, antibiotic therapy of chronic infected cows, dry antibiotic therapy and culling.

Antibiotic therapy and teat dipping are the most efficient mastitis control measures used for treatment and control of this disease (Nickerson, 2009 and 2001). However, the overuse of antibiotics in human medicine, agriculture and veterinary medicine is reported to heighten the number of multidrug resistance in pathogenic strains of human and animal origin (Shaikh *et al.*, 2015; Swatantra *et al.*, 2014). Another concern of human health significance and of economic importance are the presence of antibiotics when used and end up contaminating milk with antibiotics residues. This may cause allergic reactions in humans and the dairy farmers receive penalties when delivering milk contaminated with antibiotics (Bradley, 2002; Dagnaw, 2015; White and McDermott, 2001). In addition, almost all teat dips currently used are chemical based and side effects such as teat irritation leading to teat damage such as sores and cracks have been reported (Huber, 1988; Nickerson, 2001). Damage in cows' teat increases the chances of secondary bacterial infection from occurring and chemicals have a negative impact on the environment. Therefore, there is a need for the development of alternative innovative tools for treatment and control of bovine mastitis that are natural and of biological origin, environmental friendly and safe to humans when incidentally consumed. Bacteriophages provide a promising alternative for this and over the past years, several studies conducted in different fields have shown their potential as therapeutic agents in humans, animals and plants (Borie *et al.*, 2014; Gill and Abedon, 2003; Sillankorva *et al.*, 2012; Sulakvelidze *et al.*, 2001; Weber-Dabrowska *et al.*, 2000).

Bacteriophages are viruses specifically infecting bacteria, exist as DNA or RNA (single or double stranded), circular or linear packages with protein capsid. They occur naturally, and are the most abundant organisms on earth, approximately 10^{31} particles (Bergh *et al.*, 1989; Burrowes *et al.*, 2011; Sillankorva *et al.*, 2012; Wommack & Colwell, 2000). Phages are divided into two types as virulent or temperate based on their life cycle. The virulent phage multiply within the bacterial cell to finally lyse the cell and release phage progeny while temperate phage integrated its DNA into host chromosome where it replicates as part of the host genome (Higgins *et al.*, 2005).

Currently, there are few experimental field trial studies on phage application treatment of bovine mastitis cases and results are promising even though they vary. Gill *et al.* (2006) conducted a study treating mastitis-infected cows by intramammary injection of phage solution, in their results, they observed an increase in the SCC of treated udders and the cure rate was 16.7%. The lack of treatment efficacy was reported to be due to inhibitory effects of raw milk (whey protein shielding binding site). However, in another study by Swatantra *et al.* (2014) similar results were achieved with a much higher cure rate of 83%. In another study by Ndlela *et al.* (2016) in South Africa; phages were applied to cows as a post-milking udder spray treatment and 87% of phage-treated cows showed a lower bacterial cell count and 96% of the untreated cows showed a higher bacterial cell count compared to treated. Phage therapy provided an alternative, but Intra-mammary infusion of bacteriophages seems to be problematic at this stage and more studies are needed on interaction of phages in the mammary gland. However, the use of Bacteriophages in teat dipping may provide another alternative in preventing new intra-mammary infections in dairy cattle. The objectives of this study were to isolate lytic bacteriophages for mastitis associated causative pathogens, to characterize them morphologically and to determine their host range.

4.2 Material and Methods

4.2.1 Phage isolation, purification and propagation

Samples for phage isolation and host cultures used

Phage isolation was done on raw milk and cow manure using enrichment before isolation and direct plating methods following the procedure described by Mazzocco *et al.*, (2009); Van Twest and Kropinski, (2009) with some modification. The host cultures used were species isolated from raw milk samples from clinical and subclinical mastitis cows in Bloemfontein dairy farms. These were representatives of the species and number of strains *Coagulase-negative Staphylococcus* spp. (16), *Staphylococcus aureus* (6), *Streptococcus* spp. (11), *E. coli* (3), *Pseudomonas* sp. (1), *Proteus* sp. (1), *Enterobacter* sp. (1), *Vibrio* sp. (1), *Bacillus* spp. (3), *Corynebacterium* spp. (8), *Mycobacterium* sp. (1) and other pathogens *Acinetobacter* spp. (18) and *N. meningitides* (2).

Enrichment of bacteriophages

In brief, distilled water (dH₂O) was added to cow manure and incubated at room temperature for 2hrs with manual agitation. Thereafter, the solution was centrifuged at 10,000xg for 10 min to remove debris. The supernatant was enrichment for phages by adding 20ml cow manure or milk supernatant into a shake flask with 20ml 2x Tryptic Soya Broth (TSB) + 2 ml host culture and incubated at 37°C with shaking for 24hrs. Thereafter 1 ml of chloroform (CHCl₃) was added and incubated for 5min at room temperature and centrifuged at 8,000xg for 10min and filter sterilized using (0.22µm) membrane filters. The enrichment step was repeated three times to ensure that the samples were rich with phages of interest. The spot test was used to screen the filtrate for phages, done by spreading 200µl exponential phase host culture used for enrichment on TSA soft agar (with 3g bacteriological agar in 500ml TSB) and 10 µl filtrate spotted and incubated at 37°C for 12hrs. The formation of the clear zone indicated positive results for phage isolation.

Isolation and purification

Direct plating methods were used for isolation and purification of phages; this was done by cutting the plaque and adding it to 1ml TSB, vortex, incubated at room temperature for 2hrs. Thereafter 200 µl CHCl₃ added, incubated for 5 minutes then centrifuged at 8,000xg for 10 min and filter sterilized (0.22µm). Ten-fold dilutions of spot phage lysates were done and 200µl exponential phase host culture was added in dilutions, incubate at 37°C for 20 min for phage adsorption. The dilutions were plated by spread plate method using 200 µl of dilutions and incubated at 37°C for 24hrs. The plaques formed, were further purified by repeating the same procedure three times. Purified phages were stored in 1x Phosphate buffer (PBS) at 4°C until further use.

Phage propagation and concentration

Phages were propagated by mixing 1.5 ml TSB media, 100µl mid exponential phase host culture and 100µl phage (approximately 10¹⁰PFU/ ml) in 2ml eppendorf tubes for 5hrs at 37°C with shaking. 200 µl CHCl₃ was added incubated at room temperature for 5 min and centrifuged at 8,000xg for 10 min, supernatant harvested and pellet discarded. Phages were concentrated by precipitation using (5x) PEG-6000/NaCl. In brief, 1200 µl of supernatant was transferred to a clean microfuge tube and 300 µl of 5x PEG/NaCl was added and mix thoroughly by inversion and tubes chilled on ice for 5 minutes. After that tubes were taken out and observed for PEG-precipitated virions which are often seen by the naked eyes, if nothing was observed, incubation on ice was continued for an hour. Thereafter, the virions were pelleted by centrifugation for 3 min at 13,000 g and the supernatant removed, discarded and

centrifuged again for 1 min at 13,000 g to remove all residual supernatant. The pellet was re-suspended with 120 µl of (1x) TBS and mixed by vigorous vortexing and incubated on ice for another hour. All tubes were vortexed again and phage solution centrifuged for 1 min at 13,000 g and transferred to a clean microtube.

4.2.2 Morphology characterization by Transmission Electron Microscopy (TEM)

Samples were analyzed using negative staining with 2% uranyl acetate. 10 µl of phage suspension was added on a grid, followed by 2 drops of uranyl acetate solution before the third drop was added to the shiny side of the grid and allowed it to sit for 45 seconds. The grid was observed using Philips CM100 Transmission Electron Microscopy, the Netherlands and phage images overall length, the tail length and width, and the capsid diameter and length were determined using analysis, soft Imaging system GMBH, Germany. When necessary, additional dilutions were performed to obtain greater quality images.

4.2.3 Host range determination by spot test and efficiency of plaque formation

The host range of six phages, isolated in cow manure with a phage titre of 10^{10} CFU/ml was determined by spot test. The species applied as hosts included, *S.aureus* (6), CNS (16), *Streptococcus* spp. (11), *Acenetobacter* spp. (18), *N. meningitidis* (2), *E. coli* (3), *Bacillus* spp. (3) *Listeria* ssp. (4), *Corynebacterium* spp. (8), *Pseudomonas* spp. (2), *Proteus* sp. (1), *Salmonella* sp. (1), *Enterobacter* sp. (1), and *Vibrio* sp. (1).

This was done by spreading 200 µl exponential phase host culture on soft TSA and 10 µl of phage spotted allowed to dry and incubated at 37°C for 24hrs in duplicates. The spot test host range results were confirmed by Efficiency of plaque formation (EOP) using double agar plaque assay, following Khan Mirzaei and Nilsson, (2015) and O'Flaherty *et al.* (2005) protocol with some modifications. In brief, 200 µl of exponential phase test strain culture was mixed with 100µl of five serial ten-fold dilutions of individual phage suspensions (10^{-6} - 10^{-10}) in 4 ml of molten soft TSA top agar and the mixture was poured on top of solidified TSA plates in duplicate. The plates were incubated overnight at 37°C and the number of plaque forming units per ml (PFU/ml) was counted for each combination. Efficiency of plaque formation (EOP) of phages was determined by dividing the phage titre on the test strain by the phage titre on the reference strain (host strain). When the 10^6 dilution did not result in any plaques, a lower dilution was tried afterwards to verify that the EOP was lower than 0.001.

4.3 Results and discussion

4.3.1 Phage isolation

Phage isolation was done on raw milk and cow manure using 69 mastitis associated causative strains of bacteria as hosts. Unfortunately, no phages were successfully isolated from raw milk and this maybe due to the low number of phages usually present in raw milk. However, in cow manure, approximately more than 20 lytic phages were successfully isolated, but during purification process, they become lysogenic and we were left with 12 that remained still lytic (Table 4.1).

Raw milk and cow manure samples were chosen for phage isolation because phages are reported to co-exist in the same environment as their host as evidenced by Basdew and Laing, (2015) who were able to successfully isolate 28 *S.aureus* lytic phages from raw milk in their study. O'Flaherty *et al.* (2005) also isolated *S.aureus* lytic phages from a farmyard slurry and Li and Zhang, (2014) isolated *S.aureus* phages in udder cleaning wastewater. Lytic phages were identified by consisted formation of clear zone (plaques) in the lawn of host cells which resulted from the lysis of bacteria; while lysogenic phage were identified by having plaques with turbid centres or bulls-eye appearance due to cells being immune to lysis and continues to grow.

For success of phage therapy, strictly lytic phages are recommended for use compared to lysogenic phages. This is due to these phages life cycle properties, such as expression of genes that allows the phage to take over host machinery protein expression systems producing multiply copies of its phage genome and components. These are subsequently assembled into phage progeny and producing enzymes that lyse the host cell, killing it and releasing phage progeny, which infect other host cells (Gill and Hyman, 2010). While, on the other hand lysogenic phages life cycle results in establishment of the lysogeny which are resistant and genome may contain genes, which alter the phenotype of the host cell causing it to be more effective disease-causing agents.

These were the names designated to our phages S.A 4 and S.A 8 for *S.aureus* phages, CNS 17 for *Coagulase-negative Staphylococcus* phage, Str. 2 and Str. 11 for *Streptococcus* spp. phages, Cory.19 for *Corynebacterium* sp. phage, Ace.19 for *Acenetobacterium* sp. and *E. coli* for *E. coli*. All these phages formed clear round plaques with sizes between 1-2 mm in diameter and titers between 10^8 - 10^{12} PFU/ml (Fig.4.1).

Table 4.1 Mastitis causing pathogens phage isolation results.

Organisms	Amount of hosts	Number of phages
<i>Staphylococcus aureus</i>	6	3
Coagulase-negative <i>Staphylococcus</i> spp.	12	3
<i>Streptococcus</i> spp.	11	2
<i>E.coli</i>	18	2
<i>Corynebacterium</i> sp.	14	1
<i>Acenectobacter</i> sp.	8	1
Total	69	12

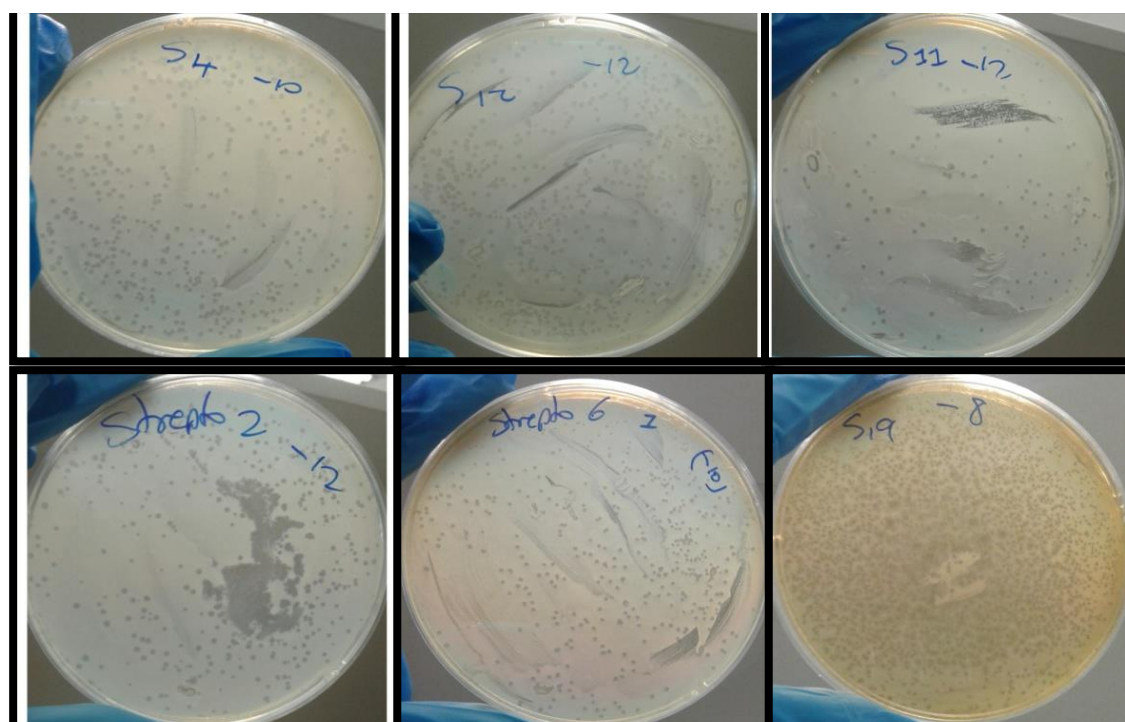


Figure 4.1 Phage plaque formation by direct plating plaque assay on Tryptic Soya Agar. S4, S11, S12- *Staphylococcus* spp., S19 – *Corynebacterium* sp., Strepto 2 and 6- *Streptococcus* spp. Phages forms clear round plaques with size 1-2 mm in diameter. Phage titer was 10^8 - 10^{12} PFU/ml.

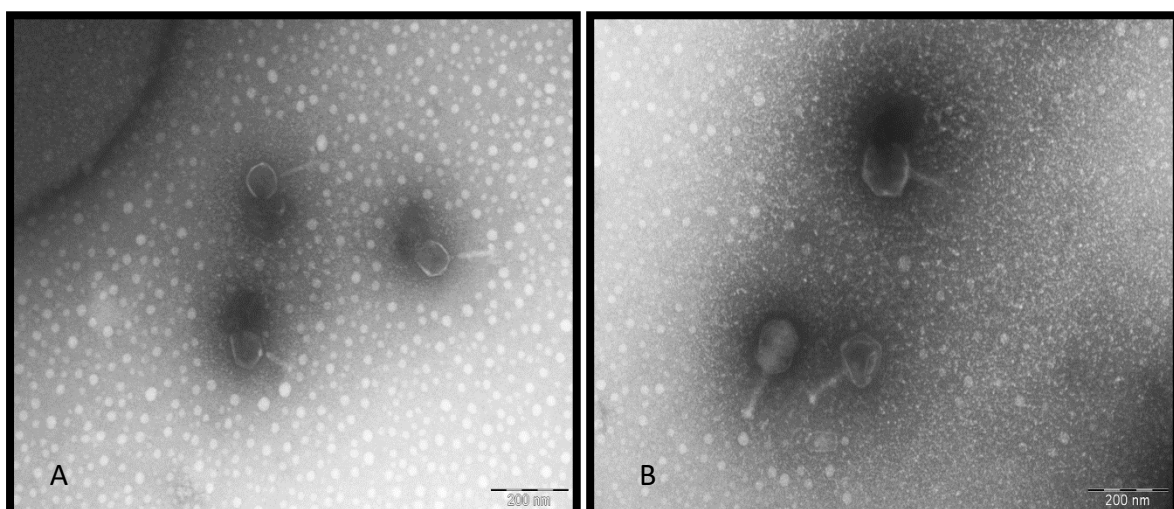
4.3.2 Morphology characterization by Transmission Electron Microscopy (TEM)

For morphological characterization, few phages were selected by observing their host range and consistence in their lytic activity. Their morphologies were determined by negative staining using Transmission Electron Microscopy (TEM). All phages examined showed to have icosahedral heads and rigid contractile sheath tails and were assigned to the family *Myoviridae*, order *Caudovirales*. Electron micrographs are presented in Figure 4.2. They were all different in their size and length between 162 ± 8 - 233 ± 3 ; Heads diameters 57 ± 5 – 117 nm and length 59 ± 1 - 121 ± 5 nm, Tail length 92 ± 7 - 114 ± 5 nm as seen in Table 4.2. Phages also differ in their morphologies

from filamentous, to icosahedral without tails and with tails and even several phages with a lipid containing or contain lipids in the particle shell, but their basic structural feature is coat (capsid) which houses its genome (Ackermann, 2007).

Phage species in the order *Caudovirales* are characterised by dsDNA genome with the virus particles that have a capsid, connector, a tail and baseplate with tail fibres and needle or tip, differentiated based on their size from 24-400 nm in length and genome length can range from 18 to 400 kb in size (Orlova, 2012). Families within this order are differentiated from one another by the difference observed in their tail morphology, *Myoviridae* phages have long rigid tails composed of inner tail tube that is surrounded by a contractile sheath, which contracts during infection of the bacterium while *Siphoviridae* phages have long, noncontractile tails and *Podoviridae* have short tails.

All these structural features play a specific role during phage life cycle, the head (capsid) houses the genome, while the connector serves as an adaptor between head and tail, participating in the packaging of DNA into the capsid. Also, functions as a gatekeeper locking the capsid exit of the phage, preventing leakage of DNA and later open releasing it into the bacterium after a signal transmitted by the tail indicating that the phage is attached (Plisson *et al.*, 2007). The tail serves as a signal transmitter and as a channel through which DNA is delivered into the host cell during infection. At the end of the tail there is a baseplate with long and short fibres and a needle. Their function is to locate specific membrane receptors of the bacterium and bind until irreversible adsorption of phage occurs which induces the opening of the phage connector and releases of the genome through the tail tube into the bacterial cell (Leiman *et al.*, 2010).



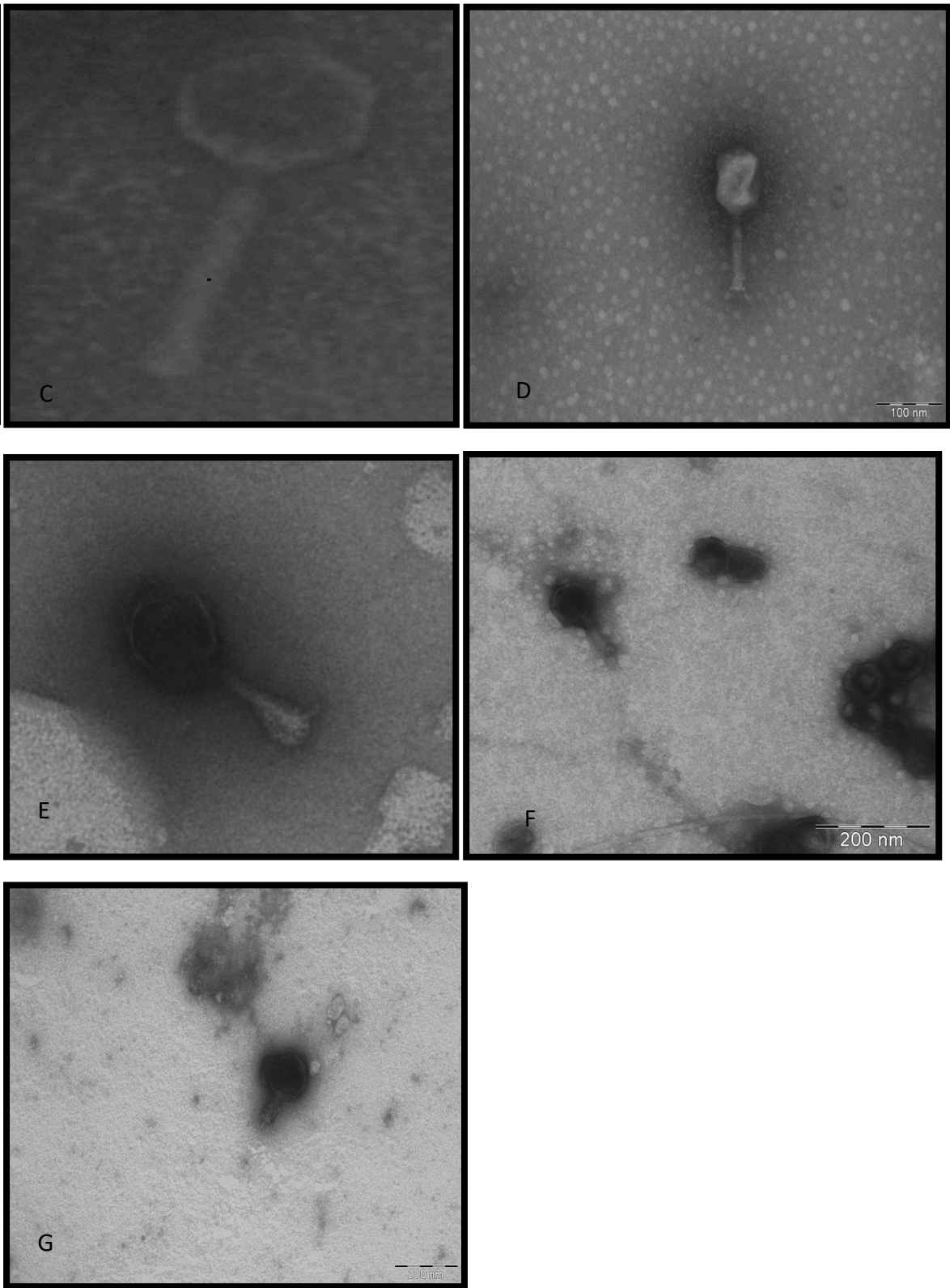


Fig. 4.2 TEM microphotography of *Staphylococcus* spp. A= S. A 4, B= S. A 8 and C = CNS 17, D = *E.coli*, *Streptococcus* spp. = E and F and G = *Acinetobacter* sp.

Phage	Family	Rationale
S. A 4	Myoviridae	Overall size 200 ± 8 nm , Icosahedral capsid with diameter 80 ± 6 nm and length 86 ± 2 nm, tail with contractile sheath, length 92 ± 7 nm, width 12 ± 3 nm

S. A 8	Myoviridae	Overall size 233±3 nm , Icosahedral capsid with diameter 93±8 nm and length 117±9 nm, tail with contractile sheath, length 114±5 nm, width 16±1nm
Str.2	Myoviridae	Overall size 204±1nm , Icosahedral capsid with diameter 70 nm and length 95±7 nm, tail with contractile sheath, length 97±3 nm, width 10±3nm
Str. 11	Myoviridae	Overall size 200±6 nm , Icosahedral capsid with diameter 80±4 nm and length 80±3 nm, tail with contractile sheath, length 90±8 nm, width 15±1nm
Ace.19	Myoviridae	Overall size 196±9 nm , Icosahedral capsid with diameter 117 nm and length 121±5 nm, tail contractile sheath, length ± nm, width 3153nm
CNS 17	Myoviridae	Overall size 162±8 nm , Icosahedral capsid with diameter 57±5 nm and length 59±1 nm, tail with contractile sheath, length 91±9 nm, width 13±nm
<i>E.coli</i>	Myoviridae	Overall size 200±8 nm , Icosahedral capsid with diameter 80±6 nm and length 86±2 nm, tail with contractile sheath, length 92±7 nm, width 12±3nm

Table 4.2 Phage Morphology characterization.

4.3.3 Phage host range determination by spot test and Efficiency of plaque formation (EOP)

Phage host range was determined by challenging each of the six phages S.A 4, S.A 8, CNS 17, Str.2, Str.11 and Ace.19 to the different strains of *S.aureus* (6), CNS (16), *Streptococcus* spp. (11), *Acenetobacter* spp. (16), *N. meningitidis* (2), *E. coli* (3), *Bacillus* spp. (3) *Listeria* spp.(4), *Corynebacterium* spp. (8), *Pseudomonas* spp. (2), *Proteus* spp. (1), *Salmonella* spp. (1), *Enterobacter* spp. (1), and *Vibrio* spp. (1). To confirm positive spot test host range results, efficiency of plaque formation was done and EOP was calculated as the ratio of phage titre of target bacterium over the phage titre of host strain and the ratios between 0.1 to 0.5=+++ represented high productive infection; 0.05 to 0.1 = ++ medium productive infection; 0.001 to 0.05= + low productive infection; <0.001=- no productive infection; bacterial strain was not susceptible to phage attack. N/T= not tested. The host range results for spot test and EOP are summarised in Table 4.3 and Table 4.4.

Host range is determined as the ability of a phage to infect numerous strains of bacteria across several genera (Gill and Hyman, 2010). Phages are known to be highly specific to their bacterial host strain; phage species can only cause infection in a certain number of bacterial strains. This has been reported to be due to adsorption specificity determined by the number of phages and host factors such as the ability of phage receptors to recognise their similar receptors on the host cell surface and receptors localization, the amount and their density on the cell surface (Rakhuba *et al.*, 2010). Also, the ability of the phage to successfully inject its genetic material into

the cell, and other bacterial mechanisms such as bacterial restriction-modification systems, abortive infection and CRISPR mechanism have been reported to be other determining factors in phage host range (Gill and Hyman, 2010).

Based on the spot test and EOP host range results, three phages S.A 4, S.A 8 and Str.11 showed the broadest host range. S.A 4 phage was able to produce infection on another *S. aureus* strain, three *Streptococcus* spp., *Acinetobacter*, *N.meningitidis* and *Corynebacterium* species. S.A 8 and Str.11 were able to lyse CNS, *Streptococcus* and *N.meningitidis* strains from the same species and strains from other species. This was unusual, as phages are known to have narrow host ranges, only able to lyse a few strains from their species of isolation and non from other genera. However, in a recent study by Shende *et al.* (2017), similar results were reported of phages isolated with broad host ranges on dairy farm waste. The host for phage isolation were *B. subtilis* and *E. coli*. These phages showed lytic activity against other strains from genera such as *Staphylococcus*, *Salmonella*, *Proteus* and *Pseudomonas*. Bielke *et al.* (2007), also reported similar findings, where *Salmonella* host range of bacteriophages were able to infect multiple genera and they concluded that phage host ranges are not always genera restricted, so phages could have wide host ranges.

Phages CNS 17, Str.2 and Ace.19 showed positive results in the spot test and failed to produce plaques in the EOP test, which showed productive infection. Many factors have been reported to be the cause of this, like phages bind to the strain and cause bacterial death through an abortive infection and or via lysis, but were not able to produce sufficient progeny phages in this strain to form plaques or bacteriocin could be causing the lysis. These results demonstrated that these phages possess strong bacteriolytic activity, which is important for use in phage therapy. A phage cocktail may be an effective tool in controlling mastitis used as teat dip.

Species ID and number	Phage ID and (Number) % of phages that infect each strains					
	S.A 4	S.A 8	CNS17	Str.2	Str.11	Ace.19
<i>S.aureus</i> (6)	2 host	1host	-	-	-	-
CNS (16)	-	2	2host	1	3	-
<i>Streptococcus</i> spp. (11)	3	1	2	2host	1host	
<i>Acinetobacter</i> spp. (16)	1	-	-	1	1	1host
<i>N. menengitidis</i> (2)	1	1	-	1	1	1
<i>E. coli</i> (3)	-	-	-	-	-	-
<i>Bacillus</i> spp. (3)	-	-	-	-	-	-

<i>Listeria</i> spp.(4)	-	-	-	1	-	-
<i>Corynebacterium</i> spp. (8)	1	-	-	-	-	-
<i>Pseudomonas</i> spp (2)	-	-	-	-	-	-
<i>Proteus</i> spp. (1)	-	-	-	-	-	-
<i>Salmonella</i> spp. (1)	-	-	-	-	-	-
<i>Enterobacter</i> spp. (1)	-	-	-	-	-	-
<i>Vibrio</i> spp. (1)	-	-	-	-	-	-
Total	8	5	4	6	6	2

Table 4.3 Phage host range testing by spot test.

Table 4.4 Efficiency of Plaque formation (EOP) of phages positive in spot test.

Species ID and number	Phage ID					
	S. A 4	S. A8	CNS17	Str. 2	Str. 11	Ace. 19
<i>S.aureus</i> 4	host					
8	-	host				
CNS spp. 2.3		-		-	-	
11			-		++	
12		-				
17			host		+++	
<i>Streptococcus</i> spp. 2	+			host		
6	-		-			
11	+++	-	-		host	
<i>Acinetobacter</i> sp. 3	+			-	-	-
<i>N. meningitidis</i> 19						host
20		+				
<i>Corynebacterium</i> sp. 12	-					
Total spot test	8	5	4	6	6	2
Total EOP	3	2	2	2	3	1
+++ , EOP 0.1 to 0.5; ++, EOP 0.05 to 0.1; +, EOP 0.001 to 0.05; - (<0.001) bacterial strain was not susceptible to phage lysis. N/T= not tested						

4.4 Conclusions

Lytic bacteriophages for mastitis-associated pathogens were successfully isolated from cow manure and they showed broad host ranges, were able to lyse other strains from the same species and strains from other species, which is important for their application as therapeutic agents or bio control agents. Their morphological classification showed that they belong to the *Myoviridae* family. They have a potential of being used as a phage cocktail in control of this disease, either as a teat dip or as an antimicrobial in teat sealants.

4.5 References

Ackermann, H.W. (2007). 5500 Phages examined in the electron microscope. *Archives of Virology* Vol.152, No.2, pp. 227-243. PMID 17051420.

Basdew I.M and Laing M.D (2015). Investigation of the lytic ability of South African bacteriophages specific for *Staphylococcus aureus*, associated with bovine mastitis, *Biocontrol Science and Technology*, 25:4, 429-443, DOI: 10.1080/09583157.2014.983458.

Bielke H, Donoghue S, Donoghue A and Hargis D.B.M, (2007). *Salmonella* Host range of bacteriophages that infect multiple genera. *Poultry Science*, 86: 2536-2540.

Borie C, Robeson J, Galarce N, (2014). Lytic bacteriophages in Veterinary Medicine: a therapeutic option against bacterial pathogens? *Archivos de Medicina Veterinaria* 46, 167-179.

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

Burrowes B, Harper D.R, Anderson J, McConville M. and Enright M.C, (2011). Bacteriophage therapy: potential uses in the control of antibiotic-resistant pathogens. *Expert Review of Anti-Infective Therapy*, Vol.9, No.9, pp. 775-85.

Dagnaw G.G, (2015). Public Health Significance of Bovine Mastitis. *World Journal of Biology and Medical Sciences*, Volume 2 (4), 20-31.

Gill J.J and Hyman P, (2010). Phage Choice, Isolation, and Preparation for Phage Therapy. *Current Pharmaceutical Biotechnology*, Vol11, No 1, pp 2-14.

Gill J.J, Pacan J.C, Carson M.E, Leslie K.E, Griffiths M.W, Sabour P.M, (2006) Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical *Staphylococcus aureus* mastitis in lactating dairy cattle. *Antimicrob Agents Chemother* 50(9):2912.

Gill J and Abedon S.T, 2003. Bacteriophage ecology and plants. APSnet Feature. [<http://www.apsnet.org/publications/apsnetfeatures/Pages/BacteriophageEcology.aspx>].

Haber W.G, (1988). In *veterinary pharmacology and therapeutics* 6th edition. Panima pub.Corp, New Delhi, pp. 765-784.

Higgins J.P., Higgins S.E, Guenther K.L, Newberry L.A, Huff W.E and Hargis B.M, (2005). Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry. Poultry Science, Vol. 84, pp. 1141-1145.

Khan Mirzaei M and Nilsson A. S, (2015). Isolation of Phages for Phage Therapy: A Comparison of Spot Tests and Efficiency of Plating Analyses for Determination of Host Range and Efficacy. PLoS ONE 10(3): e0118557. doi: 10.1371/journal.pone.0118557.

Leiman P.G, Arisaka F, van Raaij M.J, Kostyuchenko V.A, Aksyuk, A. A, Kanamaru S and Rossmann M.G, (2010). Morphogenesis of the T4 tail and tail fibers. Virology Journal, Vol. 7, pp. 355.

Li L and Zhang Z, (2014). Isolation and characterization of a virulent bacteriophage SPW specific for *Staphylococcus aureus* isolated from bovine mastitis of lactating dairy cattle. Mol Biol Rep DOI 10.1007/s11033-014-3457-2.

Mazzocco A, Waddell T.E, Lingohr E, Johnson R.P, (2009). Enumeration of Bacteriophages by the Direct Plating Plaque Assay. In: Clokie M.R., Kropinski A.M. (Eds) Bacteriophages, Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions. Methods in Molecular Biology™, vol 501. Humana Press, pp 69-76.

Ndlela M, Laing M.D and Basdew I.H, (2016). Biological control of *Staphylococcus aureus*-induced bovine mastitis in dairy cows using bacteriophages. Proc. 6th IDF Mastitis Conf., Nantes, France; 7-9 Sept.

Nickerson S. C, (2009). A comprehensive plan to control mastitis and milk quality. Animal and Dairy Science Departmental Report. <http://www.ads.uga.edu/ADS2008DepartmentalReports.html>.

Nickerson SC. (2001). Choosing the Best Teat Dip for Mastitis Control and Milk Quality. National Mastitis Council. Retrieved November 18, 2014 from <http://www.nmcoline.org/articles/teatdip.htm>.

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

Orlova E.V, (2012). Bacteriophages and Their Structural Organisation. In Ipek Kurtböke, Bacteriophages. Published by InTech Janeza Trdine 9, 51000 Rijeka, Croatia pp 3-29.

Plisson C, White, H.E, Auzat I, Zafarani A, São-José C, Lhuillier S, Tavares P and Orlova, E.V. (2007). Structure of bacteriophage SPP1 tail reveals trigger for DNA ejection. *The EMBO Journal*, Vol. 26, No.15, pp. 3720-3728.

Radostits O.M, Blood D.C, Gay C.C, Blood D.C and Hinchkliff K.W, (2000). *Veterinary Medicine*. 9th Edn, ELBS-Bailliere Tindal, London pp: 563-618.

Rakhuba D.V, Kolomiest E.I, Szwajcer Dey E and Novik G.I, (2010). Bacteriophage receptors, mechanism of phage adsorption and penetration into host cell. *Polish Journal of Microbiology*, Vol. 59, No 3, pp 145-155.

Shaikh S, Fatima J, Shakil S, Rizvi S.D, Kamal M. A, (2015). Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi Journal of Biological Sciences* 22, 90–101.

Shende R. K, Hirpurkar S. D, Sannat C, Rawat N, Pandey V, (2017). Isolation and characterization of bacteriophages with lytic activity against common bacterial pathogens, *Veterinary World*, 10(8): 973-978.

Sillankorva S. M, Oliveira H and Azeredo J, (2012). Review Article Bacteriophages and Their Role in Food Safety. *International Journal of Microbiology*; Volume 2012, Article ID 863945, 13.

Sulakvelidze A, Alavidze Z and Glenn Morris JR. J, (2001). Bacteriophage Therapy (minireview). *Antimicrobial agents and Chemotherapy*. Vol. 45, No. 3, p. 649–659.

Swatantra S, Shukla S, Tandia N, Kumar N and Paliwal R, (2014). Antibiotics residues: A global challenge. *Pharma Science Monitor* 5(3), 184-197.

Weber-Dabrowska B, Mulczyk M and Gorski A, (2000). Bacteriophage Therapy of Bacterial Infections: an Update of Our Institute's Experience (Review). *Archivum Immunologiae et Therapiae Experimentalis*, 48, 547–551.

White D. G and McDermott P. F, (2001). Emergence and transfer of antibiotic resistance. *Journal of Dairy Science* 84 (E. Suppl.), E151±E5.

Wommack K.E and Colwell R.R, (2000). Virioplankton, pp. viruses in aquatic ecosystems. *Microbiology and Molecular Biology Reviews*, Vol. 64, pp. 69–114.

Van Twest R and Kropinski A.M, (2009). Bacteriophage Enrichment from Water and Soil. In: Clokie M.R., Kropinski A.M. (eds) *Bacteriophages, Methods and*

Protocols, Volume 1: Isolation, Characterization, and Interactions. Methods in Molecular Biology™, vol 501. Humana Press, pp15-21.

CHAPTER 5

GENERAL DISCUSSION, CONCLUSION AND FUTURE WORK

The diversity of bacterial species found in this study were similar and wide, mostly were species commonly associated with mastitis cases, representing species of *S. aureus* and CNS spp. (*S. chromogenes*, *S. haemolyticus*, *S. xylois*, *S. epidermis*, *S. hominis*, *S. hycus*, *S. capitis*, *S. sciuri*), *Str. dysgalactiae*, *Str. uberis*, *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., *Enterococcus* spp., *Enterobacteriaceae* spp., *Proteus* spp., *Citrobacter* spp., *Bacillus* spp., *B. pumilus* and less common species like *Acinetobacter* spp., *Lactococcus lactis* and *Pasteurella* spp. *S. chromogenes*, *S. aureus*, *Str. dysgalactiae*, *E. coli*, *Klebsiella* spp. and *Bacillus* spp were the dominant species found on the farms. Based on this study and previous studies, results conducted in the country for the past 10 years indicated on CNS spp. to be still the most dominant pathogens causing mastitis on dairy farms, followed by *S. aureus* and *Streptococcus* spp.

Most strains were resistant to polymyxin B (53%), penicillin (53%), ampicillin (41%), bacitracin (41%) and novobiocin (41%) and intermediate resistance to neomycin (47%). Most studies reported the highest resistance to penicillin and related antibiotics, but in this study less resistance was found. It was surprising to detect that the highest sensitivity was present against aminoglycosides (streptomycin 82% and kanamycin 82%), and tetracycline (65%). For teat dip disinfectants analysed, results indicated they were all effective in inhibiting growth of all our mastitis associated causative strains tested; they had 100% lethal effect and no resistance was exhibited. However, for milking equipment, the disinfectant Perosan acid sanitizer resistance was observed for Gram-negative strains. Resistance to Gram-negative strains was not surprising, as they are generally more resistant than Gram-positive strains.

In total 12 lytic phages for mastitis associated pathogens *S. aureus*, *Coagulase-negative Staphylococcus*, *Streptococcus* spp., *E. coli* were successfully isolated from cow manure and their morphology characteristics showed that they belong to the *Myoviridae* family. Most phages showed a broad host range and were able to lyse other strains from the same species and strains from other species

Mastitis is caused by a wide variety of different bacterial species, which makes it difficult to control, but for decade's antimicrobials have been successful in controlling it until the major pathogens developed resistance. Even though the conditions are not yet life treating, it is important to develop alternative treatments and bacteriophages might provide a promising alternative.

Future research

Future research must focus on studying growth parameters (optimal replication temperature, adsorption and binding rates, latent period and burst size) of these phages and their storage conditions. All phages must be characterized based on molecular studies (genome size and gene sequence) and applied in *in vivo* studies to explore their potential as an alternative method in controlling mastitis.