



Molecular characterization of selected pathogens in milk

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DECLARATION

I, Mafisa Mosidi Elizabeth, declare that the dissertation entitled “**Molecular characterization of selected pathogens in milk**”, submitted for the degree of Master of Science in Biology (Molecular Microbiology) at the Mafikeng Campus of the North-West University is my work and design. I also declare that this dissertation has not been submitted for any other degree or is being considered for examination in any other university. All materials used and quoted herein have been duly acknowledged.

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DEDICATION

I dedicate this project to my beloved Mother (Agnes Mafisa), to my family, friends and my pastor (Apostle Abisai Ditsele) and my church (Elim Terbanacle).

“Train up a child in the way
She should go: and when she is old
She will not depart from it”

Proverbs 22:6

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ABSTRACT

Milk is a nutrient-rich liquid produced by the mammary glands of mammals including cow. It is an excellent source of vitamins, minerals and nutrients and it thus consumed by humans. The composition of milk makes it an excellent growth medium for microorganisms and thus very prone to microbial and chemical contamination. Contamination of affects its quality and this frequently occurs when standard operating procedures and farm management techniques during the production and processing procedures are not strictly enforced. The consumption of contaminated milk has been associated with a variety of health complications. The aim of this study was to isolate and characterize selected pathogens (*S. aureus*, *S. agalactiae*, and *E. coli*) in milk using phenotypic and molecular methods (PCR). One hundred and two milk samples comprising 24 raw, 37 bulk tank and 41 pasteurized milk were randomly collected from supermarkets, shops as well as both commercial and communal farms in the North-West province of South Africa in winter and spring, 2018. Target pathogens *S. aureus*, *S. agalactiae*, and *E. coli* were isolated using selective media Mannitol Salt agar (MSA) plates, Columbia agar supplemented with 5% (v/v) sheep blood and Eosin Methylene Blue agar (EMBA), respectively. Based on colonial morphologies, 62 presumptive isolates comprising 21 *S. aureus* (yellow colonies), 20 *S. agalactiae* (golden yellow) and 21 *E. coli* (metallic sheen colonies) were obtained. The presumptive isolates were subjected preliminary identification test for *E. coli* (oxidase and the triple sugar iron agar test), *S. agalactiae* and *S. aureus* serological assays, respectively. The haemolytic activity of presumptive *S. aureus* and *S. agalactiae* was determined using ox-blood agar supplemented with 5% (w/v) bovine blood. *S. aureus* (ATCC® 29213), *E. coli* (ATCC® 25922) and *S. agalactiae* (ATCC® 13813) were used as positive control strains while *K. pneumonia* (ATCC® 13883) was used as a negative control strain. Genomic DNA was

extracted from 62 presumptive isolates and bacterial 16S rRNA gene was amplified as an internal control. The identities of potential *S. aureus*, *S. agalactiae* and *E. coli* was confirmed through PCR amplification of the *nuc*, *VI* and *C1* and the *uidA* gene fragments respectively. The antimicrobial resistance profiles of all confirmed *S. aureus*, *S. agalactiae* and *E. coli* isolates was determined using the disc diffusion technique. PCR targeting classical *S. aureus* virulence genes: *sea*, *seb*, *sec*, *sta* and *Etb* genes was used to determine the clinical significance of the isolates. All *E. coli* isolates were screened for the presence virulence genes (*stx*₁, *stx*₂ and *hlyA*) using specific primers. In addition, all the 9 confirmed *S. agalactiae* isolates were screened by multiplex PCR analysis for the presence of the *Cluster PI-1*, *hylB*, *fbsA*, *cps C, D, E*, *cpsJ, K*, *eneuB* virulence genes. All the potential *S. aureus*, *S. agalactiae* and *E. coli* isolates were Gram-positive cocci appearing in clusters, Gram-positive cocci appearing in chains (presumptively) and Gram-negative rod-shaped cells, respectively. Large proportions (76.2%) of the presumptive *E. coli* isolates were oxidase positive. All the 62 presumptive isolates harboured the bacterial 16S rRNA PCR gene fragments. Despite the fact that only nine (45%) of the presumptive *S. agalactiae* were positive for the *C1* gene, large proportions (18/21; 85.7%) and (14/21; 66.7%) of the presumptive *S. aureus* and *E. coli* isolates harboured the *nuc* and *uidA* genes respectively.

Virulence gene analysis of the 18 *S. aureus* isolates indicated that 6 (33.3%) were positive for *seb* virulence gene while only 3 (16.7%) of these isolates harboured the *sec* gene. The exfoliative toxin b (*etb*) was detected in 5 (27.8%) of the *S. aureus* isolates. PCR analysis revealed that *S. agalactiae* isolates only 2 (22.2%) were positive for *cpsJ, K* and *eneuB* virulence genes. A large proportion (12; 86%) of *E. coli* isolates harboured the three STEC virulence genes assessed in this study. Generally, the majority (10; 71%) possessing the *hlyA* gene. The *stx*₂ gene was also

detected in 8 (57.1%) of the isolates. On the contrary, only four *E. coli* isolates were positive for the *stx₁* gene. Phenotypic determination of the virulence potentials (haemolytic patterns on blood agar) of *S. aureus* and *S. agalactiae* isolates revealed that 7 (38.89%) and 6 (66.6%) isolates respectively displayed beta (β) haemolytic patterns.

All the 41 PCR confirmed isolates comprising 18 *S. aureus*, 9 *S. agalactiae* and 14 *E. coli* isolates were subjected to the disc diffusion antibiotic susceptibility test in order to determine their drug resistant profiles against a panel of 8 antimicrobial agents. Results indicated that all (100%) of the *S. aureus* isolates from milk samples were resistant to Cefotaxime. Large proportions of the *S. aureus* isolates from tank milk in Mafikeng were resistant to Ertapenem (80.95%), Piperacillin (76.19%) and Ticarcillin (47.62). In addition, small proportions of isolates from pasteurized milk in Zeerust were resistant to Cefepime (33.33%) and Amoxicillin (19.05%). On the contrary, none of the *S. aureus* isolates was resistant to Aztreonam and Ceftazidime. Similarly, all (100%) the nine PCR confirmed *S. agalactiae* isolates were resistant to multiple antibiotics. Moreover, drug resistance was frequently observed against Cefotaxime, Ceftazidime, Amoxicillin, Cefepime when compared to Ertapenem and Ticarcillin. A large proportion (100%) of the isolates from tank milk in Klerksdorp were resistant to Cefotaxime. Large proportions of *S. agalactiae* from pasteurized milk from Coligny were resistant to Ceftazidime (80%), Amoxicillin (60%) and of Cefepime (55%). On the contrary, small proportion of isolates of tank milk in Rustenburg were resistant to [of Ertapenem (45%) and Ticarcillin (35%). Despite this, none of these isolates was resistant to Piperacillin and Aztreonam. Large proportions of *E. coli* isolates of raw milk from Rooigrond were resistant toof Piperacillin (71.43%), Ticarcillin (61.90%) and Amoxicillin (57.14%). On the contrary, small

proportions 23.81%) of the isolates from raw milk from Lichtenburg were resistant to Cefepime and Ertapenem respectively. All the *E. coli* isolates in this study were susceptible to Aztreonam and Ceftazidime. From the findings of this study, it is evident that these multi-drug resistant and virulent isolates may cause infections in humans when consumed in contaminated milk. Moreover, infections caused by the isolates may be difficult to treat using these antibiotics and thus amplified the need to constantly search for alternative antibacterial agents.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

The following abbreviations, acronyms and symbols have been used in this dissertation following the style recommended by the American Society for Microbiology for Journals.

AR : Antibiotic resistance genes

BTM : Bulk tank milk

BTSCC : Bulk tank somatic cell counts

BMSCC : Bulk milk SCC

CM : Clinical mastitis

CMT : California mastitis test

E. coli : *Escherichia coli*

IMI : Indicator of intramammary infections

IMM : Intramammary

IRCM : Incidence rate of clinical mastitis

MSA : Mannitol salt agar

NAP : Nutrient agar plates

NMC	: National mastitis council
PFGE	: Pulsed field gel electrophoresis
rRNA	: Ribosomal Ribonucleotide acid
SCC	: Somatic cell counts
SCM	: Subclinical mastitis
WHO	: World health organization

DEFINITION OF CONCEPTS

Antibiotic: A drug that is capable of destroying or weakening microorganisms, especially bacteria or fungi, which cause infection or infectious disease.

Antibiotic resistance: An ability of a given bacteria to survive the exposure to a defined concentration of an antimicrobial agent.

Antimicrobial: Agents that are a capable of destroying or inhibiting the growth of microorganisms and especially pathogenic microorganisms.

California mastitis test: Is a diagnostic tool to aid in the quick diagnosis of mastitis in dairy cows, and for an udder health management program.

Clinical mastitis: Characterized mainly by appearances of changes in the milk such as flakes and clots and presence of signs of inflammation on the mammary glands such as swelling, heat, pain, and oedema.

Enterobacteriaceae: Are gram-negative, rod shaped bacilli, non-spore forming and facultative anaerobic bacteria.

***Escherichia coli*:** Gram-negative, facultative anaerobic, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms.

Genes: Portion of DNA molecule that serves as the basic unit of heredity.

Inflammation: Reaction that produces redness, warmth, swelling and pain as result of infection.

Lactation: Secretion or formation of milk from the mammary gland.

Mammary gland: Exocrine gland in mammals that produces milk to feed young offspring.

Mastitis: Inflammation of the mammary gland and udder tissues, and is a major endemic disease of dairy cattle.

Microbiome: Assemblage of microorganisms associated with specific habitat or niche.

Primer: Is a strand of short nucleic acid sequences (generally about 10 base pairs) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA.

Pathogen: Biological agent that causes disease or illness to its host.

Species: Collection of bacterial cells, which share an overall similar pattern of traits in contrast other bacteria whose pattern differ significantly.

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 GENERAL INTRODUCTION

Milk is a nutrient-rich liquid food produced by the mammary glands of mammals. It is the primary source of nutrition for young mammals, including breastfed human infants before they are able to digest solid food (Van Winckel *et al.*, 2011). As an agricultural product, dairy milk is collected from farm animals with dairy farms producing approximately 730 million tonnes (800 million short tons) of milk a year (Food Outlook, 2012) (World Dairy Cow Numbers, 2014). Throughout the world, more than six billion people consume milk and milk products (Hemme and Otte, 2010). The ever-increasing rise in domestic demand for dairy products and a large demand-supply gap could lead to poor farm management practices and lack of adherence to standard operating procedures thus resulting in a zoonotic transfer of bacterial contaminants in milk to consumers.

The composition as well as the level of microbial and chemical contamination in milk determines its quality. Milk is largely made up of water, and contains a wide range of nutrients including vitamins, proteins, fats and carbohydrates (Gudeta, 1987; Godefay and Molla, 2000). Milk therefore serves as an excellent culture medium for the growth and multiplication of different types of microorganisms due to its complex biochemical composition and high water activity (Soomro *et al.*, 2002). However, despite its very rich nutritional contents milk may serve as a source for the transmission of pathogenic microbes that could cause diseases in humans (Garedew *et al.*, 2012). This is mainly associated with the quality of milk that is also dependent

largely on adherence to the standard operating procedures and farm management techniques during the production and processing procedures (Garedew *et al.*, 2012).

Mastitis in cow is an inflammation of the mammary gland caused by bacterial infections of the udder (Ferrero *et al.*, 2014). Mastitis can occur in either clinical or subclinical forms (Karimuribo *et al.*, 2008). Clinical mastitis is characterised by changes in the udder such as udder swelling or redness and are visible to the naked eye whereas the subclinical disease is characterised by an increase in the number of somatic cells in the milk, and the absence of clinical signs (Karimuribo *et al.*, 2008). Subclinical mastitis in dairy cattle can result to reduced milk production with economic consequences for the farmer (Bhatt *et al.*, 2012). In some cases of clinical mastitis, *E. coli* is observed in the milk of lactating animals is as a result of infection that may have occurred during the dry period, implying that dry periods rather than the peak of lactation can be a critical time for control of *E. coli* mastitis (Bradley and Green, 2001).

Pathogens associated with mastitis have been divided into contagious and environmental organisms based on their ability to cause persistent or transient opportunistic infections (Bradley and Green, 2001). Contagious organisms are capable of adapting to different ecological conditions within their hosts and in particular changes within the mammary gland of animals (Bradley, 2002). However, environmental pathogens of mastitis are mostly found in the environment and are capable of invading the mammary gland when proper farm management and hygiene practices are not adhered to (Kurjogi and Kaliwal, 2014). *S. aureus* and *S. agalactiae* are classified as contagious pathogens of mastitis with the milk from infected mammary glands serving as the primary reservoir of these bacteria thus can be transferred easily

to other animals within the herd during milking (Keefe, 1997; Capurro *et al.*, 2010; Elias *et al.*, 2012). On the contrary, *E. coli* is one of the main pathogens associated with environmental mastitis leading to a wide range of diseases whose severity range from acute to severe systemic complications (Günther *et al.*, 2011; Shome *et al.*, 2011). Pathogens that are associated with environmental mastitis are found in bulk milk may also be derived from non-specific contamination resulting from contact with cow skin, bedding, manure, or water (Jayarao *et al.*, 2004; Lam *et al.*, 2009). *S. agalactiae* is a contagious obligate pathogen of the mammary gland of bovine and which primarily causes subclinical mastitis (Mweu *et al.*, 2012) while *S. aureus* is regarded as important pathogenic bacteria in bovine mastitis since it is often isolated from milk of animals that present with acute to chronic cases of mastitis (Katsuda *et al.*, 2006). The complexity of the mastitis due to the different pathogens associated with the disease, the degree of economic losses and the increasing importance of the dairy industries along with public health concerns amplifies the need for constant surveillance using appropriate pathogen detection techniques that can offer rapid and accurate results.

Risk factors of mastitis comprise quarter-specific risk factors such as teat position, distance from teat to floor, presence of previous hyperkeratosis and bacterial infection that are responsible for the difference in the occurrence of mastitis in different quarters within the same animal (Neijenhuis *et al.*, 2001; Green *et al.*, 2007; Leelahapongsathon *et al.*, 2014). Cow-specific risk factors such as parity, month of lactation, season of the year, somatic cell count (SCC) in previous lactation and history of mastitis account for differences in the incidence of California mastitis among cows. Inadequate suction pressure is responsible for differences in the incidence of California mastitis among dairy farms and this has direct link to lack in the implementation of

proper farm management techniques (Barkema *et al.*, 1999; Peeler *et al.*, 2000; Nyman *et al.*, 2007). Despite the wide spectrum of bacterial species that are known to cause mastitis, only a few species notably; *S. aureus*, *S. agalactiae* and *E. coli* are of economic and epidemiological importance (Zecconi *et al.*, 2003; Taverna *et al.*, 2007; Shome *et al.*, 2011). Moreover, a study by Petzer *et al.*, (2009) revealed that mastitis did not decrease in South Africa after analysis of milk samples obtained from South African dairy herds during the period 1996 to April 2007. This study also reported that although *S. aureus* remains the principal pathogen of mastitis in South Africa, owing to its chronic nature and resultant economic losses, *S. agalactiae* was also highly prevalent among the samples (Petzer *et al.*, 2009). This therefore explains why this study focuses on these pathogens. This study aimed to determine the antibiotic resistance and virulence gene profiles of *S. aureus*, *S. agalactiae* and *E. coli* isolates from pasteurised and unpasteurised milk samples. Data generated may be of great epidemiological importance.

1.2 PROBLEM STATEMENT

Bacteria pathogens, *S. aureus*, *S. agalactiae* and *E. coli* especially strains that are resistant to antibiotics are generally associated to mastitis worldwide (Pitkälä *et al.*, 2004; Olsen *et al.*, 2006; Hendriksen *et al.*, 2008). Mastitis in cow is an inflammation of the mammary gland caused by bacterial infections of the udder (Ferrero *et al.*, 2014). Mastitis in dairy cattle most often results to reduced milk production with economic consequences for the farmer (Bhatt *et al.*, 2012). The quality of milk may be affected by a numbers of factors such as adulteration, contamination during and after milking and the presence of infections in the udder (Esron *et al.*, 2005). The quality of milk is negatively affected as a result of mastitis and the disease also negatively affects the longevity of dairy cows (Seegers *et al.*, 2003; Halasa *et al.*, 2007).

The high incidence of clinical mastitis (CM) in cattle has often been associated with lack of control programs that are designed based on specific risk factors for a given area (Oliveira *et al.*, 2015). Mastitis causes discomfort to the animal, makes milking difficult, and reduces the yield and quality of milk and increases the rate of culling and veterinary costs (Miles *et al.*, 1992; Calvino, 1999; Bradley, 2002; Seegers *et al.*, 2003; Halasa *et al.*, 2007). Antimicrobial resistance is an increasingly important problem among several bacterial species causing infections in animals and humans in recent years (Aarestrup, 1999; Levy, 2001). The situation is aggravated by the fact that a number of resistant bacteria such penicillin-resistant strains of *S. aureus* (PRSA), methicillin resistant *S. aureus* and Vancomycin resistant *S. aureus* are of great concern both in animal production and human medicine (Østerås *et al.*, 1999b).

Against this background, the implementation of standard mastitis prevention programs may significantly contribute in the eradication of the causative agents such as *S. agalactiae*, *S. aureus* and *E. coli* from cattle farms (Shome *et al.*, 2012). Moreover, due to the effects of globalization, increase in food supply demand due to human population growth and climate change (Nader-Macías *et al.*, 2013) constant monitoring of these pathogens is of prime importance. Aside from the economic losses, mastitis impairs animal welfare, and foodborne diseases may also affect human health (Seegers *et al.*, 2003). This even becomes a more severe public health issue when circulation mastitis pathogens harbour multi-drug resistant determinants (Pitkälä *et al.*, 2004; Olsen *et al.*, 2006; Hendriksen *et al.*, 2008).

1.3 RESEARCH AIM AND OBJECTIVES OF THE STUDY

1.3.1 Aim of the study

The aim of the study was to isolate and characterize antibiotic and pathogenic *S. aureus*, *S. agalactiae* and *E. coli* from milk.

1.3.2 Objectives of the study

The specific objectives of the study were:

- To isolate *S. aureus*, *S. agalactiae* and *E. coli* from milk samples obtained from farms and retail shops.
- To confirm the identities of the isolates using phenotypic and genotypic techniques (specific PCR).
- To determine the antibiotic resistance profiles of the isolates.
- To determine the virulence profiles of the isolates using phenotypic and genotypic assays.

CHAPTER 2

LITERATURE REVIEW

2.1 IMPACT OF MILK CONSUMPTION

2.1.1 Background

Milk is one of the widely consumed products and important source of nutrients for human as well as animals. The South African milk-producing herd was estimated to be approximately 2474 dairy herds in 2012, with an average herd size of 238 and an average production of 20.2 L of milk per cow per day (Milk, 2013). South Africa is a developing country in southern Africa, with a population of 55 million people, the average annual milk consumption has been estimated to be approximately 36 L of milk per capita, which is well below the 200 L per capita annually recommended by the World Health Organization (Lassen, 2012). Milk components take part on human metabolism in several ways, providing essential amino acids, vitamins, minerals and fatty acids, or by affecting absorption of nutrients (Nader-Macías *et al.*, 2013). Consumption of 0.5 litre milk daily supplies a significant amount of many of the nutrients that are required daily (Haug *et al.*, 2007).

2.2 MASTITIS RELATED PATHOGENS

The bacterial pathogens responsible for infection of the mammary gland may be split into two main categories: major and minor pathogens, infection with major pathogens generally results in clinical illness or strong inflammatory responses and reduced milk yields, whereas minor pathogen infection is usually subclinical (White *et al.*, 2006).

Pathogens found both in humans and dairy cattle cause mastitis in dairy cows (Haran *et al.*, 2012). Among the various pathogens causing mastitis, *S. agalactiae* causes mainly subclinical infections which are not identified by the herdsmen and is of particular importance because it is highly infectious (Meiri-Bendek *et al.*, 2002). Contagious pathogens, such as *S. aureus* and *S. agalactiae* generally come from IMI, but due to dilution, latent infections, and intermittent shedding, sensitivity of a single bulk milk BC is low (Lam *et al.*, 2009). *S. aureus* is an important human pathogen and one of the most important causes of bovine mastitis worldwide (Olsen *et al.*, 2006). In most countries, the major mastitis pathogens are *S. aureus*, *S. agalactiae* and *E. coli* which have a considerable impact on cow health, milk quality and productivity (Zadoks and Fitzpatrick, 2009). Classically, the mastitis pathogens have been divided into contagious and environmental organisms, based on their proclivity to cause persistent or transient opportunistic infection (Blowey and Edmondson, 1995). *S. aureus* is the most significant pathogen causing intra-mammary infection (IMI) in dairy (Graber *et al.*, 2007; Kuang *et al.*, 2009).

2.3 ECONOMIC COSTS ASSOCIATED WITH MASTITIS DISEASES

A study was conducted that economic calculation of the mastitis costs or economic implications vary between countries and even between regions within the country (Nader-Macías *et al.*, 2013). Further, it was also reported that the results of these calculations change with time, based on the modifications of the milk quality regulations and market circumstances (Lescourret and Coulon, 1994). Mastitis increases the rate of culling and veterinary costs and causes discomfort to the animal, makes milking difficult, reduces the yield, quality of milk (Miles *et al.*, 1992; Calvinho, 1999; Bradley, 2002; Seegers *et al.*, 2003; Halasa *et al.*, 2007). There are costs associated with production losses, culling, veterinarian intervention, antibiotics and other

therapies, discarded milk, milk quality penalties, and labor (Stevenson and Lean, 1998; Huijps and Hogeveen, 2007; Bar *et al.*, 2008). It has been observed that mastitis discarded milk and costs of treatment and culling and reduces dairy farm profitability, with losses stemming from milk production decreases (Gröhn *et al.*, 2005). In another study, it was established that the dairy producer incurs the cost of these negative outcomes that includes pain, decreased production, culling, and death, through reduced quality and quantity of milk, as well as increased production costs (Rollin *et al.*, 2015). Clinical mastitis has direct economic effects including diagnostic costs, treatment costs, the cost of non-saleable milk, labor and veterinary costs, and death, but CM also has long term health effects that influence future milk production (Wilson *et al.*, 2004; Schukken *et al.*, 2009), future culling risk (Beaudeau *et al.*, 1995; Gröhn *et al.*, 1998), and future reproductive efficiency (Santos *et al.*, 2004).

2.4 RISK FACTORS ASSOCIATED WITH BOVINE MASTITIS

2.4.1 Host or Quarter- level risk factors (cow-level) variables

Examples such as breed (Heringstad *et al.*, 2000), milk production level (Schukken *et al.*, 1990), and nutrition (Hogan *et al.*, 1993) are risk factors related to host resistance. Vitamin E and Se are essential nutrients that share common biological activities (Hogan *et al.*, 1993). Nutritional food that are considered as milk are rich in carbohydrate, proteins, fats, vitamins and minerals (Mekibib *et al.*, 2010). A study was conducted that increase milk production cross breeding of indigenous zebu with exotic breeds particularly with Holsteins is widely practiced, which resulted in a larger portion of the dairy cattle population specially in urban areas to be with a high level of exotic blood (Almaw *et al.*, 2008).

The incriminated biological causes include the low genetic potential of the animals, poor nutrition and prevalence of diseases (Lakew *et al.*, 2009). The biological efficient system common that cause for antibacterial use is a dairy production in lactating dairy that converts feed and roughages to milk (Tadesse and Chanie, 2012). In an another study, it was established that research works showed that on the average of the affected quarter suffers a 30% reduction in productivity and affected cow a 15% loss its production (Radostits *et al.*, 2007). A study was conducted that, to increase milk production cross breeding of indigenous zebu with exotic breeds particularly with Holstein Friesian is widely practiced which resulted in a larger portion of the dairy cattle population especially in urban areas to be with a high level of exotic blood (Mekonnin *et al.*, 2016).

A study was conducted with 500 lactating buffaloes of different age, parities and stage of lactation belonging to different organized or un-organized dairy farms in, Chhattisgarh State, India and found that the higher prevalence of Sub-Clinical Mastitis (SCM) in buffaloes was recorded in 5 to 9 years old animals and in 3rd and 4th parities (Sharma *et al.*, 2007). A study suggests that a longer dry period (>40 days) increased the risk of clinical mastitis (Peeler *et al.*, 2000). The influence of an irregular interval between morning and evening milking (<12 or >12 hours/day) on the prevalence of mastitis may have been the consequence ends and streak canals during the longer milking intervals. Risk factors that affect health status of mammary gland resulting in bovine mastitis are shown in Figure 2.1.

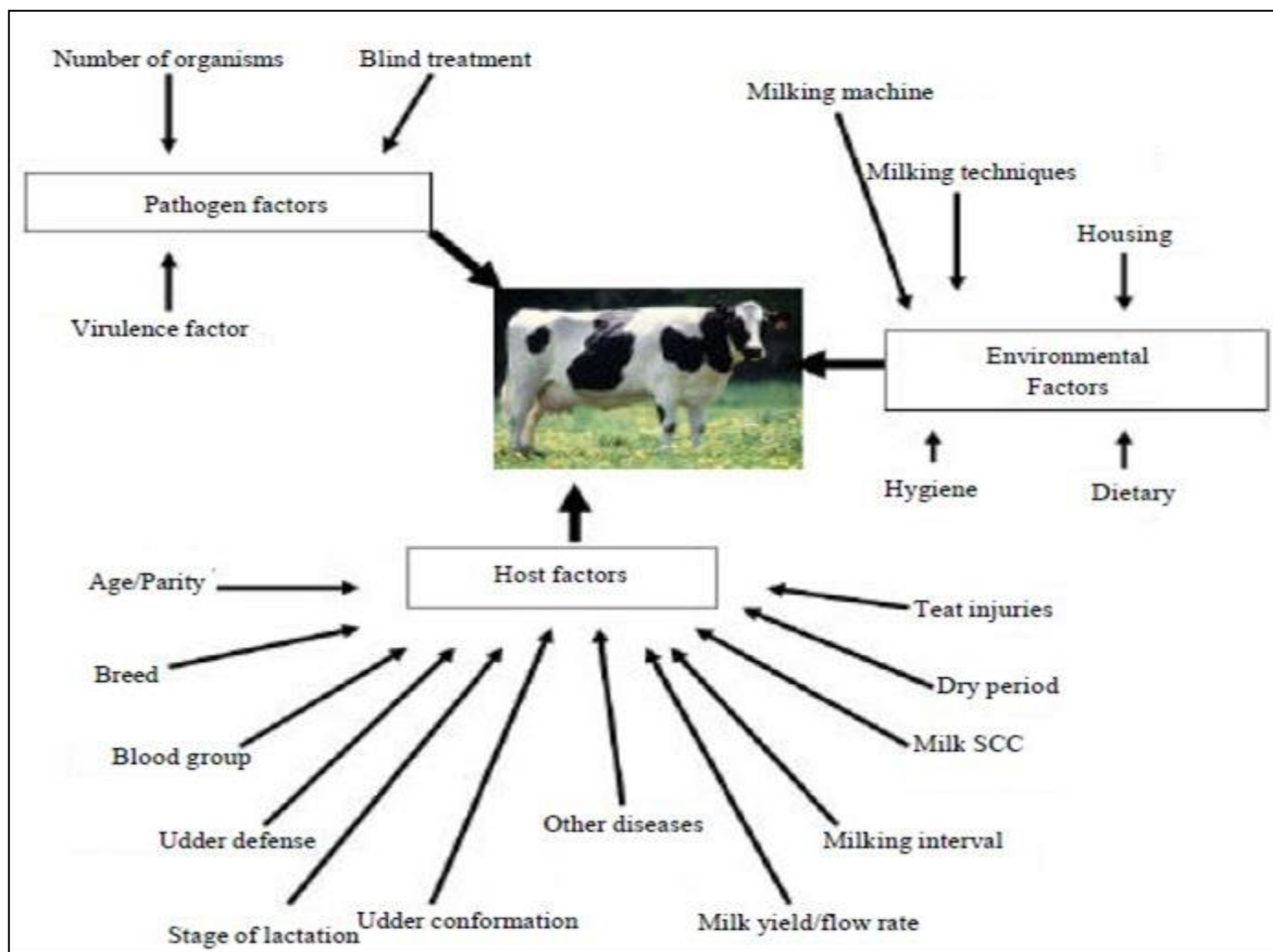


Figure 2.1: Risk factors that affect health status of the mammary gland resulting from bovine mastitis (Sharma *et al.*, 2012).

2.4.2. Farm based risk factors (farm-level) variables

A complex and costly disease which known as mastitis, of dairy cows, that results from microorganism and the interaction of the cow and environment including milking machine (Hawari and Al-Dabbas, 2008). On farms with conventional milking systems (CMS), risk factors within the first category are, associated with hygiene of cow, milking machine, and housing (Schukken *et al.*, 1990; Schreiner and Ruegg, 2003; Breen *et al.*, 2009), milking procedures

(Barkema *et al.*, 1999), post milking teat disinfection (Lam *et al.*, 1997; Santman-Berends *et al.*, 2016), and milk leakage (Klaas *et al.*, 2005). Risk factors for mastitis at farm level included no dry cow therapy and poor milking management such as failure to teat dip, use of common cloths for udder preparation, improper milking machine function, (Dargent-Molina *et al.*, 1988; Mungube *et al.*, 2004).

Various environmental factors in the occurrence of mastitis include housing system, climate, season, heat stress, milking hygiene, udder hygiene, milking technique and milking machine (Sharma *et al.*, 2012). Heat stress can lead to a higher udder temperature which may affect the udder leading to mastitis (Igono *et al.*, 1988). Heat stress can weaken immune system of the cattle, which will eventually facilitate the mastitogenic udder infection (Giesecke, 1985). Figure 2.2 outlines the impact of heat stress on milk production in dairy cattle affected with mastitis.

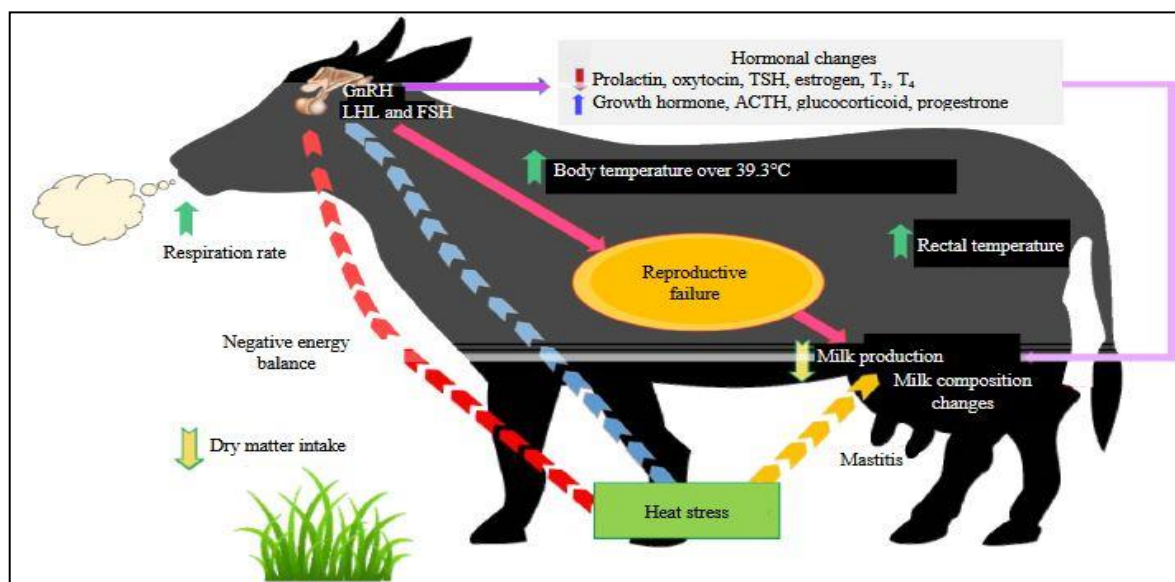


Figure 2.2: The impact of heat stress on milk production in dairy cattle as reported by Pragna *et al.*, (2017).

2.5 RISK FACTORS ASSOCIATED WITH CLINICAL (ENVIRONMENTAL) MASTITIS OF *ESCHERICHIA COLI*

2.5.1 Quarter- level risk factors (cow-level) variables

Gram-negative mastitis caused by *E. coli* is generally transient and disease outcome largely depends on host factors, for example, lactation stage (Burvenich *et al.*, 2003). Cow characteristics that influence the susceptibility to mastitis include parity, stage of lactation, and genetic make-up (Barkema *et al.*, 1998; Schukken *et al.*, 1999; Busato *et al.*, 2000). Previous bacterial infection, teat position, and teat end hyperkeratosis have been reported as quarter-level risk factors for mastitis (Zadoks *et al.*, 2001; Green *et al.*, 2002). A great number of individual cow-specific risk factors for CM have been identified, including breed, parity, period of lactation, udder and teat morphology, age at first calving, milk leakage, udder oedema, milk production, number of milk somatic cells and reproductive disorders (Peeler *et al.*, 2000; Nyman *et al.*, 2007).

Risk indicators for IRCM can be categorized into three groups of factors: 1) resistance of the cow to IMI, 2) exposure to pathogens, and 3) cure of IMI or inflammation (Barkema *et al.*, 1999). Previous bacterial infection, teat position, and teat end hyperkeratosis have been reported as quarter-level risk factors for mastitis (Zadoks *et al.*, 2001; Green *et al.*, 2002). Cow-level risk factors for CM include breed (Elbers *et al.*, 1998), lactation number (Zadoks *et al.*, 2001), leaking milk between milking's (Schukken *et al.*, 1990; Elbers *et al.*, 1998; O'Reilly *et al.*, 2006), per parturient disease (Peeler *et al.*, 1994), and cow cleanliness (Ward *et al.*, 2002).

Management practices and risk indicators that are associated with IRCM and that have been reported to be related to resistance to IMI are nutrition (Schukken *et al.*, 1990), milk production (Schukken *et al.*, 1990), leaking of milk (Schukken *et al.*, 1990), breed of cows (Schukken *et al.*, 1990), and post milking teat disinfection (Lam *et al.*, 1997). Most risk factors at the cow or quarter level have been identified in cross-sectional or experimental studies, or in studies on clinical mastitis (Zadoks *et al.*, 2001). Cow-level risk factors for mastitis include breed, lactation number, leaking milk between milkings, and periparturient disease (Zadoks *et al.*, 2001; Steeneveld *et al.*, 2008; Breen *et al.*, 2009).

2.5.2. Farm based risk factors (farm-level) variables

Intramammary infections that are present during the dry period can be partitioned into those that were carried into the dry period from the previous lactation, existing infections, and those that enter between the time of drying off and calving new infections (Bradley and Green, 2004). The probability of a new dry-period IMI is influenced by the rate of exposure to potential pathogens (e.g., from the environment), factors that affect an individual cow's susceptibility to infection and the effectiveness of protection from medical interventions such as antibiotic dry-cow therapy or teat sealants (Dingwell *et al.*, 2004). Agent-specific risk factors for clinical mastitis associated with environment factors like housing, but also to individual cow factors, have been identified in other countries (Barkema *et al.*, 1999; Riekerink *et al.*, 2008). Any economic benefits of dry-cow therapy would, however, arise from better milk price because of lower somatic cell counts (SCC), lower clinical treatment costs, lower livestock replacement costs, and more efficient production (Østerås and Sandvik, 1996).

2.6 RISK FACTORS ASSOCIATED WITH SUBCLINICAL (CONTAGIOUS) MASTITIS OF *Staphylococcus aureus*

2.6.1 Quarter-level risk factor (cow-level) variables

Risk factors for bacteriological cure of *S. aureus* infections following antibiotic treatment were reported including age, number of infected quarters, quarter location, stage of lactation, and severity of the inflammation (Sol *et al.*, 1997). High prevalence of *S. aureus* (IMI) in dairy herds worldwide and the negative impact of this pathogen on the dairy industry, measuring its effects on milk production and composition at cow and quarter levels would help the dairy industry establish control measures to avoid losses both in milk yield and quality of dairy products due to the pathogen. Cow characteristics that influence the susceptibility to mastitis include parity, stage of lactation, and genetic make-up (Barkema *et al.*, 1998; Schukken *et al.*, 1999; Busato *et al.*, 2000).

2.6.2 Farm based risk factors (farm level)

An approach using a blanket dry cow therapy program is still preferred until better diagnostic tests and data management methods become available (Radostits *et al.*, 1994). Norway is the only Nordic country in which there has long been a tradition of using lactation preparations in dry cow therapy, and, as a consequence, no dry cow preparations have, to date, been officially approved (Østerås *et al.*, 1999b). The historical argument against the dry cow therapy has been that there is too little penicillin to achieve therapeutic dose levels, and the consequence of having the antibiotics available over a long time is a risk of developing resistant strains of mastitis bacteria, particularly *S. aureus*. Inselective dry cow therapy, cows that are not expected to be cured after therapy could be recommended for slaughter without treatment, and cows expected to

self cure could also be left without antibiotic therapy (Østerås *et al.*, 1999a). Most veterinarians practice selective dry cow therapy on quarter base in order to minimise the use of antibiotics. It is commonly believed that in order to establish a rational selective dry cow therapy it is essential to know what cow and quarters to treat (Østerås *et al.*, 1991). The intensity of bacterial shedding was negatively associated with *S. aureus* cure following antibiotic treatment at dry-off (Dingwell *et al.*, 2003).

2.7 IMPROVEMENT OF MASTITIS MANAGEMENT

Cleaning and renewing the bedding of the cubicles and yards should ideally be carried out during milking, so that as cows exit from the parlour they are able to walk back along clean passage ways, past fresh food, and then lie down in clean cubicles. If the management of mastitis is improved, there is a reduction in the incidence of clinical mastitis (Mungube *et al.*, 2004). As with most infectious diseases, mastitis risk factors depend on three component: exposure to microbes, cow defence mechanisms, and environmental and management factors (Suriyasathaporn *et al.*, 2000). In addition to the presence of bacteria, dairy herd management and cow or quarter characteristics that increase the risk of infection can be identified in risk factor studies to develop preventive measures to control spread of disease (Zadoks *et al.*, 2001). Management is considered to be one of the most effective means to control mastitis (Seegers *et al.*, 2003). Management strategies, such as teat disinfection, milking technique, and segregation of infected cows, affect the incidence of IMI (Lam *et al.*, 1996). Culling and antibiotic therapy are methods used to shorten the duration of IMI in cows infected with *S. aureus*, which are important component of a program for mastitis management (Sol *et al.*, 1997). Although antimicrobial therapy improves animal health and well-being, the economic losses associated

with additional labour costs and discarded milk are significant (Erskine *et al.*, 2003). The prevalence of mastitis and its associated pathogens in animals can be reduced by improving on the farm management techniques within the dairy industry (Pitkälä *et al.*, 2004). Shortening the duration of IMI is an important component of a program for mastitis management (Sol *et al.*, 1997).

Treatment of infected quarters during lactation may become more important to shorten the duration of IMI and to have an immediate impact on BMSCC. In addition, most smallholder dairy farms have their own styles of farm management, for example, farm sizes of 15–20 milking cows, bathing cows before milking, individuals taking care of their milking cows, whole year tiled stall barn, open-wall barn type, farmers' poverty, no culling plan for mastitis cows, and using the bucket-type milking machine.

2.8 PATHOGENIC BACTERIA (*ESCHERICHIA COLI*) IN RAW MILK

Raw milk (RM) may cause food-borne diseases which contains microorganisms (Adesiyun *et al.*, 1995). *E. coli* as indicate that other microorganisms of faecal origin and index of recent faecal contamination may be present (Cardoso *et al.*, 2001). *E. coli* making microorganisms useful indicators of faecal contamination and are excreted by faeces, however, many strains (intestinal but also extra intestinal pathogens) can cause human diseases (Zago *et al.*, 2007). The possibility of *E. coli* disseminating into raw milk meant for human consumption is normally high due to the presence of animal faeces in a milking environment, unclean dairy parlour environment or equipment as well as unhygienic farm workers (Van Kessel *et al.*, 2011). the dissemination of faeces into milk accompanied by improper hygiene in the farm are considered the major reasons

for the presence of *E. coli* in such milk being produced (Guh *et al.*, 2010). Raw milk may also get contaminated through contact with manure, and bedding soiled with pathogens (Connor and Charles, (1995).

2.9 *Escherichia coli* SPECIES ASSOCIATED WITH CLINICAL (ENVIRONMENTAL) MASTITIS

Whole genome comparison of *E. coli* strains associated with different disease syndromes may reveal genes that are specific to mastitis associated *E. coli* (Zadoks and Fitzpatrick, 2009). Some cases of clinical *E. coli* mastitis that are observed in lactation are the results of infection that took place in the dry period, implying that the dry period rather than peak lactation can be a critical time for control of *E. coli* mastitis (Bradley and Green, 2001). Numerous studies have been conducted to identify virulence factors of *E. coli* isolated from cows with clinical mastitis (Kaipainen *et al.*, 2002). *E. coli* is one of the main pathogens associated with environmental mastitis leading to a wide range of disease severity from acute to severe systemic disease (Günther *et al.*, 2011; Shome *et al.*, 2011).

2.10 *STAPHYLOCOCCUS AUREUS* SPECIES ASSOCIATED WITH SUBCLINICAL (CONTAGIOUS) MASTITIS

S. aureus cause a variety of diseases in their hosts and are facultative pathogenic organisms in both animals and humans (Bissong and Ateba, 2020). *S. aureus* is a contagious pathogen that causes mastitis in dairy cattle (Cremonesi *et al.*, 2015; Boss *et al.*, 2016), and is an opportunistic pathogen in humans and many other animal species (Nübel *et al.*, 2011; Sangvik *et al.*, 2011; Zadoks *et al.*, 2011). Contaminated milk and milk products have frequently been implicated in

staphylococcal food poisoning (De Buyser *et al.*, 2001). Staphylococci are the bacteria most commonly isolated from cases of subclinical mastitis (Taponen and Pyörälä, 2009). Among the mastitis pathogens, *S. aureus* is considered an agent of major concern due to the low cure rate of *S. aureus* infections by antibiotic treatment and its ability to persist in a herd in the form of undetected, subclinical infections (Gill *et al.*, 2006). Furthermore, *S. aureus* is a contagious pathogenic bacterium that resists elimination by antibiotic therapy and persists for long periods without overt symptoms (Katsuda *et al.*, 2006). *S. aureus* is the cause of a variety of infections in both humans and animals (Mørk *et al.*, 2005). *S. aureus* is an important human pathogen and one of the most important causes of bovine mastitis worldwide (Olsen *et al.*, 2006).

S. aureus is a major pathogen of the mammary gland and a common cause of bovine, ovine, and caprine mastitis (Bergonier *et al.*, 2003). *S. aureus* isolates produce several virulence factors including surface associated secretory products, leukotoxins and enterotoxins (Dego *et al.*, 2002; Peacock *et al.*, 2002), representing potential target for vaccine development (Zecconi *et al.*, 2006). Recurrent and persistent mammary infections by *S. aureus* pathogens are also a serious problem for women, owing to their eventual severity and difficulties in curing due to the antibiotic resistance of the pathogens (Günther *et al.*, 2011). *S. aureus* can produce numerous putative virulence factors that allow adhesion to membranes and resistance to phagocytosis (Hogeveen *et al.*, 2011). *S. aureus* is generally a clonal organism as the populations in dairy herds often consist of groups of genetically related strains (Hogeveen *et al.*, 2011).

2.11 *Streptococcus agalactiae* SPECIES ASSOCIATED WITH SUBCLINICAL (CONTAGIOUS) MASTITIS

S. agalactiae is a major contagious mastitis pathogen and continues to be a major cause of mastitis in dairy cattle and buffaloes (Shome *et al.*, 2012). Herds with contagious mastitis generally have high somatic cell count (SCC) and marked reduction in milk production and quality (Elias *et al.*, 2012). *S. agalactiae* is well known worldwide as a major contagious pathogen causing bovine subclinical mastitis, which may have a substantial impact to the quantity and quality of produced milk (Merl *et al.*, 2003). *S. agalactiae* is a contagious obligate pathogen of the bovine mammary gland, which primarily causes subclinical mastitis (Mweu *et al.*, 2012).

Streptococci are one among the major mastitis pathogens, which have a considerable impact on cow health, milk quality and productivity (Mungube *et al.*, 2004). Among the various pathogens causing mastitis, *S. agalactiae* causes mainly subclinical infections which are not identified by the herdsmen and is of particular importance because it is highly infectious (Meiri-Bendek *et al.*, 2002). *S. agalactiae* is a contagious pathogen of bovine mastitis, and the mammary gland is considered as the main reservoir of these bacteria in dairy herds (Tomazi *et al.*, 2018). *S. agalactiae*, also known as Group B Streptococcus (GBS), following the classification of Lancefield (Lancefield, 1933). This is a highly contagious agent and commonly found in the mammary gland of cattle (Fonseca, 2000; Hillerton *et al.*, 2004).

2.12 OUTBREAK OF MILK CONTAMINATION

In South Africa, based on investigation of routine milk samples, the prevalence of mastitis increased from 8.1% in 2002 to 15.4% in 2006 (Petzer *et al.*, 2009). Mastitis has also been identified as the most common cause of death in adult dairy cows (Esslemont and Kossaibati, 1997). After ingestion of contaminated water and food, including milk and dairy products, *E. coli* was responsible for several outbreaks of diarrhoea in children and adults (Feresu and Nyati, 1990). In Japan, the most recent large scale outbreak occurred during June 2000 was caused by consumption of low-fat milk produced from skim milk powder contaminated with *S. aureus* enterotoxin A (Asao *et al.*, 2003). Raw milk and dairy products made from non-pasteurized milk have been responsible for *S. aureus* and *E. coli* outbreaks and could represent a potential hazard for consumers (Little *et al.*, 2008). *S. aureus* is responsible for various foodborne outbreaks and a gram positive, catalase and coagulase positive microorganism (Abraha *et al.*, 2018). *E. coli* is an important food-borne disease organism and enteropathogenic type which can cause diarrhea, even cause complications, resulting in fatalities (pasteurized milk)-from supermarkets (Anderson *et al.*, 2011). Raw milk and dairy products made from non-pasteurized milk have been responsible for *S. aureus*, *E. coli* outbreaks and could represent a potential hazard for consumers (Shome *et al.*, 2011).

Empirical evidence for the importance of native bacterial populations within the mammary gland, comes from the reports of clinical *E. coli* mastitis outbreak following “blitz” therapy (simultaneous antibacterial treatment of all infected mammary glands within a herd to eliminate *S. agalactiae* infection from a herd (Boyer, 1997). Contaminated milk and milk products have frequently been implicated in staphylococcal food poisoning (Haran *et al.*, 2012). Besides

causing mastitis, the genus *Staphylococcus* contains etiologic agents responsible for approximately 45% of toxin infections worldwide that causes toxic shock syndrome and food poisoning (Balaban and Rasooly, 2000).

2.13 PREVENTION, TREATMENT AND CONTROL OF BOVINE MASTITIS INFECTION

Pharmacodynamics of the antimicrobial is another aspect which should be considered for treatment (Pyörälä, 2009). Penetration of substances into milk when administered parenterally or absorption and distribution throughout the udder when infused intramammarily (IMM) depends on their pharmacokinetic characteristics (Pyörälä, 2009). The efficiency of CM (clinical mastitis) control can be improved by using information about cow-specific risk factors by allowing a farmer to identify the cows that have a higher risk of CM and to subsequently provide a higher level of care for these cows (Oliveira *et al.*, 2015). In addition to cow-specific risk factors, knowledge of the pathogens involved in CM is also useful to improve the efficiency of control measures (Steenefeld *et al.*, 2008). Probiotics administered to the cow for mastitis prevention would be also in contact with the calf during the first days of its life and would stimulate the immature immune system and prevent calf diarrhoea (Figure 2.5). Control methods to reduce mastitis in dairy herds must be directed to decrease the exposure of the teat ends to contagious pathogens and can be achieved by reducing the spread from cow to cow during the milking operation, eliminating the reservoir of the pathogens in the dairy herd and/or working on the genetic selection of dairy cattle resistant to mastitis (Nader-Macías *et al.*, 2013). Ability to control mastitis will depend on access to knowledge, diagnostic facilities, and animal health

products (Zadoks and Fitzpatrick, 2009). The mastitis treatment products on the US market fall in the first and third cephalosporin generations (Schukken *et al.*, 2013).

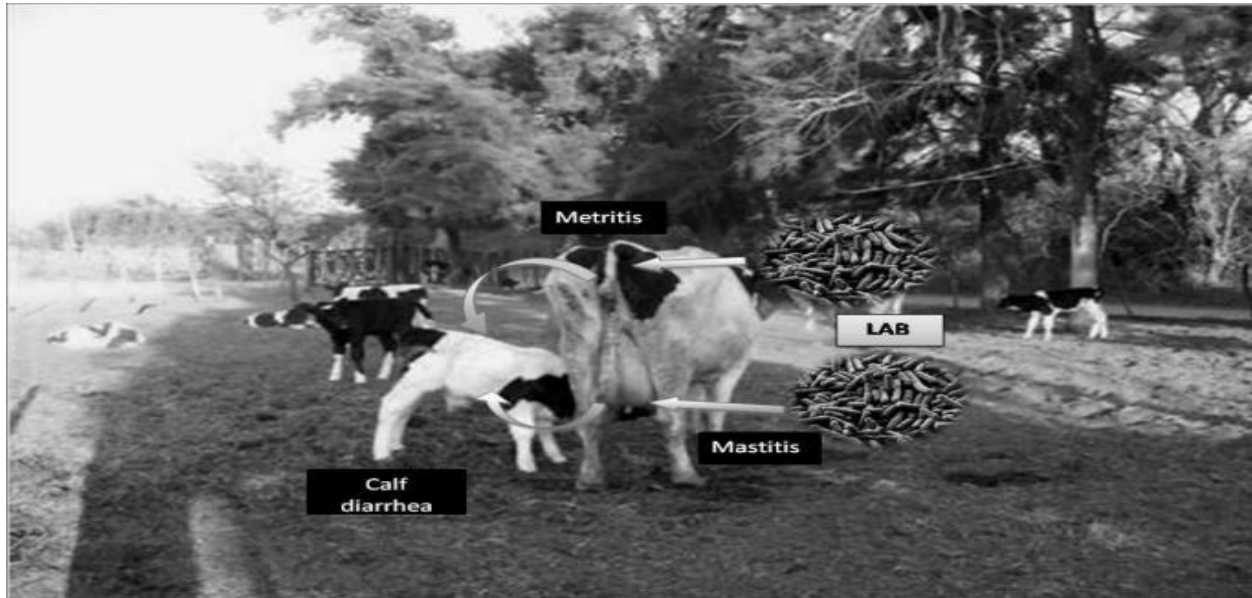


Figure 2.1: Proposed colonization of lactic acid bacteria as probiotic in bovines: Adapted from Nader-Macías *et al.*, (2013).

2.14 PREVENTION, TREATMENT AND CONTROL OF *STAPHYLOCOCCUS AUREUS* INFECTION

Contagious transmission of *S. aureus* can largely be prevented through good herd management (Sommerhäuser *et al.*, 2003). The prevalence of mastitis and its associated pathogens in animals can be reduced by improving on the farm management techniques within the dairy industry and thus of paramount importance to ensure that proper hygienic practices are enforced in both the area where the animals are kept and the milking environment (Pitkälä *et al.*, 2004). Antibiotics on dairy operations are used to treat highly prevalent infections, such as subclinical mastitis, and as a preventive measure during dry cow therapy (Haran *et al.*, 2012). Cure rates for mastitis caused by penicillin-resistant strains of *S. aureus* seem to be inferior to those of mastitis due to

penicillin-susceptible strains (Ziv and Storper, 1985; Pyörälä and Pyörälä, 1998; Sol *et al.*, 2000; Taponen *et al.*, 2003). The control of bovine mastitis caused by *S. aureus* is very difficult (Katsuda *et al.*, 2006).

The emergence of antibiotic-resistant microorganisms in farm animal environments poses a potential public health concern to treat highly prevalent infections, such as subclinical mastitis, and as a preventive measure during dry cow therapy (Haran *et al.*, 2012). Antibiotics on dairy operations are used to treat highly prevalent infections, such as subclinical mastitis, and as a preventive measure during dry cow therapy. Using an in vitro β -lactamase test for determining resistance to penicillin G of staphylococci before treatment is recommended (Olsen *et al.*, 2006). Strains of *S. aureus* resistant to β -lactam antibiotics are known as methicillin-resistant *S. aureus* (MRSA). *S. aureus* strains have been observed for resistance against different antibiotics viz. beta-lactam, aminoglycosides, fluoroquinolones, lincosamides, macrolides and streptogramins that are commonly used by veterinarians (Moon *et al.*, 2007; Hendriksen *et al.*, 2008; Wang *et al.*, 2008). Control of *Staphylococcus aureus* intramammary infection (IMI) was based on hygiene at milking that aims to limit the spread of IMI in herds, particularly pre-milking washing and post-milking drying of udders with individual cloths, dipping teats with antiseptic solutions after milking, and cleaning of milking machines (Sutra and Poutrel, 1994).

2.15 PREVENTION, TREATMENT AND CONTROL OF *Streptococcus agalactiae* INFECTION

S. agalactiae can be treated economically by 'blitz therapy with penicillin or a penicillin/novobiocin combination during lactation (Erskine and Eberhart, 1990; Erskine *et al.*,

1996). Knowledge of this transmission characteristic and demographic parameters can be used in modelling the effectiveness of control strategies against *S. agalactiae* and in the estimation of the basic reproductive ratio, R_0 (the average number of secondary infections caused by one infectious individual, throughout its infectious period), in an entirely susceptible population, which reflects a pathogen's potential for spread (Keeling and Rohani, 2008). These measures were supplemented with improved milking practices and hygienic measures to control within-herd spread of infection (Mweu *et al.*, 2012).

Mastitis causing streptococci have remained susceptible to penicillin G, but emerging resistance to macrolides and lincosamides has been detected (Pitkälä *et al.*, 2004; Loch *et al.*, 2005). Measures to control mastitis such as improved milking hygiene have reduced the prevalence of contagious major pathogens such as *S. agalactiae* (Hillerton *et al.*, 1995; Myllys *et al.*, 1998; Makovec and Ruegg, 2003a; Pitkälä *et al.*, 2004). Because of its subclinical manifestation and contagious nature, control of *S. agalactiae* requires early diagnostic identification of infected cows (Ahmadi *et al.*, 2009). Because of its subclinical manifestation and contagious nature, control of *S. agalactiae* requires early diagnostic identification of infected cows (Ahmadi *et al.*, 2009).

2.16 PREVENTION, TREATMENT AND CONTROL OF *ESCHERICHIA COLI* INFECTION

Preventing the growth of contaminating bacteria in milk involves limiting contamination levels, cooling immediately after milking, and maintenance of cold storage temperatures (Garedew *et al.*, 2012). For prevention of environmental mastitis, it is important to determine whether CM in

early lactation is due to infections during the dry period or during lactation (Klaas and Zadoks, 2018). Environmental controls, that is, insect control, may reduce the risk of vector borne mastitis, but an investigation of the impact of fly control in heifers on early lactation CM yielded results that depended on selection of the outcome variable of interest (Green *et al.*, 2007). Limitation of bacteria primarily includes cleaning, sanitizing and drying cow's teats and udder before milking and using sanitized milking equipments (Garedew *et al.*, 2012). Control measures need to target the relevant infection risks, for example, poor environmental hygiene or non-use of teat-sealants in the dry period, versus inadequate hygiene or nutrition in lactation.

The efficiency of CM control can be improved by using information about cow-specific risk factors that allows a farmer to identify the cows that have a higher risk of CM and to subsequently provide a higher level of care for these cows (Oliveira *et al.*, 2015). In addition to cow-specific risk factors, knowledge of the pathogens involved in CM is also useful to improve the efficiency of control measures (Steeneveld *et al.*, 2008). The use of the 3/4GC ceftiofur and cefquinome, however, improved treatment outcomes in comparison with first-generation cephalosporin or no treatment, respectively (Schukken *et al.*, 2011a; Schukken *et al.*, 2013). Throughout the world, several intramammary products for the treatment of CM in dairy cattle contain cephalosporins (Schukken *et al.*, 2013).

2.17 DISEASES, SYMPTOMS AND SIGNS OF BOVINE MASTITIS

Lumpy skin disease, foot and mouth disease and ephemeral fever are the most important viral causes of mastitis (Radostits *et al.*, 2007). Severe cases of mastitis leads to mammary atrophy, and is associated with local or systemic clinical signs such as increased rectal temperature,

changes in milk composition and yield, swelling and redness of the infected mammary glands (Fogsgaard *et al.*, 2012).

2.18 DISEASES, SYMPTOMS AND SIGNS OF *Escherichia coli* SPECIES

Clinical mastitis gives abnormal milk and swelling of the udder (Ferrero *et al.*, 2014). Bovine mastitis resembles urinary tract infection in that the infection is ascending and caused by bacteria from the environment (Kaipainen *et al.*, 2002). Flakes or clots, chronic infection in the milk and swelling of infected quarters are common signs of clinical mastitis, while subclinical mastitis, in absence of any clinical sign, usually remains unnoticed (Bhatt *et al.*, 2012). In *E. coli* mastitis, severity of clinical signs is considered to depend mainly on the host response (Hogeveen *et al.*, 2011). However, in acute mastitis when the gland is swollen and milk ducts are occluded by abnormal secretions, many drugs are poorly absorbed and may not penetrate throughout the mammary gland and, in some cases, increased rectal temperature, lethargy, anorexia and even death (Steeneveld *et al.*, 2008). *E. coli* in dairy cows around parturition and during early lactation with striking local and sometimes severe systemic clinical symptoms causes infection that causes inflammation of the mammary gland (Burvenich *et al.*, 2003). *E. coli* in dairy cows around parturition and during early lactation with striking local and sometimes severe systemic clinical symptoms causes infections in the mammary gland (Burvenich *et al.*, 2003).

2.19 DISEASES, SYMPTOMS AND SIGNS OF *Staphylococcus aureus* SPECIES

Contamination of food with enterotoxigenic *S. aureus* causes staphylococcal enterotoxins (SEs) intoxication hence the associated symptoms like vomiting and diarrhoea (Veras *et al.*, 2008). Strains of *S. aureus* in intramammary dissemination often produce incurable severe intra-herd

infections (Moon *et al.*, 2007; Kumar *et al.*, 2010). Additionally, through the production of toxins, *S. aureus* can cause specific toxin-mediated conditions, such as scalded skin syndrome, staphylococcal food poisoning and toxic shock syndrome (Becker *et al.*, 2015). In animals, *S. aureus* is a common etiological agent of mastitis, an infectious disease condition responsible for significant financial losses to dairy farmers worldwide (Petrovski *et al.*, 2006; Fitzgerald, 2012). Although *Staphylococcus aureus* is known to be one of the most common causes of bovine mastitis and other severe animal diseases such as septicaemia and wound, bone, and joint infections (Juhász-Kaszanyitzky *et al.*, 2007). *S. aureus* is considered the world's third most important cause of food-borne illnesses (Schmidt and Tirado, 2001).

However, the advent of molecular techniques, together with the availability of DNA sequence information for all the described SEs, have led to the use of the polymerase chain reaction (PCR) method to detect specific enterotoxin gene sequences. Symptoms typically have a rapid onset (1–6 h) and often include nausea, vomiting, diarrhoea and abdominal pain (Jablonski, 1997). Milk is a good substrate for *S. aureus* growth, and dairy products are a known source of intoxication (De Buyser *et al.*, 2001; Jørgensen *et al.*, 2005). An efficient screening to detect the prevalence of enterotoxic strains in foods is required because of the importance of these toxins in the public health and food sectors (Morandi *et al.*, 2007).

2.20 DISEASES, SYMPTOMS AND SIGNS OF *Streptococcus agalactiae* SPECIES

S. agalactiae mostly causes subclinical mastitis which tends to be chronic (Elias *et al.*, 2012). *S. agalactiae* causes disease in both humans and cattle, while in cattle, it causes bovine clinical mastitis (CM) and subclinical mastitis (SCM), and in humans, it is associated with infections

among neonates and adults (Pinto *et al.*, 2013). *S. agalactiae* is one of the main pathogens causing mastitis, invasive disease (Jain *et al.*, 2012). Mastitis in *S. agalactiae* is an inflammation of the mammary gland caused by microorganisms and their toxins, myiasis, physical trauma or chemical irritants (Arpini *et al.*, 2016). Mastitis caused by *S. agalactiae* is extremely costly once these bacteria usually infect many quarters of the mammary gland, causing extensive damage to alveolar cells and resulting in decrease of milk production (Edmondson, 2011; Keefe, 2012).

2.21 VIRULENCE FACTORS ASSOCIATED WITH *Escherichia coli* SPECIES

PCR as one of the molecular biological DNA-based diagnostic methods has been considered and established for rapid and precise identification of *E. coli* species. The only virulence characteristic consistently associated with *E. coli* isolated from cows with clinical mastitis was serum resistance (Bradley and Green, 2001; Wenz *et al.*, 2006). Cytotoxic necrotizing factors 1 and 2 (CNF1 & CNF2) were found in only a few mastitic strains, whereas CNF2 was very commonly found in faecal strains (Pohl *et al.*, 1993; Burns *et al.*, 1996). Molecular biological techniques have been used to analyze the production of F17 Fimbriae, enterotoxins (LT & ST1) and the genes for verotoxins and TraT (Lipman *et al.*, 1995).

2.22 VIRULENCE FACTORS ASSOCIATED WITH *Streptococcus agalactiae* SPECIES

PCR as one of the molecular biological DNA-based diagnostic methods has been considered and established for rapid and precise identification of *Streptococcus* species. The *fbsA* gene is responsible for encoding the protein FbsA, which allows the binding of *S. agalactiae* to fibrinogen, soluble or mobilized from extracellular matrix of the host organism (Jacobsson, 2003; Sukhnanand *et al.*, 2005). The gene is responsible for *hlyB* protein called hyaluronate

lyase [HlyB], which is very important for the pathogenesis of *S. agalactiae* (Glaser *et al.*, 2002). The cps cluster is responsible for the formation of the polysaccharide capsule and its sialidation (Arpini *et al.*, 2016).

In order to identify streptococci of bovine origin, several PCR techniques that employ amplifications of species-specific sequences have been used, including the gene *tuf* (Picard *et al.*, 2004), the gene of factor CAMO (Merl *et al.*, 2003), the gene 16S rRNA (Jayarao *et al.*, 1992; Meiri-Bendek *et al.*, 2002; Merl *et al.*, 2003), the gene 23S rRNA (Kawata *et al.*, 2004) and the intergenic space between the latter two genes (Phuektes *et al.*, 2001). Major virulence factors of *S. agalactiae* include fibrinogen binding protein (*fnb*), laminin-binding protein (*lmb*), fibronectin-binding protein (*pavA*), β -C protein (*cba*), capsule, C5a peptidase (*scp*), hyaluronate lyase, α -C protein, β -hemolysin/cytolysin, and CAMP factor (*cfb*) (Chen *et al.*, 2005). *S. agalactiae*, possesses a variety of virulence factors that contribute to pathogenicity and surface proteins and polysaccharide capsules were identified such as *scpB* gene encodes for surface enzyme ScpB (C5a peptidase), the *bca* gene encodes for a-protein, the *lmb* gene encodes for laminine-binding protein, and *bac*, *cyl*, *glnA*, *cfb*, *hylB*, and *scaA* encode for b-antigen, b-hemolysin, glutamine synthetase, the Christie–Atkins–Munch–Peterson (CAMP) factor, hyaluronidase, and aggregation factor, respectively (Dmitriev *et al.*, 2002).

2.23 VIRULENCE FACTORS ASSOCIATED WITH *Staphylococcus aureus* SPECIES

Virulence factors of *S. aureus* have been investigated both by measuring phenotypic expression of products assumed to be associated with virulence and by screening genes encoding these products (Taponen and Pyörälä, 2009). The study used PCR to analyse the distribution of genes

encoding sea, sec, sed, seg, seh, sei, sej and sel in *S. aureus* isolated from bovine, goat, sheep and buffalo raw milk and milk products (Morandi *et al.*, 2007). Various virulence factors, including production of haemolysins, leucocidins, exfoliative toxins, enterotoxins, toxic-shock syndrome toxin, and ability to form slime and biofilm have been found in *Staphylococcus aureus* strains isolated from bovine mastitis (Fitzgerald *et al.*, 1997; Zecconi *et al.*, 2006). Virulence factors from *S. aureus* include an exo protein that exhibits the qualities of a super antigen which are categorized as staphylococcal enterotoxins (SEs) and a toxic shock syndrome toxin (TSST-1) known to be involved in several human and animal diseases (Dinges *et al.*, 2000).

2.24 TRANSMISSION OF (CONTAGIOUS) *Streptococcus agalactiae* SPECIES

2.24.1 Cow-cow transmission

The primary method of spread for these pathogens is contagious (cow to cow) with infected cows within a herd acting as a reservoir of infection (Keefe, 2012). Transmission of the pathogen within dairy herds may occur during milking via milkers' hands, liners and use of common udder cloths contaminated with milk from infected cows (Keefe, 1997; Mweu *et al.*, 2012). Transmission of this pathogen occurs directly from infected udder to an uninfected udder during the milking process, via milker's hands, liners, and common udder cloths (Keefe, 1997). An often used parameter to quantify transmission is the transmission rate, γ which is defined as the average number of secondary infections caused by one infectious individual per unit of time (Halasa *et al.*, 2007; Keeling and Rohani, 2008). Contagious pathogens are also noted for their ability to be spread between quarters and cows during the milking process (Bradley and Green, 2001).

In contagious bacteria, the reservoirs of these pathogens are the infected quarters and the transmission occurs mainly during milking, by their presence in the milking machines, washing water or hands of the operators (Nader-Macías *et al.*, 2013). Among the various pathogens causing mastitis, *S. agalactiae* is of particular importance because it is highly infectious (spreading from cow to cow by machine milking, unless care is taken to disinfect the teat cups), and causes mainly subclinical infections, which are not identified by the herdsman (Berry and Hillerton, 2007). *S. agalactiae* can spread widely within a herd, causing immediate loss due to reduced milk yield, and eventual large losses as listed above, when it is finally recognized (Ahmadi *et al.*, 2009).

2.25 TRANSMISSION OF (CONTAGIOUS) *Staphylococcus aureus* SPECIES

2.25.1 Cow-cow transmission

S. aureus spread from cow to cow, primarily during milking, and tend to result in chronic sub-clinical infections with flare-ups of clinical episodes (Abebe *et al.*, 2016). Raw unpasteurized milk may become contaminated with enterotoxigenic coagulase-positive *S. aureus* either through contact with the cow's udder during milking or by cross contamination during processing (Ekici *et al.*, 2004; Normanno *et al.*, 2005). Transmission occurs from cow to cow at or around the time of Milking through contaminated milking machines, clothes and hands of milkers or machine operators (Radostits *et al.*, 1994). Mode of transmission of *Staphylococcus aureus* (Figure 2.4)

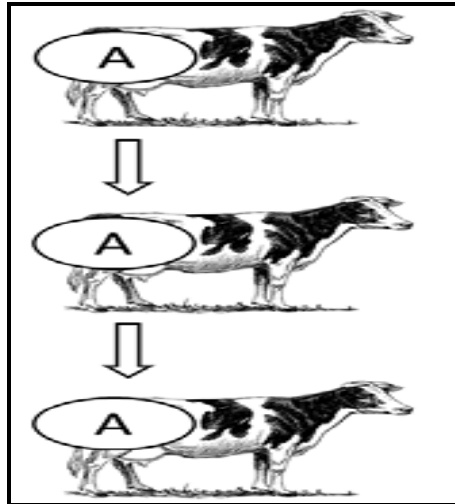


Figure 2.4: Modes of transmission of *Staphylococcus aureus* (contagious pathogen): Adapted from Klaas and Zadoks (2018).

2.25.2 Transmission from contact with hands of the milking personnel

Additionally, hand milking may introduce possibilities for transmission between farmers and their cattle (Mekonnen *et al.*, 2005). Resistant *S. aureus* (MRSA) infection has emerged recently among animals and can be spread between cows and humans, posing a potential risk both for human and animal health (Juhász-Kaszanyitzky *et al.*, 2007). The transmission of bovine MRSA to humans is possible and may contribute to outbreaks in animal and human populations (Lee, 2003). Bacterial contamination of milk usually occurs during the milking process and this depends on the sanitary condition of the environment, utensils used for milking and the milking personnel and also result from micro-organisms that enter the udder through the teat opening canal (Farzana *et al.*, 2004).

2.25.3 Vector and flies transmission

Mastitis pathogens are rarely vector-transmitted (Klaas and Zadoks, 2018). Insect vectors such as flies and wasps may play a role in transmission of some mastitis pathogens, notably

Staphylococcus aureus pathogens associated with summer mastitis (Chirico *et al.*, 1997). The pieces of tissue taken by the workers from the teats are used to feed larvae, adults do not produce protease and therefore cannot degrade protein (Braverman *et al.*, 1991).

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2.26 TRANSMISSION OF CLINICAL (ENVIRONMENTAL) OF *Escherichia coli* SPECIES

2.26.1 Environmental transmission route

Environmental bacteria cannot be removed from the environment, and although they can move from one quart to another, this route of transmission does not contribute to the development of new infections and occurs in the dry period and around deliver (Donkor *et al.*, 2007; Nader-Macías *et al.*, 2013). Bacterial contamination could arise from the cow's udder, barn, milk collection materials, various ingredients added to dairy products and dairy farm workers (Teka, 1997). Gram-negative staining bacteria, which are the target of the present study, have been reported to contaminate milk as a consequence of milking cows affected by mastitis and from poorly sanitized utensils used during milking, transportation and storage processes (Ledenbach and Marshall, 2009). Figure 2.5 indicates the mode of transmission of *E. coli* to cattle from environmental sources.

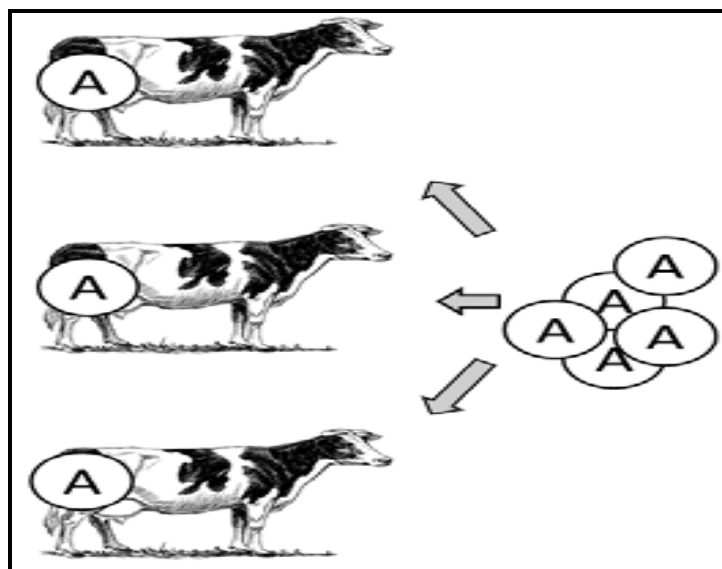


Figure 2.5: Modes of transmission of *Escherichia coli* to cattle from environmental sources: Adapted from Klaas and Zadoks, (2018).

2.27 LABORATORY DIAGNOSIS AND DETECTION OF BOVINE MASTITIS

Currently, bacterial culture is the gold standard method for identification of mastitis-causing microorganisms (Oikonomou *et al.*, 2012). Sequencing and analysis of hyper variable regions within the 16S rRNA gene can provide relatively rapid and cost-effective methods for assessing bacterial diversity and abundance and may be useful for pathogen discovery and identification (Kolbert and Persing, 1999). Methods such as real-time PCR (Koskinen *et al.*, 2010; Shome *et al.*, 2011)], denaturing gradient gel electrophoresis (DGGE) PCR are now being used to identify bacterial DNA in milk samples (Kuang *et al.*, 2009).

2.28 LABORATORY DIAGNOSIS AND DETECTION OF *Escherichia coli* SPECIES

Advances in next-generation sequencing allow the in-depth investigation of clinical samples' microbiome and determining their taxonomic composition including unculturable species (Loeffelholz and Fofanov, 2015). *E. coli* were identified using morphologic characteristics of

colonies on MacConkey agar, production of indole, motility, and utilization of citrate (Oikonomou *et al.*, 2014). The gold standard for identification of the causative pathogen is by bacterial culture, which uses standards set by the National Mastitis Council (Oultram *et al.*, 2017). A multiplex PCR, ‘real-time’ The pyrosequencing of PCR amplicons has been used to represent specific regions of 16S rRNA genes (rDNA) directly from milk to characterize the microbiota of “culture negative” clinical mastitis samples (Kuehn *et al.*, 2013).

Intramammary infections are defined as identified predominantly by aerobic culture, modified anaerobic and in some cases by molecular diagnostics (Koskinen *et al.*, 2010; Shome *et al.*, 2011). Identifying the microorganisms responsible for culture negative, clinical mastitis and assessing changes in bacterial populations throughout infection has been improved understanding of the disease process allowing us to identify more effective intervention strategies (Kuehn *et al.*, 2013). In general, methods based on 16S rRNA gene analysis provide extensive information about the taxa and species present in an environment; however, these data usually provide only little, if any, information about the functional role of different microbes within the community and the genetic information they contain about microbial niches (Streit and Schmitz, 2004).

PCR assay that can simultaneously detect different mastitis-causing organisms in milk samples is already commercially available in a number of countries (Hogeveen *et al.*, 2011). Knowledge on the infectious status of mammary glands, however, can also be very helpful to prevent transmission of pathogens by diagnosing a reservoir at an early stage (Lam *et al.*, 2009). Culture, however, has an inherent bias toward organisms that are able grow on the selected media (Oultram *et al.*, 2017). Shotgun sequencing is still prohibitively expensive in a commercial

clinical setting, whereas a metataxonomic (16S rRNA gene sequencing) approach could be a relatively rapid and cost-effective method for assessing bacterial diversity and abundance (Oikonomou *et al.*, 2012). The most frequently used diagnostic methods are somatic cell counting (SCC) and bacteriological culturing (BC) of milk to solve mastitis problems (Lam *et al.*, 2009). Testing the presence of a specific bacterial species, a part of the DNA of that pathogen is amplified and subsequently visualised (Lam *et al.*, 2009). For a number of mastitis pathogens, PCR-based techniques have been described (Lee *et al.*, 1998).

2.29 LABORATORY DIAGNOSIS AND DETECTION OF *Streptococcus agalactiae* SPECIES

Current methods for identifying *S. agalactiae* are based on bacteriological examination of blood agar plates including the hemolysis caused by an exocellular product [the Christie, Atkins, and Munch-Peterson (**CAMP**) test], Or the lack of ability to hydrolyze esculin, and on the production of colored colonies when grown anaerobically on starch (Keefe, 1997). Serological methods based on surface polysaccharide antigens are often used to confirm the biochemical identification. The development of molecular biological techniques, PCR provide a method for direct detection and identification of pathogens (Ahmadi *et al.*, 2009). PCR represents an innovative diagnostic tool for the direct detection of *S. agalactiae* in milk (Ahmadi *et al.*, 2009).

The high specificity of the molecular diagnosis is based on the use of species-specific DNA sequences for bacterial detection; thus, the sequence 16S RNA, which is commonly present as multiple copies and has highly conserved segments, is greatly useful for this technique (Elias *et al.*, 2012). The present study evaluated *S. agalactiae* prevalence, total bacterial count in tank milk, and PCR efficiency in detecting *S. agalactiae* as a diagnostic test relative to the

microbiological culture (Elias *et al.*, 2012). Of these methods, molecular detection of pathogenic microorganisms based on DNA amplification of the target pathogen based on unique DNA sequences within the 16S subunit of the ribosomal RNA (rRNA) gene has been proven to be an accurate, reliable, and fast diagnostic method (Meiri-Bendek *et al.*, 2002; Elias *et al.*, 2012). The development of molecular biological techniques provides a method for direct detection and identification of pathogens. PCR is one of these techniques, which has high specificity, high sensitivity and can be carried out in less than 24 hour.

2.30 LABORATORY DIAGNOSIS AND DETECTION OF *Staphylococcus aureus* SPECIES

Next-generation platforms are helping to open entirely new areas of biological inquiry, including the investigation into ancient genomes, characterization of ecological diversity and identification of unknown aetiological agents (Bhatt *et al.*, 2012). Depending on the strain under analysis, *Staphylococcus aureus* grow well at temperatures between 62 and 95°C. PCR is a rapid and sensitive method to identify bacteria, it was used in this preliminary study to assess its applicability as an alternative tool for monitoring mastitis caused by *S. aureus* (Phuektes *et al.*, 2003).

The study used PCR to analyse the distribution of genes encoding sea, sec, sed, seg, seh, sei, sej and sel in *S. aureus* isolated from bovine, goat, sheep and buffalo raw milk and milk products (Morandi *et al.*, 2007). To improve the diagnostics for *S. aureus* in mastitis, we developed a novel methodology including a fast and reliable preparation procedure for bacteria from bovine milk, DNA extraction, and specific detection by real-time quantitative PCR (QPCR). The

described method showed a high analytical sensitivity and specificity as well as a high repeatability (Graber *et al.*, 2007). Testing for the presence and type of hemolysis on blood agar-based plates represented a first simple and rapid method for the detection of *Staphylococcus aureus* in milk samples (Lam *et al.*, 1995).

2.31 PATHOGENICITY OF MASTITIS

Normally, the teat canal is tightly closed by sphincter muscles, preventing the entry of pathogens (Viguié *et al.*, 2009). A waxy material derived from stratified squamous epithelium that obstructs the migration of bacteria and contains antimicrobial agents, such as long-chain fatty acids, that assist in combating the infection was lined with keratin. Strains originating from bovine mastitis mostly represent a genetically different cluster than e.g. human strains, suggesting host specificity (Hogeveen *et al.*, 2011). The degree of changes depend on the pathogenicity of the mastitis causing bacteria and the amount of affected tissue in the gland; especially the affected epithelial area (Pyörälä, 2003).

The main changes in the udder include (1) leaking of ions, proteins and enzymes from the blood into the milk due to an increased permeability, (2) invasion of phagocytosing cells into the milk compartment and (3) a decrease of the synthetic capacity of the gland, resulting in decreased concentrations of certain (Korhonen and Kaartinen, 1995). Vaccination is one of the main strategy to control mastitis, as its principal objective is to increase the concentration of opsonic antibodies in blood and milk against a particular microorganism to inhibit their growth and toxin production (Nader-Macías *et al.*, 2013)

2.32 PATHOGENESIS OF *Escherichia coli* SPECIES

Bacterial virulence factors are required to fight the host's selection pressure and for the bacteria to colony, multiply and survive in the udder (Kaipainen *et al.*, 2002). Several virulence factors which include (toxins, adhesions, invasins, capsule production and the ability to resist serum complement and scavenger iron) detected from pathogenic *E. coli* bacteria cause urinary tract infections, diarrhea, septicemia and meningitis in humans and animals (Kaipainen *et al.*, 2002). Enterotoxigenic *E. coli* (ETEC) strains, the colonisation of intestinal mucosa is related to the expression of several different proteinaceous surface structures, i.e. colonisation factors, which recognise specific receptors on the intestinal epithelial surface (Sears and Kaper, 1996; China and Goffaux, 1999). The pathogenicity in these animals mainly depends on expression of particular virulence factors important during the pathogenesis of *E. coli* is the endotoxin or lipopolysaccharide (LPS) (Dosogne *et al.*, 2002). Disease severity is determined by interactions between the host, the environment, and the infectious agent (Wenz *et al.*, 2006). Neutrophils move rapidly from the blood stream into the secretions of infected quarters, causing an increase in somatic cells in milk (Suriyasathaporn *et al.*, 2000). As a results of neutrophil infiltration into the invaded area of the mammary gland, changes in blood cell composition occur, due to bone marrow stimulation and alteration of the normal venular equilibrium that controls attachment, rolling and diapedesis (Burvenich *et al.*, 2003).

The *eaeA* gene encodes for the intimin protein, which is associated with attaching and effacing lesions and bacterial adherence to epithelial cells (Jerse *et al.*, 1990). *Escherichia coli* with increasing numbers of neutrophils appearing invades the udder through the teat canal where it grows and initiates a prompt inflammatory reaction (Burvenich *et al.*, 2003). This neutrophil

infiltration is one of the first steps of the inflammatory reaction. is often characterized by an acute and severe inflammation, which, however, may eventually lead to pathogen clearance (Vangroenweghe *et al.*, 2005). Antibody-mediated immunity and neutrophil phagocytosis play a major role in the host response to *E. coli* mastitis, which may explain why vaccination against *E. coli* mastitis has been more successful than vaccination against other mastitis pathogens (Schukken *et al.*, 2011b).

2.33 PATHOGENESIS OF *Staphylococcus aureus* SPECIES

During intramammary infection (IMI) that induces inflammation of the mammary gland, the milk contains less casein proteins and an increased amount of serum proteins and host cells (Brouillette and Malouin, 2005). The importance of surface components (proteins and capsular polysaccharides) and exotoxins in the pathogenesis of bovine mastitis is well-known (Taverna *et al.*, 2007). Therefore, mapping of surface proteins provides a benchmark for strain comparison of pathogens with different combination of pathogenic characteristics and antibiotic resistance mechanism and can aid in defining vaccine and therapeutic targets (Taverna *et al.*, 2007). Indeed, during *S. aureus* intramammary infections surface components take part in bacterial adhesion to host mammary tissues and to resistance to phagocytosis by milk cells (Sutra and Poutrel, 1994). The pathogenicity of *S. aureus* is usually associated with its ability to produce toxins or through the invasion and destruction of host tissues (Le Loir *et al.*, 2003). Mapping of surface proteins provides a benchmark for strain comparison of pathogens with different combination of pathogenic characteristics and antibiotic resistance mechanism and can aid in defining vaccine and therapeutic targets (Kumar *et al.*, 2011).

2.34 MECHANISMS OF ANTIMICROBIAL AGENT OF BOVINE MASTITIS

Antimicrobial resistance is an increasingly important problem among several bacterial species causing infection in animals and humans in recent year, while there is few treatment (Aarestrup, 1999; Levy, 2001). Resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows (Owens *et al.*, 1997; Erskine *et al.*, 2002; Makovec and Ruegg, 2003b; Pitkälä *et al.*, 2004). Resistance to antimicrobial agents in mastitis pathogens has relevant aspects which is a reduction in cure rates after treatment of clinical mastitis cases (Owens *et al.*, 1997; Sol *et al.*, 2000) and the issue is the potential impact of transmission of resistant bacteria to humans via the food chain (Ungemach, 1999). The World Health Organization (WHO) has stated that any use of antimicrobial agents is associated with the risk of inducing resistance to antimicrobial agents among bacteria (Organization, 1997).

2.34.1 The use of antimicrobial and antibiotic resistance in bovine mastitis

Antimicrobial agents are widely used for the treatment of bovine mastitis, respiratory tract infections and diarrhoea in cattle (Botrel *et al.*, 2010). The use of antimicrobial agents on dairy farms as well as in other food animal production systems is a major concern in the emergence of resistant zoonotic bacterial pathogens (PIDDOCK, 1996). Resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows (Owens *et al.*, 1997; Erskine *et al.*, 2002; Makovec and Ruegg, 2003b; Pitkälä *et al.*, 2004). The risk of emerging antimicrobial resistance among bovine mastitis pathogens has been low, particularly for drugs with high therapeutic value in human medicine (Lindeman *et al.*, 2013; McDougall *et al.*, 2014).

2.35 MECHANISMS OF ANTIMICROBIAL AGENT OF *Staphylococcus aureus*

It is believed that these infections respond poorly to therapy with antimicrobial agents, whether given parenterally or via the intra-mammary route (Sutra and Poutrel, 1990). Antimicrobial resistance is an important health problem worldwide (Cosgrove, 2006). Antimicrobial resistance has become a major public health problem (Akindolire *et al.*, 2018).

2.35.1 The use of antimicrobial and antibiotic resistance in *Staphylococcus aureus*

To study antimicrobial resistance in humans and animals is important for detecting changing patterns of resistance, implementing control measures on the use of antimicrobial agents and preventing the spread of multidrug-resistant strains of bacteria (Abraha *et al.*, 2018). The use of antimicrobial agents on dairy farms as well as in other food animal production systems is a major concern in the emergence of resistant zoonotic bacterial pathogens. In *S. aureus* isolated from humans, there is a trend of increasing antimicrobial resistance (AMR) (Moges *et al.*, 2014). The use of antimicrobials is needed on dairy farms, similar to the stewardship of antimicrobial resources advocated in human medicine (Weber, 2006). In recent years, there has been development of resistance has been attributed to the extensive therapeutic use of antimicrobials or to their administration as growth promoters in food animal production (Normanno *et al.*, 2007). Isolates of *S. aureus* are frequently resistant to methicillin and essentially all other β -lactam antibiotic (Tassew *et al.*, 2016). The occurrence and transmission of antimicrobial resistant *S. aureus* itself or its gene has been suggested as one of the reasons for difficulty in antibiotic therapy (Goh and Byrne, 1992; Kreiswirth *et al.*, 1993). The development of resistance, both in human and animal bacterial pathogens, has been ascribed to the extensive therapeutic use of antimicrobials or with their use as growth promoters in animal feed production (Rogues *et al.*, 2007).

2.36 MECHANISMS OF ANTIMICROBIAL AGENT OF *Streptococcus agalactiae*

Antimicrobial resistance in pathogenic bacteria has become a real problem and may be partially due to the use of antimicrobials as growth promoters or prophylactic agents in animal agriculture (Tian *et al.*, 2019). Antimicrobial treatment should take into account previous information such as the minimum inhibitory concentrations (MIC) of the available agents for individual pathogens (Sheldon *et al.*, 2004). The increase of antimicrobial residues in milk pose a serious threat, as such residues may cause reactions in individuals who are allergic to antimicrobials (Nemati *et al.*, 2008).

2.36.1 The use of antimicrobial and antibiotic resistance in *Streptococcus agalactiae*

The use of antimicrobial agents on dairy farms as well as in other food animal production systems is a major concern in the emergence of resistant zoonotic bacterial pathogens (Haran *et al.*, 2012). The development of resistance, both in human and animal bacterial pathogens, has been ascribed to the extensive therapeutic use of antimicrobials or with their use as growth promoters in animal feed production (Rogues *et al.*, 2007; Silbergeld *et al.*, 2008).

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS AND METHODS

3.1.1 Area of the study

The research study was conducted at the Antimicrobial Resistance and Phage Biocontrol Research Laboratory in the Department of Microbiology, North-West University (Mafikeng Campus), North West province of South Africa.

3.1.2 Study design

This cross-sectional study was used to determine the antibiotic resistance and virulence potentials of *S. aureus*, *S. agalactiae* and *E. coli* isolated from raw milk from dairy farms and pasteurized milk from selected supermarkets in the North West Province, South Africa.

3.1.3 Ethical clearance

Ethical clearance for the study was obtained from the Faculty of Natural and Agricultural Sciences (FNAS) Ethics Committee and an ethics number (NWU-01225-19-S9) was assigned to the study. Verbal administrative authorizations was obtained from the farmers and managers of farms. The names of dairy farms and supermarkets were coded to ensure confidentiality.

3.1.4 Sample size determination

Milk samples (raw, tank and pasteurised) were randomly collected from supermarkets, shops as well as both commercial and communal farms. The number of samples that was collected during the study was determined using the formula outlined below:

$$\text{Sample (n)} = \frac{Z_{1-\alpha/2}^2 P (1-P)}{d^2}$$

$$d^2$$

$Z_{1-\alpha/2}$ = is standard normal variable (at 5% type I error ($P < 0.05$) it is 1.96)

P = Expected prevalence in population based on previous studies

d = Absolute error or precision (which is 5%)

$$\text{Sample size (n)} = \frac{(1.96)^2 (0.281) (1-0.281)}{(0.05)^2} = 185.0011$$

For estimation of the prevalence of *S. aureus*, *S. agalactiae* and *E. coli* pathotypes, the sample size for this study was determined using a prevalence of 28.1 % obtained in the study by (Arthur *et al.*, 2007) in South Africa. The expected prevalence with the 95% confidence level and desired precision of 5% using the formula described by (Charan and Biswas, 2013) was calculated. Accordingly, the minimum sample size required for the study was 62 milk samples.

3.1.5 Collection of samples and sample sites

A total of 102 milk samples (raw, tank and pasteurized) were randomly collected from supermarkets, shops as well as both commercial and communal farms in the North-West province of South Africa on three consecutive days in winter and spring, 2018. Samples for winter were collected in the months of June and July, while samples for spring were collected in

September and October. Approximately, 50 mL of each milk sample of was collected using conical centrifuge tubes, labeled accordingly, and stored in ice. The samples were transported to the Antimicrobial Resistance and Phage Biocontrol Research Laboratory in the Department of for analysis. The number of samples collected from the different areas are shown in Table 3.1.

Table 3.1: Areas from which milk samples were collected for isolation of *S. aureus*, *S. agalactiae* and *E. coli*.

Sampling area	Number of the different types of milk samples collected		
	Raw	Shops/Supermarkets	Bulk tank Milk
Mafikeng			4
Zeerust	2	2	3
Vryburg		2	3
NWU-Molelwane	4		
Rustenburg	2	3	3
Koster		3	3
Swartruggens	4	2	2
Coligny		4	3
Ventersdorp		2	4
Lichtenburg		3	3
Rooigrond 1	4	2	2
Rooigrond 2	4	3	3
Potchefstroom		2	2
Klerksdorp		4	2
Taung	4	3	2
Lehurutshe		2	2
Total	24	37	41

Total number of samples = 102

3.2 BACTERIAL ISOLATION AND ANALYSIS OF MILK SAMPLES

3.2.1 Selective isolation of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* species

Upon arrival in the laboratory, the milk samples comprising 24 raw, 37 bulk tank and 41 pasteurized samples were analysed immediately. Ten-fold serial dilutions (10^{-1} to 10^{-5}) were prepared using 2% (w/v) buffered peptone water (BPW). Aliquots of 100 μ L from dilutions (10^{-5}) for each sample was spread-plated Mannitol Salt agar (MSA) plates, Columbia agar with sheep blood plates and Eosin Methylene Blue agar (EMBA) plates for selective for *S. aureus* (yellow colonies), *S. agalactiae* (golden yellow) and *E. coli* (metallic sheen colonies). The plates were incubated aerobically at 37°C for 24 hours to allow the bacterial growth. Pure colonies with characteristics colonial morphologies (golden yellow colonies on MSA plates, β -haemolytic yellow colonies on Columbia agar with sheep blood and metallic sheen colonies on EMBA) were purified by sub-culturing on the respective selective media. The plates were incubated aerobically at 37°C for 24 hours. These isolates were stored at room temperature and used for bacterial identification tests.

3.3 BACTERIAL IDENTIFICATION

Presumptive (*S. aureus*, *S. agalactiae* and *E. coli*) isolates were subjected to the following bacteria identification tests:

3.3.1 Preliminary Biochemical Tests for Identification of *S. aureus*, *S. agalactiae* and *E. coli*

3.3.1.1 Cellular morphology

The cellular morphology of the isolates was determined using Gram staining based on a previous protocol (Cruickshank *et al.*, 1975). All isolates that satisfied this criterion were subjected to preliminary biochemical identification tests.

3.4 SECONDARY IDENTIFICATION TESTS FOR *S. aureus* AND *S. agalactiae* SPECIES

3.4.1 Slide agglutination test (serology) for *S. aureus* and *S. agalactiae*

The slide agglutination tests for confirming the identities *S. aureus* and *S. agalactiae* was performed according to the manufacturer's instructions. The Prolex™ Streptococcal and *Staphylococcus* Grouping Latex test kit obtained from Pro-Lab Diagnostics, UK was used for serological identification of *S. aureus* and *S. agalactiae*, respectively. Cells of pure *S. aureus* and *S. agalactiae* colonies were placed on clean slides using sterile toothpicks and a drop of Prolex™ STREP (Pro-Lab Diagnostics, UK) and STAPH-PLUS (Pro-Lab Diagnostics, UK) reagents for *S. agalactiae* and *S. aureus* was added to the slides. The contents of the slides were mixed and the slides were rocked for 1 to 2 minutes. The detection of clots indicates a positive reaction and vice versa.

3.4.2 Haemolysis activity

The haemolysin activity of pure *S. aureus* and *S. agalactiae* was determined by sub-culturing the isolates on ox-blood agar supplemented with 5% (w/v) bovine blood (Türkyilmaz and Kaya, 2006). After inoculation, the plates were incubated aerobically at 37°C for 24 hours. The plates were observed for the presence of zones of clearing around the colonies.

3.5 BACTERIAL CONTROL STRAINS

In all the experiments, *S. aureus* (ATCC[®] 29213), *E. coli* (ATCC[®] 25922) and *S. agalactiae* (ATCC[®] 13813) were used as positive control strains while *K. pneumonia* (ATCC[®] 13883) was used as a negative control strain.

3.6 BACTERIAL STORAGE

All the bacteria isolates were stored by adding 500 µL of the overnight cultures of *S. aureus*, *S. agalactiae* and *E. coli* to 500 µL of 50% (v/v) glycerol in a 2 mL screw top tubes. The contents of the tubes were homogenised by vortexing gently and the cultures were stored at -80°C until further use.

3.7 MOLECULAR CHARACTERISATION OF *S. aureus*, *S. agalactiae* AND *E. coli* SPECIES

3.7.1 DNA extraction

Genomic DNA was extracted from all presumptive *S. aureus*, *S. agalactiae* and *E. coli* isolates using the cetyltrimethyl ammonium bromide (CTAB) method as described earlier by Doyle and Doyle (1990). The DNA was resolved by electrophoresis on a 2% (w/v) agarose gel on a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X, Amersham Pharmacia biotech, Sweden) for 1 hour at 80 V, 250 mA using 1X TAE buffer. A 100 bp DNA molecular weight marker (Fermentars, USA) was included in each gel and was used to confirm the size of the amplicons. The gels were stained in ethidium bromide (0.1µg/mL). The amplicons were visualised under short wave length UV transilluminator (Sambrook *et al.*, 1989). A ChemiDoc Imaging System (Bio-RAD ChemiDoc[™] MP Imaging System, UK) was used to capture the

images using Gene Snap (version 6.00.22). The DNA samples were stored at -20°C for future use.

3.7.2 Determination of DNA quality and quantity

The quality of the genomic DNA extracted from the isolates was determined using a UV Visible spectrophotometer (model S-22, Boeco, Germany) at the wavelength of 260 nm and the purity measured at 280 nm as previously described (Sambrook, 1989).

3.8 PCR FOR THE AMPLIFICATION OF BACTERIAL 16S rRNA GENE FRAGMENTS IN *S. aureus*, *S. agalactiae* AND *E. coli* ISOLATES

The 16S rRNA gene is a conserved universal gene found in all bacteria species and has a variable portion, which possesses a signature unique to each bacterium (Song, 2005). It has a high discriminatory power for the identification of different pathogens and thus highly utilised in research laboratories. In this study, bacterial 16S rRNA gene fragments were amplified as an internal control for all presumptive isolates. Bacteria 16S rRNA gene fragments were amplified using oligonucleotide primer 27F and 1492R (Table 3.2) to amplify a 1420 bp gene fragment (Korzeniewska and Harnisz, 2013).

DNA samples from 62 presumptive isolates were used for amplification of 16S rRNA universal genes. Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler, Johannesburg South Africa). The reactions were prepared in 25 µL volumes that constituted 12.5 µL of 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM MgCl₂ and loading buffer), 11 µL nuclease free distilled

water, 0.5 µL mixture of the forward and reverse primers and 1 µL of template DNA. All the PCR reagents used in this study were Fermentas USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The cycles and conditions for 16S rRNA amplifications are specified on Table 3.2. The PCR amplicons were resolved by electrophoresis on a (2% w/v) agarose gel. A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software and to determine the relative sizes of amplicons.

3.9 GENUS SPECIFIC PCR

3.9.1 Molecular identification of *S. aureus*, *E. coli* and *S. agalactiae* through PCR amplification of *uidA*, *nuc* and *VI and C1*) gene fragments respectively

The specie-specific primers were used to confirm the identities of presumptive *S. aureus* (Martinon and Wilkinson, 2011), *S. agalactiae* (Meiri-Bendek *et al.*, 2002; Ahmadi *et al.*, 2009) and *E. coli* (Anbazhagan *et al.*, 2011) isolates using PCR. The primer sequences used for PCR amplification of the target genes, the amplification conditions as well as the amplicon sizes are shown in Table 3.2.

Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch™ Thermal Cycler, Johannesburg South Africa). The reactions were prepared in 25 µL volumes that constituted 12.5 µL of 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4 µM MgCl₂ and loading buffer), 11 µL nuclease free distilled water, 0.5 µL mixture of the forward and reverse primers and 1 µL of template DNA. All the PCR reagents used in this study were Fermentas USA products, supplied by Inqaba Biotechnical

Industry Ltd, Sunnyside, South Africa. The PCR amplicons were resolved by electrophoresis on a 2% (w/v) agarose gel on a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X, Amersham Pharmacia biotech, Sweden) for 1 hour at 80 V, 250 mA using 1X TAE buffer for 50 mins. A 100 bp DNA molecular weight marker (Fermentars, USA) was included in each gel and was used to confirm the size of the amplicons. The gels were stained in ethidium bromide (0.1µg/mL). A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software and to determine the relative sizes of amplicons.

Table 3.2: Oligonucleotide primers used for amplification of PCR amplification of the different genes.

Primers	Sequence (5'-3')	Target Organism	Targeted gene	Amplicon size (bp)	PCR conditions and cycles	Reference
27F	AGAGTTTGATCATGGCTCAG	All bacteria species	16S rRNA	1420	94°C for 3 min1 (1X); 94°C for 1 min (25X); 55°C for 1 min; 72°C for 2 min	(Korzeniewska and Harnisz, 2013)
1492R	GGTACCTTGTTACGACTT					
UidA-F	TGGTAATTACCGACGAAAACGGC	<i>E. coli</i>	<i>uidA</i>	600	95°C for10 min (1X); 95°C for 45s; 59°C for 30s; 72°C for 90s (35X); 72°C for 10min (1X)	(Anbazhagan <i>et al.</i> , 2011)
UidA-F	ACGCGTGGTTACAGTCTTGCG					
NUC-F	GCGATTGATGGTGATACGGT	<i>S. aureus</i>	<i>nuc</i>	279	95°C for 6 min; (28X); 65°C for 30s; 62°C for 15s; 72°C for 30s	(Martinon and Wilkinson, 2011)
NUC-R	AGCCAAGCCTTGACGAACTAAAGC					
V-F V-R	TTTGGTGTTTACACTAGACTG TGTGTTAATTACTCTTATGCG	<i>S. agalactiae</i>	Species specific 16S rRNA	121	94°C for 4 min; (30X); 55°C for 2min; 72°C for 45s; 72°C for 2min	(Elhaig and Selim, 2015)
C-F C-R	GCGTGCCTAATACATGCAA TACAACGCAGGTCCATCT					
		<i>S. agalactiae</i>	Species specific 16S rRNA	208	94°C for 4 min; (5X); 60°C for 45s; 72°C for 45s; 72°C for 5 min; (20X)	(Ahmadi <i>et al.</i> , 2009)

3.10 ANTIMICROBIAL SUSCEPTIBILITY TEST

3.10.1 Antimicrobial susceptibility test (AST)

The disc diffusion test was used for *in-vitro* determination of the antimicrobial resistance profiles of the *S. aureus*, *S. agalactiae* and *E. coli* isolates based on the Clinical and Laboratory Standards Institute guidelines (CLSI, 2007). The antibiotic susceptibility test was performed using the Kirby-Bauer disc diffusion method (Bauer, 1966). In performing the test, a pure colony from each *S. aureus*, *S. agalactiae* and *E. coli* isolate was transferred into eppendorf tubes containing 500 µL saline solution and vortexed. The suspension was adjusted to 0.5 McFarland standard solution (approximately 1×10^6). An aliquot of 100 µL of the suspension was spread-plated onto Mueller-Hinton agar. The antibiotic resistant profiles of the isolates was determined against a panel of twelve (12) different antimicrobial agents obtained from Mast Diagnostics, South Africa (Table 3.3). The discs impregnated with therapeutic concentrations of the different drugs were placed at equitable distances on the inoculated plates. The plates were incubated aerobically at 37°C for 24 hours. After incubation, the growth inhibition zone diameters around the discs were measured in millimetres. Growth inhibition zone diameter data were compared with standard reference values in order to classify isolates as susceptible, intermediate resistant or resistant to a particular antibiotic (Wikler, 2007).

Table 3.3: Details of antibiotics used in the study to determine the antibiotic resistant profiles of the isolates.

Group	Antibiotic	Abbr.	Disc conc.(µg)	Reference values		
				Sensitive	Inhibition	Resistant
Penicillin	Amoxicillin	AM	10	≥17	14-16	≤13
	Amoxicillin clavulanic acid	AMC	10	≥18	14-17	≤13
	Piperacillin	PIP	100	≥21	18-20	≤17
	Ticarcillin	TIC	75	≥20	15-19	≤14
	Tazobactam	TZP	10	≥18	14-17	≤13
Cephalosporin ¹	Cefalothin	KF				
Cephalosporin ²	Cefuroxime		30	≥23	15-22	≤14
Cephalosporin ³	Cefotaxime	CTX	30	≥26	23-25	≤22
	Ceftiofura					
	Ceftazidime	CAZ	30	≥21	18-20	≤17
Cephalosporin ⁴	Cefepime	FEP	30	≥18	15-17	≤14
	Cefquinome	FEP	30	≥25	19-24	≤18
Cephameycin	Cefoxitin	FOX				
Monobactam	Aztreonam	ATM	30	≥21	18-20	≤17
	Ertapenem	ETP	10	≥22	19-21	≤18

S=susceptible; I=intermediate resistant; R=resistant; 1 = first generation antibiotics; 2 = second generation antibiotics; 3 = third

generation antibiotics and 4 = fourth generation antibiotics

3.12 DETECTION OF VIRULENCE GENES IN *S. aureus* ISOLATES

PCR targeting classical *S. aureus* virulence genes: *sea*, *seb*, *sec*, *sta* and *Etb* was conducted using specific primers that appear in Table 3.4 (Peles *et al.*, 2007). Amplifications were performed using DNA thermal cycler (model- Bio-Rad C1000 Touch TM Thermal Cyclers, Johannesburg South Africa). The reactions were prepared in 25 μ L volumes that constituted 12.5 μ L of 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4 μ M MgCl₂ and loading buffer), 11 μ L nuclease free distilled water, 0.5 μ L mixture of the forward and reverse primers and 1 μ L of template DNA. PCR cycling conditions for the different target genes are shown in Table 3.4. All the PCR reagents used in this study were Fermentas USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa.

Table 3.4: Oligonucleotide primers used for the amplification of virulence genes of *S. aureus* isolates

Primers	Sequence (5'-3')	Target gene	Amplicon size (bp)	PCR conditions and cycles	Reference
Seb	GTATGGTGGTGTAAGTGAAGC	seb	164	95 °C for 60 s (30X); (55 °C for 60 s; 72 °C for 60 s) 72 °C for 10 min	(Peles <i>et al.</i> , 2007)
	CCAAATAGTGACGAG TTAGG				
Sec	AGATGAAGTAGTTGATGTGTATGG	sec	451	94°C for 5 min (35X);(94°C for 2 min, 57°C for 2 min, 72°C for 1 min), 72°C for 7 min	(Mehrotra <i>et al.</i> , 2000)
	CACACTTTTAGAATCAACCG				
Etb	CACACATTACGGATAATGCAAG	Etb	604	95 °C for 30 s (25X); (52°C for 30s), 1.5min of extension at 72°C	(Noguchi <i>et al.</i> , 2006)
	TCAACCGAATAGAGTGAACCTTATCT				

3.13 DETECTION OF *S. agalactiae* VIRULENCE GENES

The presence of genes coding for virulence determinants *Cluster P1-1*, *hyB*, *fbsA*, *cpsC,D,E* and *cps J, K*, *eneu B* in *S. agalactiae* was determined using specific primer pairs (Arpini *et al.*, 2016; Pang *et al.*, 2017). The primer sequences, target genes, amplification conditions and amplicon sizes are listed in Table 3.5. Amplifications were performed using DNA thermal cycler (model-Bio-Rad C1000 Touch TM Thermal Cycler, Johannesburg South Africa).

The reactions were prepared in 25 µL volumes that constituted 12.5 µL of 2X Dream Tag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4 mM MgCl₂ and loading buffer), 11 µL nuclease free distilled water, 0.5 µL mixture of the forward and reverse primers and 1 µL of template DNA. All the PCR reagents used in this study were Fermentas USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. The PCR cycling conditions for the different target genes are shown in Table 3.4.

Table 3.5: Oligonucleotide primers used for the amplification of virulence genes in *S. agalactiae* isolates

Primers	Sequence (5'-3')	Amplicon size (bp)	PCR conditions and cycles	Reference
<i>Cluster P1-1</i>	CTCATCAGTTGACGATTGTTC	751	95 °C for 60 s (30X); (55 °C for 60 s; 72 °C for 60 s); 72 °C for 10 mins	Pang <i>et al.</i> , 2017
	CCATTGCCTGTTGCTCAC			
<i>CpsJ,K, eneu B</i>	GGATTAGCCTTTATCACACTT	668	94°C for 5 (30X); 94°C for 30s, 57°C for 1 min, 72°C for 2 min; 72°C for 10 mins	Arpini <i>et al.</i> , 2016
	GCAACTTCTTTAGTATTGTATA			
<i>fbsA</i>	GCTTTGGCTTTATATGGGAG	1662	94°C for 5 (30X); 94°C for 30s, 57°C for 1 min, 72°C for 2 min. 72°C for 10 mins	
	GCTACATTAGTAACCTGAGA			

3.12 DETECTION OF VIRULENCE GENES IN *E. coli* ISOLATES

All *E. coli* isolates were screened for the presence of virulence genes (*stx*₁, *stx*₂ and *hlyA*) using specific primers that appear in Table 3.6 (Ateba and Bezuidenhout, 2008). Amplifications were performed using a DNA thermal cycler (model- Bio-Rad C1000 Touch TM Thermal Cycler, Johannesburg South Africa). The reactions were prepared in 25 µL volumes that constituted 12.5 µL of 2X DreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4 µM MgCl₂ and loading buffer), 11 µL nuclease free distilled water, 0.5 µL mixture of the forward and reverse primers and 1 µL of template DNA. The amplifications were performed at 95 °C for 300 s; 45 cycles of 95 °C for 30 s; 62 °C for 60 s; 72 °C for 60 s and a final elongation step at 72 °C for 300 s. All the PCR reagents used in this study were Fermentas USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa.

Agarose gel electrophoresis

PCR amplicons were resolved by electrophoresis on a 2% (w/v) agarose gel and on a horizontal Pharmacia biotech equipment system (model Hoefer HE 99X, Amersham Pharmacia biotech, Sweden) for 1 hour at 80 V, 250 mA using 1X TAE buffer for 50 mins. A 100 bp DNA molecular weight marker (Fermentas, USA) was included in each gel and was used to confirm the size of the amplicons. The gels were stained in ethidium bromide (0.1 µg/mL). A ChemiDoc Imaging System (Bio-RAD ChemiDocTM MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software and to determine the relative sizes of amplicons.

Table 3.6: Oligonucleotide primers used for the amplification of virulence genes in *E. coli* isolates

Primers	Sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
stx1	ATAAATCGCCATTCGTTGACTAC	stx ₁	180	Ateba <i>et al.</i> , 2008
	AGAACGCCCACTGAGATCATC			
stx2	GGCACTGTCTGAAACTGCTCC	stx ₂	255	
	TCGCCAGTTATCTGACATTCT			
hlyA	GCATCATCAAGCGTACGTTCC	hlyA	534	
	AATGAGCCAAGCTGGTTAAGCT			

CHAPTER 4

RESULTS

4.7 BACTERIAL STRAINS AND DNA EXTRACTED

A total of 62 presumptive isolates of (21 *S. aureus*, 20 *S. agalactiae* and 21 *E. coli*) from milk samples were obtained through DNA extraction. The presence of DNA was confirmed by electrophoresis on a 2% (w/v) agarose gel. The DNA was of good quality without any fragmentation. Figure 4.2 indicates an agarose gel image of genomic DNA extracted from the isolates in the study.

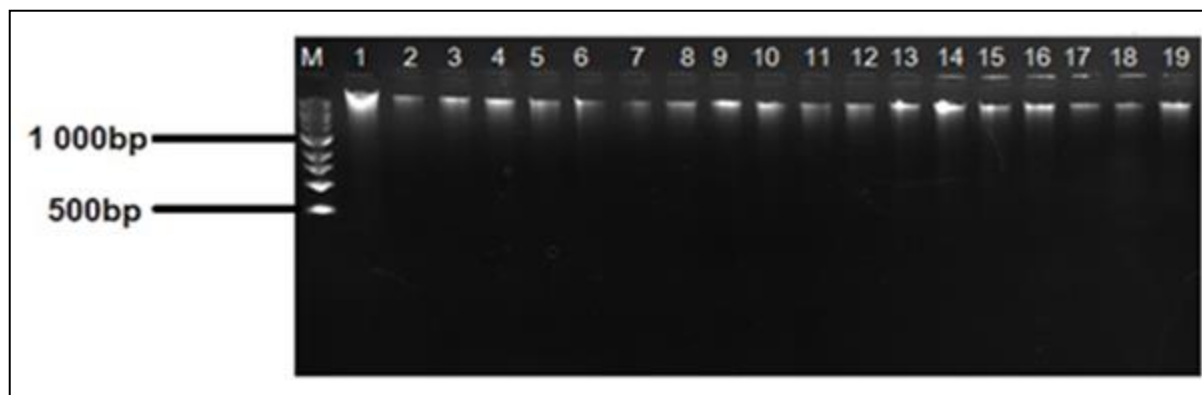


Figure 4.2: Agarose gel (2% (w/v)) image depicting DNA extracted from *S. aureus*, *S. agalactiae* and *E. coli* isolates from (milk samples). Lane M = DNA marker (O'GeneRuler 1 kilo base pairs DNA Ladder), lane 1-7 = Genomic DNA from *S. aureus* isolates, Lane 8-14 = Genomic DNA from *S. agalactiae* isolates and Lane 15-19 = genomic DNA from *E. coli*.

4.8 THE 16S rRNA GENE PCR ANALYSIS

The identities of presumptive *S. aureus*, *S. agalactiae* and *E. coli* isolates from milk samples using DNA extraction were obtained through amplification of species-specific 16S rRNA gene. Of the 62 presumptive isolates, comprising 21 *S. aureus*, 20 *S. agalactiae* and 21 *E. coli*, all (62;

100%) possessed the bacterial rRNA gene. The expected amplicon size of 1 420bp was obtained and Figure 4.3 indicates a 2% (w/v) agarose gel of bacterial 16S rRNA gene fragments amplified from the presumptive *S. aureus*, *S. agalactiae* and *E. coli*) isolates in the study.

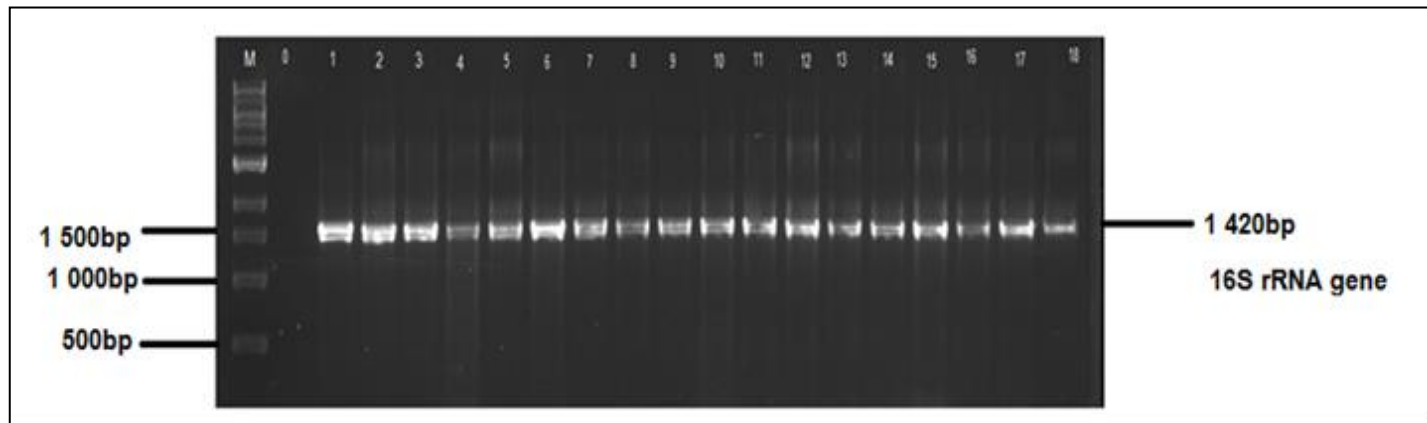


Figure 4.3: Representative image of a 2% (w/v) agarose gel depicting the 16S rRNA universal gene fragments amplified from DNA extracted from the *S. aureus*, *S. agalactiae* and *E. coli* isolates. Lane M = DNA marker (O'GeneRuler 1 kilo base pairs DNA Ladder), Lanes 1-6 = bacterial 16S rRNA gene fragments amplified from *S. aureus* isolates, Lanes 7-12 = bacterial 16S rRNA gene fragments amplified from *S. agalactiae* isolates, Lanes 13-18 = bacterial 16S rRNA gene fragments amplified from *E. coli* isolates.

4.9 MOLECULAR IDENTIFICATION OF *S. aureus*, *S. agalactiae* AND *E. coli* ISOLATES

4.9.1 Proportion of *S. aureus* isolates in the study

The identities of all 21 presumptive *S. aureus* isolates that were positive for bacterial 16S rRNA gene PCR was screened for the *nuc* gene that is specific for *S. aureus*. Of the 21 isolates a large proportion (18; 85.7%) were positive for the *S. aureus* nuclease (*nuc*) gene. A large proportion (8; 44.4%) of the isolates that harboured the *nuc* gene were from raw milk in Rooigrond. In

addition, 6 (33.3%) of the isolates from tank milk in Rustenburg also harboured the *nuc* gene. On the contrary, only a small proportion (4; 22.2%) of the isolates from pasteurized milk in Potchefstroom were positive for the *S. aureus* species-specific gene. Data in Table 4.6 indicates the proportion of isolates that were positive for *nuc* gene fragment. Amplicons with the expected size (279 bp) was obtained and Figure 4.4 indicates a 2% (w/v) agarose gel image of the thermo nuclease (*nuc*) gene fragments amplified from the *S. aureus* isolates in the study.

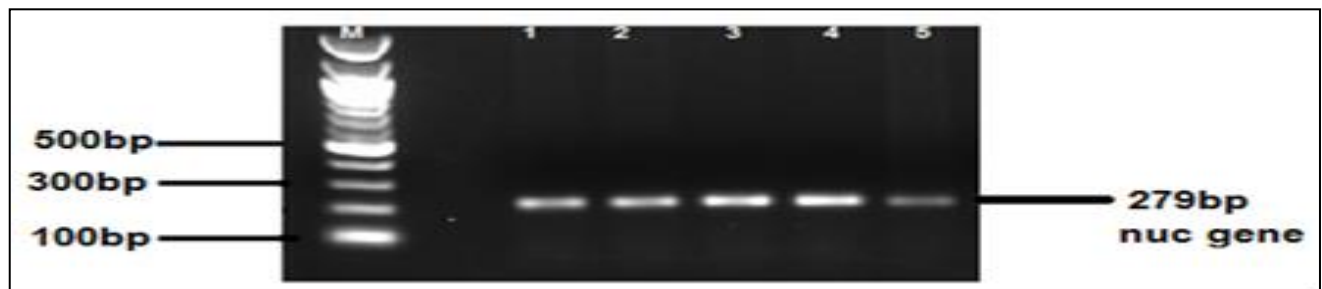


Figure 4.4: Representative image of a 2% (w/v) agarose gel depicting thermo nuclease (*nuc*) gene amplified from all *S. aureus* isolates. Lane M = 100bp DNA molecular weight marker. Lane 1-5 = gene fragments of *S. aureus* isolates from milk samples.

4.9.2 Proportion of isolates *S. agalactiae* species

The presumptive *S. agalactiae* of isolates that were positive for the 16S rRNA gene were screened for the *S. agalactiae* species-specific 16S rRNA gene fragments using primer pairs (C1 and C2) and (V1 and V2). Of the 20 presumptive *S. agalactiae* isolates, 9 (45%) were positive for the 16S rRNA *C1* gene. From these a large proportion (5; 55.56%) were isolates from raw milk in NWU, Molelwane farm, (3; 33.3%) were isolates from tank milk in Klerksdorp while only 1 (11.11%) isolate was from pasteurized milk in Zeerust. In addition, 3 (33.3%) isolates were obtained from raw tank milk in Klerksdorp. Amplicons with the expected size of

208 bp was obtained and Figure 4.4 indicates a 2% (w/v) agarose gel image of the *S. agalactiae* species-specific 16S rRNA (C1) gene fragments amplified from *S. agalactiae* isolates in the study.

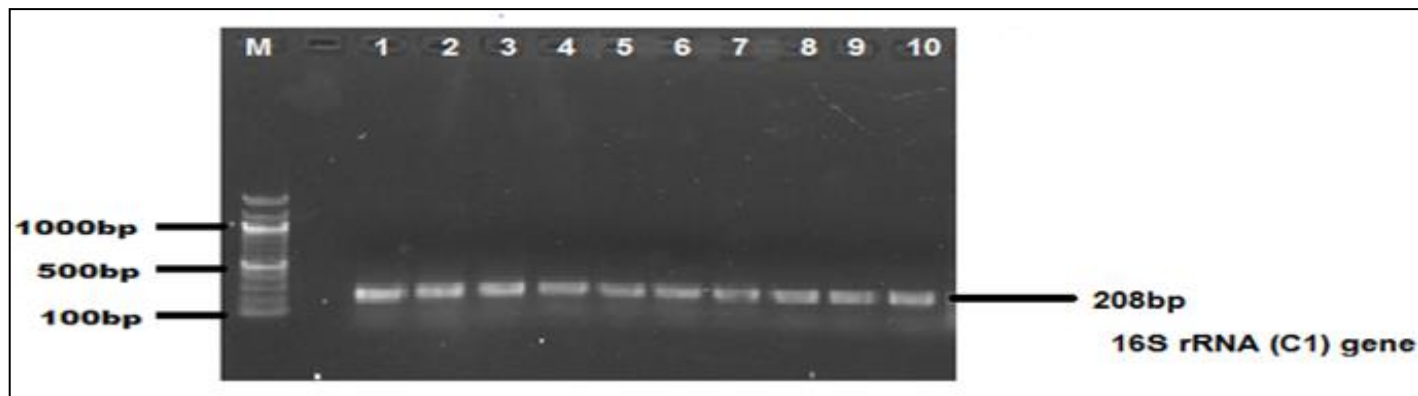


Figure 4.6: Representative image of a 2% (w/v) agarose gel depicting *S. agalactiae* species-specific 16S rRNA (C1) gene amplified from all *S. agalactiae* isolates. Lane M = 100 bp DNA molecular weight marker. Lane 1-10 = gene fragments of *S. agalactiae* isolates from milk samples.

4.9.3 Genus specific amplification of *E. coli* species

All the 21 presumptive *E. coli* isolates were subjected to species-specific PCR assay designed to amplify the *uidA* *E. coli* housekeeping gene. A large proportion (14; 66.7%) of presumptive isolates harboured the *uidA* gene. The positive *E. coli* isolates comprised 8 (57.1%) isolates from raw milk collected at the NWU Molelwane farm, 4 (28.6%) isolates from tank milk in Mafikeng and 2 (14.3%) isolates from pasteurized milk in Potchefstroom. Bands with the expected

amplicon size of 600 bp was obtained and Figure 4.4 indicates a representative image of a 2% (w/v) agarose gel of the *uidA* gene fragments amplified from *E. coli* isolates in the study.

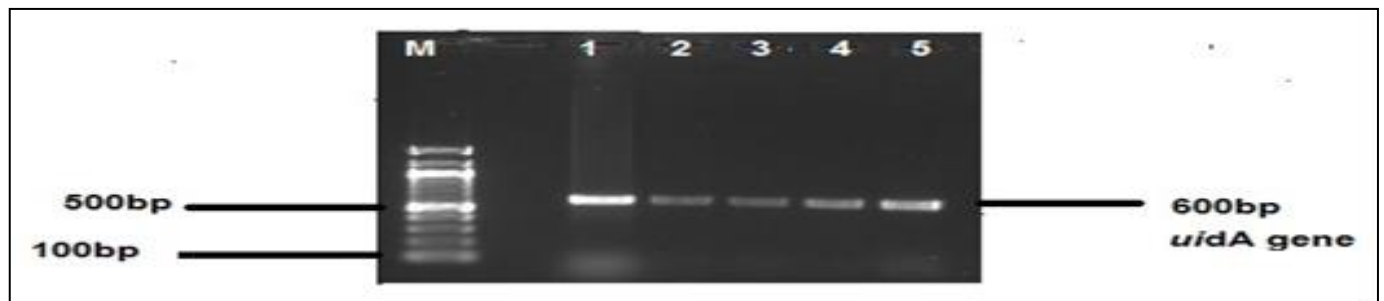


Figure 4.8: Representative image of a 2% (w/v) depicting the *uidA* gene fragments amplified from *E. coli* isolates. Lane M = DNA marker (O'GeneRuler 1 kilo base pairs DNA Ladder). Lane 1-5 = gene fragments of *E. coli* isolates from milk samples.

Table 4.6. Proportion of isolates from milk that were positive for the different target genes in *S. aureus*, *S. agalactiae* and *E. coli* isolates respectively.

Sampling area / sites	No Tested/No positive	Proportions of the various target genes detected in the different isolates			
		16S rRNA	<i>S. aureus</i> (nuc gene)	<i>S. agalactiae</i> 16S rRNA (C1)	<i>E. coli</i> (uidA)
NWU-Molelwane raw milk	NT	9	9	9	9
	NP	9	0	5 (55.56%)	8 (57.1%)
Mafikeng tank milk	NT	7	7	7	7
	NP	7	0	0	4 (28.6%)
Potchefstroom pasteurised milk	NT	11	11	11	11
	NP	11	4 (22.2%)	0	2 (14.3%)
Rooigrond raw milk	NT	9	9	9	9
	NP	9	8 (44.4%)	0	0
Rustenburg tank milk	NT	9	9	9	9
	NP	9	6 (33.3%)	0	0
Zeerust pasteurized milk	NT	9	9	9	9
	NP	9	0	1 (11.11%)	0
Klerksdorp tank milk	NT	8	8	8	8
	NP	8	0	3 (33.3%)	0
Total	NT	62	21	20	21
	NP	62	18 (85.7%)	9 (45%)	14

NT = number tested; NP = number positive: % = percentage

4.10 DETECTION OF VIRULENCE GENES IN *S. aureus* ISOLATES

A total of 18 *S. aureus* isolate were screened by PCR analysis for the presence of virulence genes as indicated in Section 3.13 (Chapter 3).

4.10.1 Proportion of isolates positive for the *seb* virulence gene

Of the 18 *S. aureus* isolates, 6 (33.3%) were positive for *seb* virulence gene. The composition of these isolates comprised 3 (33.3%) from raw milk from Rooigrond raw milk, 2 (33.3%) of pasteurized milk in Potchefstroom and 1 (16.7%) pasteurized milk in Mafikeng. A summary of the isolates that possessed the *seb* virulence genes are shown in Table 4.7. Figure 4.10 indicates a 2% (w/v) agarose gel image of *seb* virulence gene fragments (164 bp) amplified from *S. aureus* isolates.

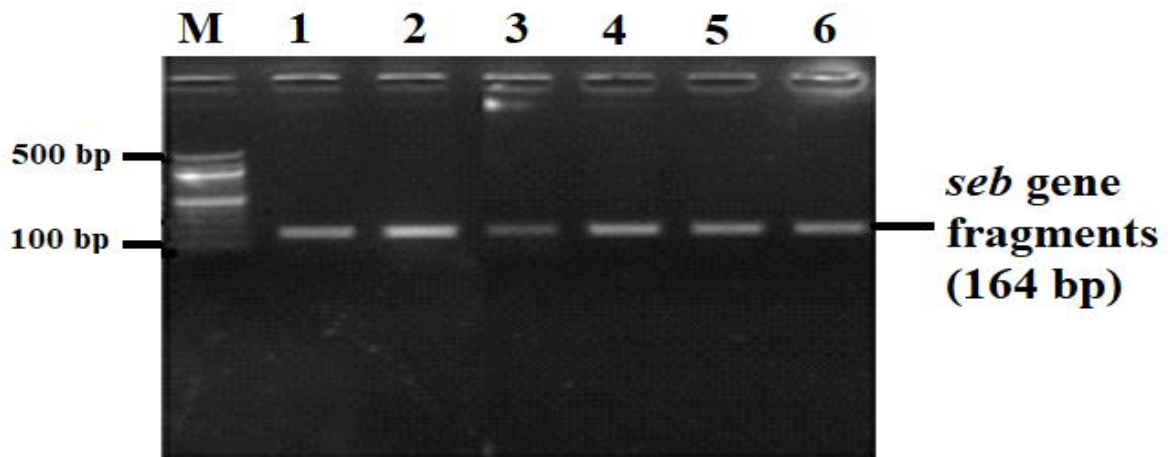


Figure 4.10: Representative image of a 2% (w/v) agarose gel of *seb* gene fragments amplified from confirmed *S. aureus* isolates. Lane M = 100 bp DNA molecular weight marker. Lane 1- 6 = *seb* gene fragments amplified from *S. aureus* isolates Obtained from milk samples.

4.10.3 Proportion of *S. aureus* isolates with the *sec* virulence gene

All the 18 *S. aureus* isolates were also screened for the presence of the *sec* virulence gene and only 3 (16.7%) isolates were positive for this gene. The composition of isolates that harboured the *sec* gene sequence comprised 2 (11.1%) isolates from raw milk in Rooigrond and 1 (50%) from pasteurized milk in Rustenburg. In this study, amplicons with the expected size of 451bp was obtained. Figure 4.11 is a 2% (w/v) agarose gel image of *sec* virulence gene fragments amplified from *S. aureus* isolates from the milk samples.

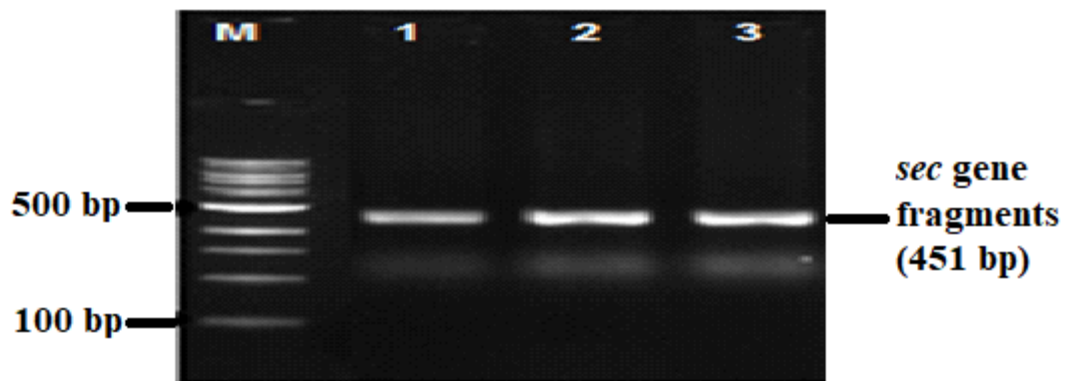


Figure 4.11: Image of a 2% (w/v) agarose gel depicting the *sec* gene fragments amplified from *S. aureus* isolates. Lane M = 100 bp DNA molecular weight marker. Lane 1- 3= *sec* gene fragments amplified from *S. aureus* isolates from milk samples.

4.10.4 Prevalence of the *etb* virulence gene among *S. aureus* isolates

All the 18 confirmed *S. aureus* isolates were also screened for the exfoliative toxin b (*etb*) and only 5 (27.8%) were positive for this gene. A large proportion (2; 40.00%) each of the *etb* positive isolates were from pasteurized milk obtained from Potchefstroom and Rustenburg, respectively while 1 (20%) was from pasteurized milk collected in Rooigrond . Figure 4.12 indicates a 2% (w/v) agarose gel image of the *etb* virulence gene fragments amplified from *S. aureus* isolates.

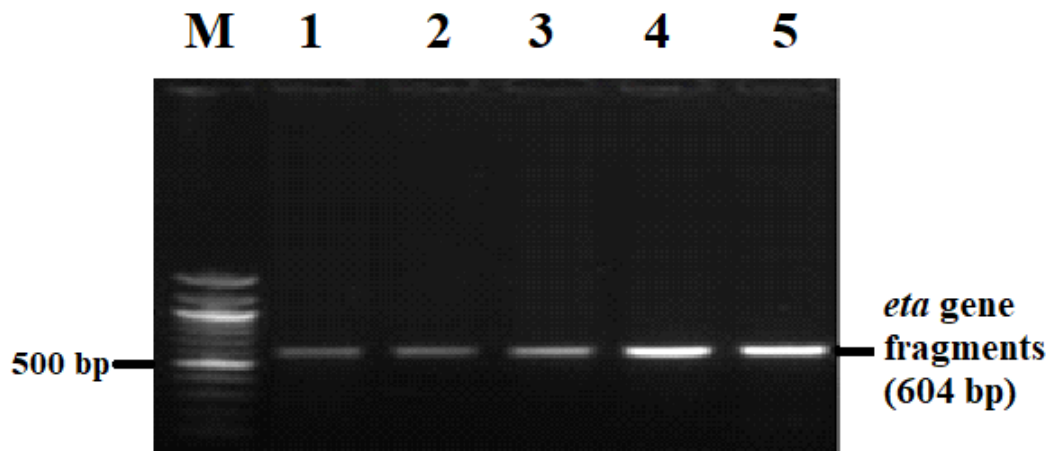


Figure 4.12: A 2% (w/v) agarose gel image of the exfoliative toxin b (*etb*) gene fragment amplified from *S. aureus* isolates. Lane M = 100 bp DNA marker, Lane 2- 5 = *etb* gene fragments amplified from *S. aureus* isolates obtained from milk samples.

Table 4.7: Proportion of milk isolates that were positive for virulent genes *S. aureus*

Sample area / site	Proportion of isolates positive for the <i>S. aureus</i> virulence genes			
		<i>Seb</i>	<i>Sec</i>	<i>Eta</i>
NW-Molelwane Raw milk	NT	0	0	0
	NP	0	0	0
Potchefstroom Pasteurized milk	NT	4	4	4
	NP	2 (33.3%)	0	2 (40.00%)
Coligny Tank milk	NT	0	0	0
	NP	0	0	0
Rooigrond raw milk	NT	8	8	8
	NP	3 (33.3%)	2 (11.1%)	1 (20%)
Rustenburg Pasteurized milk	NT	6	6	6
	NP	1 (16.7%)	1 (50%)	2 (40.00%)
Zeerust pasteurized milk	NT	0	0	0
	NP	0	0	0
Mafikeng Pasteurized milk	NT	0	0	0
	NP	0	0	0
Klerksdorp Tank milk	NT	0	0	0
	NP	0	0	0
Total	NT	18	18	18
	NP	6 (33.3%)	3 (16.7%)	5 (27.8%)

NT = number tested; **NP** = number positive; % = percentage of that were positive for the different virulence genes.

4.11 DETECTION OF VIRULENCE GENES IN *S. agalactiae* ISOLATES

4.11.2 Proportion of *S. agalactiae* positive for virulence genes

A total of 9 confirmed *S. agalactiae* isolates were screened by multiplex PCR analysis for the presence of the *Cluster PI-1*, *hylB*, *fbsA*, *cps C, D, E*, *cpsJ, K*, *eneuB* virulence genes. Of all the genes, only the *cpsJ, K* and *eneuB* virulence genes were detected in this study. In addition, of the 9 *S. agalactiae* isolates, only 2 (22.2%) were positive for *cpsJ, K* and *eneuB* virulence genes. The number of isolates that were positive for the *cpsJ, K*, *eneuB* virulence genes are shown in Table 4.8. Figure 4.11 indicates a 2% (w/v) agarose gel image of the *cpsJ, K*, *eneuB* virulence gene fragments amplified from *S. agalactiae* isolates from milk.

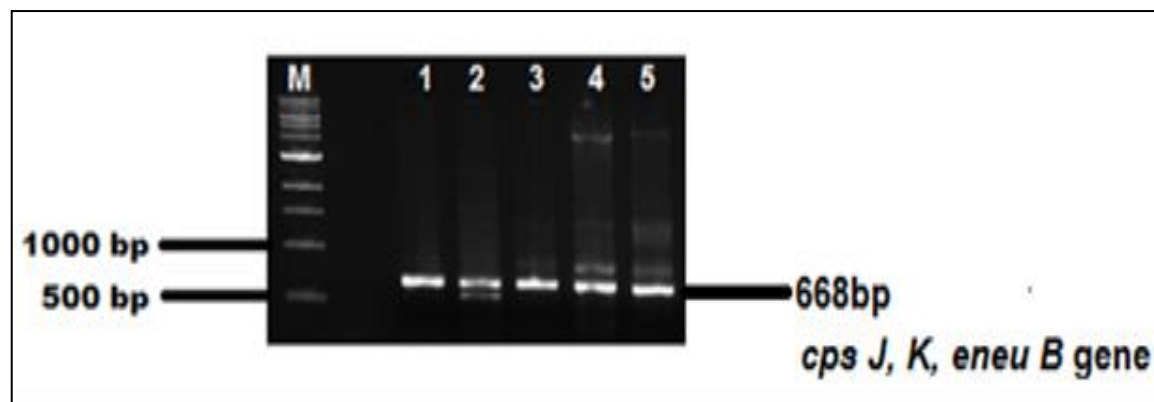


Figure 4.13: A 2% (w/v) agarose gel image of the *cps J, K, eneu B* gene fragments amplified from *S. agalactiae* isolates from milk. Lane M = 100 bp DNA marker, Lane 1-5 = *cps J,K, eneu B* gene fragments amplified from *S. agalactiae* isolates from milk samples.

4.12 DETECTION OF VIRULENCE GENES IN *E. coli* ISOLATES

All the 14 *E. coli* isolates that were positive for the *uidA* gene were screened by PCR for the presence of three virulence genes; *stx*₁ *stx*₂, and *hlyA*. Generally, a large proportion (12; 86%) of the isolates were positive for these virulence genes and the majority (10; 71%) possessing the *hlyA* gene. The *stx*₂ gene was also detected in 8 (57.1%) of the isolates. On the contrary, only four *E. coli* isolates were positive for the *stx*₁ gene. Figures 4.14, 4.15 and 4.16 indicate 2% (w/v) agarose gels showing the *hlyA*, *stx*₁, and *stx*₂ gene fragments, respectively that was amplified from the isolates.

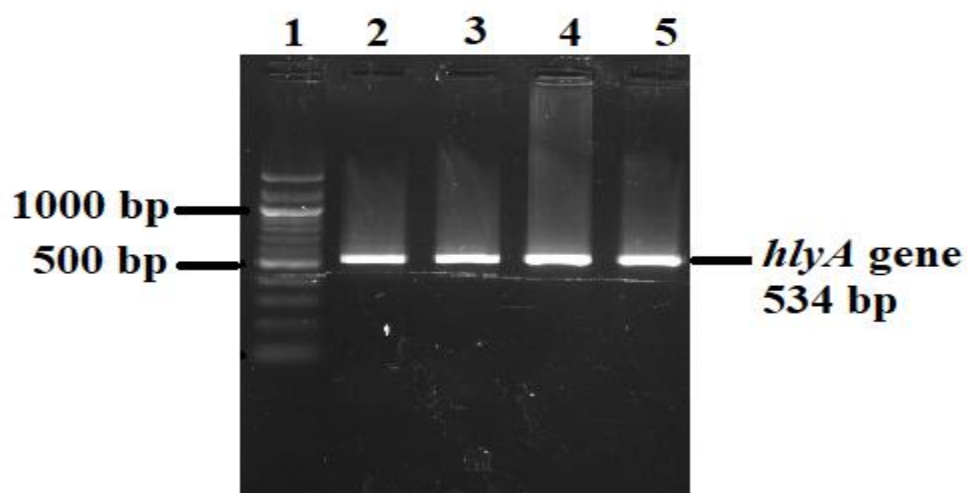


Figure 4.14: Agarose [2% w/v] gel image depicting *hlyA* gene fragments amplified from DNA samples extracted from *E. coli* isolates. Lane 1 = 100 bp molecular marker; Lanes 1-5 = 534 bp fragments of the *hlyA* gene amplified from *E. coli* isolates.

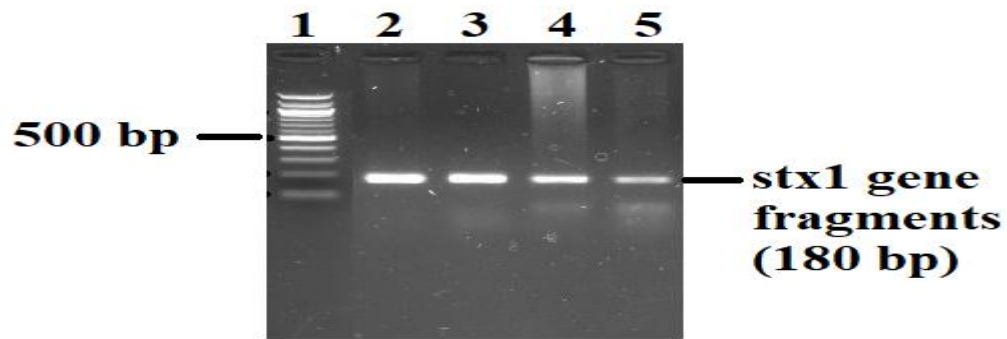


Figure 1.15: Agarose [2% w/v] gel image depicting *stx₁* gene fragments amplified from *E. coli* isolates. Lane 1 = 100 bp molecular marker; Lanes 2-5 = *stx₁* (180 bp) gene fragments amplified from *E. coli* isolates.

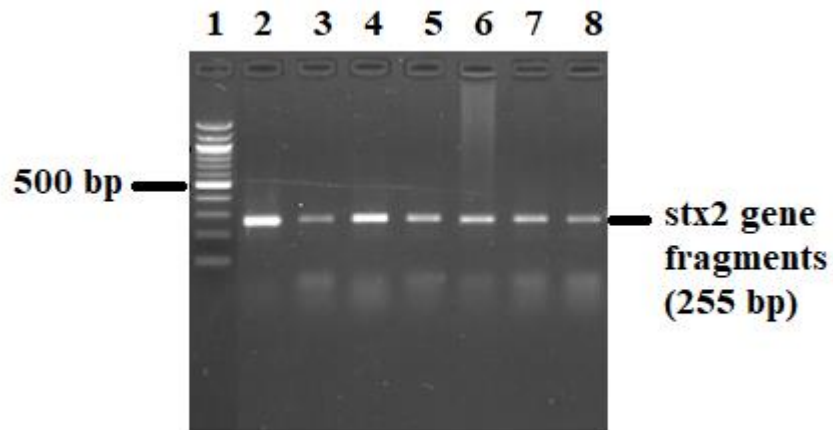


Figure 4.16: Agarose [2% w/v] gel image depicting *stx₂* gene fragments amplified from *E. coli* isolates. Lane 1 = 100 bp molecular weight marker; Lanes 2-8 = *stx₂* (255 bp) gene fragments amplified from *E. coli* isolates.

4.12 Haemolytic patterns of *S. aureus* and *S. agalactiae* isolates

4.12.1 Haemolytic patterns of *S. aureus* isolates

All the 18 *S. aureus* isolates were screened phenotypically to assess their potential to produce haemolytic patterns on blood agar. Among the isolates, 7 (38.89%) *S. aureus* displayed beta (β) haemolytic patterns (Table 4.9). The make-up of this isolates comprise 2 (28.6%) each from raw milk in Rooigrond and pasteurised milk in Potchefstroom. and In addition, 3 (42.9%) isolates were obtained from raw tank milk in Rustenburg.

4.11.2 Haemolytic patterns of *S. agalactiae* isolates

Of the 9 *S. agalactiae* isolates that were screened phenotypically to assess their potential to produce haemolytic patterns on blood agar, 6 (66.6%) isolates, were beta (β) haemolytic as shown in Table 4.9. Beta haemolytic *S. agalactiae* comprised 2 (33.3.0%) isolates each from raw milk collected from the North West University, Molelwane farm, pasteurized milk from Zeerust and unpasteurised tank milk from Klerksdorp.

Table 4.9: Proportion of *S. aureus* and *S. agalactiae* that displayed haemolytic patterns.

Sampling area / sites	Proportions of isolates positive for haemolytic patterns	
	<i>S. aureus</i> (β haemolysis)	<i>S. agalactiae</i> (β haemolysis)
NWU- Molelwane raw milk	None tested	NT=5
		NP=2 (33.3.0%)
Mafikeng tank milk	None tested	None Tested
Potchefstroom pasteurised milk	NT=4	None Tested
	NP=2 (28.6%)	
Rooigrond raw milk	NT=8	None Tested
	NP=2 (28.6%)	
Rustenburg tank milk	NT=6	None Tested
	NP=3 (42.9%)	
Zeerust pasteurized milk	None Tested	NT=3
		NP=2 (33.3.0%)
Klerksdorp tank milk	None Tested	NT=3
		NP=2 (33.3.0%)
Total	NT=18	NT=9
	NP=7 (38.89%)	NP=6 (66.6%)

NT = Number tested; **NP** = Number positive; % = percentage of isolates that produced haemolytic patterns

4.13 ANTIMICROBIAL RESISTANT PROFILES OF *S. aureus* AND *S. agalactiae* AND *E. coli* ISOLATES

A total of 41 confirmed isolates comprising 18 *S. aureus*, 9 *S. agalactiae* and 14 *E. coli* isolates from milk samples were subjected to the disc diffusion antibiotic susceptibility test in order to determine their drug resistant profiles against a panel of 8 antimicrobial agents.

4.13.1 Antibiotic resistance pattern in *S. aureus* isolates

All 18 *S. aureus* isolates were subjected to antibiotic resistance susceptibility tests and results indicated that all of 100% the isolates from milk samples were resistant to Cefotaxime. Large proportions of the *S. aureus* isolates of tank milk in Mafikeng were resistant to Ertapenem (80.95%), Piperacillin (76.19%) and Ticarcillin (47.62). In addition, small proportions of isolates from pasteurized milk in Zeerust were resistant to Cefepime (33.33%) and Amoxicillin (19.05%). On the contrary, none of the *S. aureus* isolates was resistant to Aztreonam and Ceftazidime.

4.13.2 Antibiotic resistance pattern in *S. agalactiae* isolates

All the nine PCR confirmed *S. agalactiae* were subjected to antibiotic susceptibility test and all (100%) of these isolates were resistant to multiple antibiotics. Moreover, drug resistance was frequently observed against Cefotaxime, Ceftazidime, Amoxicillin, Cefepime when compared to Ertapenem and Ticarcillin. A large proportion (100%) of the isolates from tank milk in Klerksdorp were resistant to Cefotaxime. Large proportions of *S. agalactiae* from pasteurized milk from Coligny were resistant to Ceftazidime (80%), Amoxicillin (60%) and of Cefepime (55%). On the contrary, small proportion of isolates of tank milk in Rustenburg were resistant to [of Ertapenem (45%) and Ticarcillin (35%). Despite this, none of these isolates was resistant to Piperacillin and Aztreonam.

4.13.3 Antibiotic resistance profiles of *E. coli* isolates

The antibiotic resistant profiles of 14 confirmed *E. coli* isolates from milk samples was determined and a large proportion (%) were multidrug resistant. In general, all (100%) the isolates from raw milk in North West University farm in Molelwane were resistant to Cefotaxime. Large proportions of *E. coli* isolates of raw milk from Rooigrond were resistant toof Piperacillin (71.43%), Ticarcillin (61.90%) and Amoxicillin (57.14%). On the contrary, small proportions 23.81%) of the isolates from raw milk from Lichtenburg were resistant to Cefepime and Ertapenem respectively. All the *E. coli* isolates in this study were susceptible to Aztreonam and Ceftazidime.

Table 4. 11: The Antimicrobial susceptibility of *S. aureus*, *S. agalactiae* and *E. coli* isolates from milk samples.

Antibiotic class	Antibiotics (disc content)	<i>S. aureus</i> (n=18)			<i>S. agalactiae</i> (n=20)			<i>E. coli</i> (n=21)		
		R	I	S	R	I	S	R	I	S
Penicillin	Amoxicillin (AM 10)	4 (19.09%)	0 (0%)	17 (80.95%)	12 (60%)	0 (0%)	8 (40%)	12 (57.14%)	0 (0%)	9 (42.86%)
	Piperacillin (PIP 100)	16 (76.19%)	0 (0%)	5 (23.81%)	0 (0%)	1 (5%)	19 (95%)	15 (71.43%)	0 (0%)	6 (28.57%)
	Ticarcillin (TIC 75)	10 (47.62%)	3 (14.29%)	8 (38.10%)	7 (35%)	0 (0%)	13 (65%)	13 (61.90%)	0 (0%)	8 (38.10%)
Cephalosporin ³	Cefotaxime (CTX 30)	18 (100%)	0 (0%)	0 (0%)	20 (100%)	0 (0%)	0 (0%)	21 (100%)	0 (0%)	0 (0%)
Cephalosporin ⁴	Ceftazidime (CAZ 30)	0 (0%)	5 (23.81%)	16 (76.19%)	16 (80%)	0 (0%)	4 (20%)	0 (0%)	6 (28.57%)	15 (71.43%)
	Cefepime (FEP 30)	7 (33.33%)	2 (9.52%)	12 (57.14%)	11 (55%)	0 (0%)	9 (45%)	8 (38.10%)	0 (0%)	13 (61.90%)
Monobactam	Aztreonam (ATM 30)	0 (0%)	6 (28.57%)	15 (71.43%)	0 (0%)	4 (20%)	16 (80%)	0 (0%)	5 (14.29%)	16 (76.19%)
	Ertapenem (ETP 10)	17 (80.95%)	0 (0%)	4 (19.05%)	9 (45%)	3 (15%)	8 (40%)	5 (23.81%)	2 (9.52%)	14 (66.90%)

R = Resistant; **I** = Intermediate; **S** = Susceptible; **n** = number of isolates resistant to the different antibiotics

CHAPTER 5

DISCUSSION

5.1 GENERAL DISCUSSION

Milk is a nutrient-rich liquid food produced by the mammary glands of mammals. It is the primary source of nutrition for young mammals, including breastfed human infants before they are able to digest solid food (Van Winckel *et al.*, 2011). Due to the fact that it is an agricultural product that is collected from farm animals, (World Dairy Cow Numbers, 2014) milk is also prone to cross-contamination with pathogens. Given that more than six billion people consume milk and milk products (Hemme and Otte, 2010) worldwide and this accounts for the ever-increasing demand for dairy products coupled with a large demand-supply gap, this provides opportunities for lack of adherence to standard milk production protocols. The implication is that milk supplied to consumers may be contaminated with pathogenic microorganisms and thus present severe public health complications on consumers.

The composition as well as the level of microbial and chemical contamination in milk determines its quality. The components of milk that comprise water, vitamins, proteins, fats and carbohydrates (Gudeta, 1987; Godefay and Molla, 2000) make it an excellent culture medium for the growth and multiplication of different types of microorganisms mainly due to its complex biochemical composition and high water activity (Soomro *et al.*, 2002). Milk may therefore serve as a potential source for the transmission of pathogenic microbes to humans (Garedew *et al.*, 2012). This amplifies the need to adherence to the standard operating procedures and farm management techniques during the production and milk processing procedures (Garedew *et al.*, 2012).

In addition to the public health implications of contaminated milk on humans, in cow mastitis which is the inflammation of the mammary gland caused by bacterial infections of the udder (Ferrero *et al.*, 2014) presents severe challenges to both animals and the farmer. When present, mastitis may result to reduced milk production with severe economic consequences to the farmer (Bhatt *et al.*, 2012). Amongst the bacterial pathogens, *S. aureus*, *E. coli* and *S. agalactiae* are frequently associated with mastitis and milk from infected mammary glands may serve as a potential source for their transfer to other animals within the same herd (Keefe, 1997; Capurro *et al.*, 2010; Elias *et al.*, 2012) or to humans. Moreover, in South Africa, some preliminary data have shown that *S. aureus* and *S. agalactiae* are the pathogens that most commonly associated with mastitis in dairy cattle (Karzis *et al.*, 2018). It is on these basis that the quality of milk should be constantly assessed.

According to Bissong and Ateba, (2020), *S. aureus* infections in milk and dairy sectors were present major economic and severe public health challenges around Mafikeng area. Transmission of *S. agalactiae* pathogen occurs directly from an infected udder to an uninfected udder during the milking process, via milkers' hands, liners, and common udder cloths (Keefe, 1997). Microorganisms which are *S. aureus*, *S. agalactiae* and *E. coli* are bacterial origin and pathogenic and are responsible for mastitis and spoilage of milk (Mungube *et al.*, 2005). A wide range of disease severity, from mild, with only local inflammatory changes of the mammary gland, to severe, with significant systemic signs including rumen stasis, dehydration, shock, and even death was exhibited by cattle with clinical mastitis caused by *E. coli* (Wenz *et al.*, 2006). In a previous study, suggestions were made that previous data have shown that even the antibiotics which are banned in other countries, such as growth promoters, were still being used in South

Africa (Eagar et al., 2012). In a previous study, investigations were made that most of the dairy farms located in the Mafikeng area around North West Province are small-scale farms owned by independent farmers who usually supply fresh milk to processing plants, as well as to the local communities for direct consumption (Bissong and Ateba, 2020). However, such cases, if proper hygienic practices are not observed during the milking process, milk intended for human consumption may become contaminated and eventually pose serious health problems to the consumers. According to (Ateba *et al.*, 2010), previous studies have suggested that knowledge on the occurrence of *S. aureus* remains limited in South Africa especially in the North-West province and determination of its prevalence in milk may provide an indication of the health risks associated with the consumption of milk or milk products has been reported. Feeding untreated mastitis milk can provoke disease in calves and facilitate the transmission of infectious pathogens (Godden *et al.*, 2005). Milk from cows with udder infections has to be discarded thus, is not healthy for both human and animal consumption (Abb-Schwedler *et al.*, 2014).

According to the results obtained indicates that the level of contamination in milk (raw cow's, tank and pasteurized) samples were higher. The presence of *E. coli* in raw milk (75%) is higher than pasteurized milk (2.44%) and tank milk (5.41%). However, it can be said that the possibility of *E. coli* disseminating into raw milk meant for human consumption is normally high due to the presence of animal faeces in a milking environment, unclean dairy parlour environment or equipment as well as unhygienic farm worker (Girma *et al.*, 2014). Raw milk may contain a whole host of pathogens and are dangerous (Girma *et al.*, 2014). Milk pasteurization was introduced as a public health measure in order to destroy human pathogens and eliminate or reduce the activities of spoilage microorganisms (Gunasekera *et al.*, 2002). Further, results

obtained in screening proportion indicate that presence of *E. coli* in pasteurized milk (2.44%) were lower, it can be concluded that after increasing temperature and timing in heating process, the growth of microorganisms were decreasing, liquid (Redmond, 2005). Furthermore, pasteurization value increased with increasing heating time. However, activities of pasteurized milk products can also be contaminated by poor processing and handling conditions and/or poor worker hygiene (Brock *et al.*, 2003). Preliminary identification tests revealed that the large proportion of the *S. aureus*, *S. agalactiae* and *E. coli* isolates of (lactose, sucrose and glucose) fermenting, H₂S and gas, gram negative identified as an *E.* species and gram positive identified as an *S. aureus* and *S. agalactiae* sp. 100% catase positive and oxidase positive/ negative, based on the biochemical results. *Enterobacter* species are members of the *Enterobacteriaceae* family, whose presence in milk indicates fecal contamination since they are inhabitants of the large intestine.

Furthermore, results indicate that 62 (21 of *S. aureus*, 20 of *S. agalactiae* and 21 of *E. coli*) isolates were harboured the bacterial 16S rRNA. It has been reported that the 16S rRNA sequence is of small-subunit rRNA varies in an orderly manner across phylogenetic lines and contains segments that are conserved at the species, genus, or kingdom level (Wilson *et al.*, 1990). Similar to the findings of this study, the use of oligonucleotide primer sequences that target these conserved regions throughout the eubacterial kingdom facilitates amplification of bacterial 16S ribosomal DNA sequences by PCR analysis (Wilson *et al.*, 1990). The 16S rRNA amplification can be used for broad range of applications, including the detection and identification of known pathogens that are difficult to culture, especially if combined with sequence analysis (Rampini *et al.*, 2011).

In this study, the thermo nuclease gene (*nuc*) that is specific for *S. aureus* was reliable for the identification of these isolates with a large proportion (88.89%) from raw milk when compared to tank milk (66.67%) pasteurized milk (36.36%). Similarly, a number have studies have used the *nuc* gene as a specific target sequence for the detection of *S. aureus* isolates (Brakstad *et al.*, 1990; Kateete *et al.*, 2010; Akindolire *et al.*, 2015). Although, a battery of tests including culture on selective media such as MSA and assessment of the potential of presumptive isolates to produce specific enzymes such as Dnase, are required for ideal identification of *S. aureus* especially in clinical samples (Kateete *et al.*, 2010), PCR identification through the detection of the *nuc* gene is a very reliable (Akindolire *et al.*, 2015).

Although a number of phenotypic and serological assays are used in the identification of *S. agalactiae* some of the methods are labour intensive, they have very low sensitivity and results are obtained only after at least 2 to 3 days. Recently, a number of PCR-based methods for have been developed for the identification of group B streptococci including *S. agalactiae* (Ke *et al.*, 2000). Of particular interest in this study, is the use of the 16S rRNA sequences (*C1* gene). Compared with the time-consuming and costly procedures that was also used in this study to identify *S. agalactiae*, the PCR-based assay was highly sensitive in the detection of this pathogen with the proportion in raw milk higher (55.56%) than those from tank milk (37.5%) and pasteurized milk (11.11%). Martinez *et al.*, (2001) and Meiri-Bendek *et al.*, (2002) reported similar observations.

Although extensive work has been carried out previously to detect the presence of pathogenic *E. coli* serovars in water and food samples in the study area (Wose Kinge *et al.*, 2010), the detection

and confirmation of these pathogens is a time consuming process. A few rapid PCR-based techniques are useful for the identification of *E. coli* in both clinical and environmental samples, amplification of the *uspA* and *uidA* gene sequences are unique targets for the detection of *E. coli* (Bej *et al.*, 1991; Chen and Griffiths, 1998; Maheux *et al.*, 2009). Similar to these reports, in this study only 14 *E. coli* isolates were positively identified through PCR amplification of the *uidA* housekeeping gene. In general, the findings of this study indicate that special attention must be directed at limiting and reducing microbial contamination of both raw and pasteurized milk.

Another objective of this study was to determine the antibiotic resistance profiles of pathogenic *S. aureus*, *S. agalactiae* and *E. coli* species isolated from milk samples. According to the findings of this study, motivation was made by the fact that presence of multiple antibiotic resistant *S. aureus*, *S. agalactiae* and *E. coli* strains were revealed. The emergence of antibiotic-resistant microorganisms poses a potential public health concern to treat highly prevalent infections (Haran *et al.*, 2012). Of the various veterinary uses of antibiotics, the high levels of resistance seen in mastitis-causing organisms has been a concern in South Africa (Karzis *et al.*, 2018). Consequently, there is a need to enhance mastitis control measures and to prevent the occurrence of foodborne infections in human's regulatory pathogenic *S. aureus*, *S. agalactiae* and *E. coli* isolates. Antimicrobials are compounds produced to inhibit the growth of microorganisms and especially pathogenic microorganisms. Antibiotic treatment may help in minimizing the economy losses but may lead to drug resistance. In this study, it was found that majority of *S. aureus* isolates showed resistance to penicillin (amoxicillin, ticarcillin and piperacillin), cephalosporin⁴ (cefepime), cephalosporin³ (cefotaxime) and monobactam (ertapenem) while few showed susceptibility to cephalosporin³ (ceftazidime) and monobactam (aztreonam). In a study

involving *S. aureus* and *E. coli* isolates obtained from meat in the formal and informal sectors in South Africa, *S. aureus* isolates were most often resistant to clindamycin (74.3%) and ampicillin (59.5%) penicillin (83.8%) and tetracycline (82.1%) (Jaja *et al.*, 2020). These findings differ from those in the current study. However, the findings of the previous is similar to those involving *S. aureus* from poultry meat in South Africa that reported high resistance to tetracycline when compared to clindamycin (Geornaras and von Holy, 2001). However, these findings are similar to a previous report in which large proportions (60%–100%) of the isolates was resistant to penicillin G, ampicillin, oxacillin, vancomycin, teicoplanin and erythromycin when compared to gentamicin, kanamycin and sulphamethoxazole (8.3%–40%).

A large proportion of *S. agalactiae* isolates showed resistance to amoxicillin, cephalosporin³ (ceftazidime and cefotaxime), cephalosporin⁴ (cefquinome) and monobactam (ertapenem) when compared to susceptibility to piperacillin and monobactam (aztreonam). A large proportion of of the isolates was resistant to ertapenem (45%), ceftazidime (80%), amoxicillin (60%) and cefquinome (55%) while small proportions of the isolates were resistant to Ticarcillin (35%). *E. coli* isolates were most often resistant to penicillin (amoxicillin, ticarcillin and piperacillin) and cephalosporin³ (cefotaxime), when compared to cephalosporin⁴ (ceftazidime) and monobactam (aztreonam).

A further objective of the study was to determine the pathogenicity of the *S. aureus*, *S. agalactiae* and *E. coli* isolates obtained through the amplification of genus-specific virulence genes. In this study, two different types of enterotoxin (*seb* and *sec*) and the the exfoliative toxin (*etb*) virulence genes were detected in the *S. aureus* isolates. The enterotoxin genes are the major

virulence factors of *S. aureus* and therefore responsible for most of the pathogenicity observed in humans, especially when the pathogen is consumed in food products such as milk (Le Loir *et al.*, 2003). These toxins are known to modulate immune response through super antigen activity and various diseases caused by strains that produce them (Peacock *et al.*, 2002). On the contrary, in *S. aureus*, the exfoliative toxin genes are associated with bullous impetigo (Rogolsky, 1979). These findings are different from those of a previous report in which except for the *sec* gene none of the other virulence determinants were detected (Akindolire *et al.*, 2015). However, previous reports revealed great diversity in the occurrence of these toxin genes (enterotoxins, *eta* and *etb*) among *S. aureus* isolates (Fueyo *et al.*, 2001; Mašlanková *et al.*, 2009). The findings of this study are of great epidemiological importance since the *S. aureus* strains harboured the *sec* virulence gene and isolates with this virulence gene have the potential to cause either sporadic cases or food borne outbreaks of infections (Kérouanton *et al.*, 2007).

Group B streptococcus including *S. agalactiae* is a leading cause of neonatal morbidity and mortality in many countries worldwide (Lamy *et al.*, 2006). The infections such as septicemia, pneumonia and meningitis associated with *S. agalactiae* are due to the presence of several virulence genes (Bobadilla *et al.*, 2021). In this study, polysaccharide capsule, encoded by the *cps* gene. These results are different from those of a previous study in which three distinct virulence gene comprising the *cylE* operon encoding a β -hemolysin hyaluronidase gene, the laminin binding protein Lmb (*lmb* gene), and protein C, which includes the α -subunit of C protein (*bca* gene) were identified (Seyed Aboutorabi *et al.*, 2021). These virulence factors are necessary for host-bacterial cell interaction and therefore facilitate the disease-causing ability of these *S. agalactiae* isolates.

The findings of this study showed that raw, bulk and pasteurised milk in the North-West Province is contaminated with toxigenic and multi-drug resistant *S. Aureus*, *S. agalactiae* and *E. coli* strains. There is a need to implement appropriate control measures to reduce contamination as well as the spread of these virulent strains and the burden of disease in humans.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION AND RECOMMENDATIONS

Based on the results of this study, which demonstrated poor quality of raw, cow's milk produced by communal and commercial dairy farmers in Mafikeng area and (tank and pasteurized) milk from shops and supermarkets in the North-West Province of South Africa, it was therefore concluded that such milk is not safe for human consumption. For genus specific genes, thermo nuclease (*nuc*), the 16S rRNA (*C1*) and housekeeping (*uidA*) were successfully isolated. For the growing children and expectant mothers daily diet milk is an essential part and is nutritious food for human beings, so the presence of pathogenic bacteria can harm the whole community. Milk is such a complete mix of nutrients, it is also an ideal breeding ground for bacteria. This is why milk spoils quickly, particularly when it's left out of the fridge.

To our knowledge, this is the first study applying to characterize and detect the presence of *S. aureus*, *S. agalactiae* and *E. coli* species associated with mastitis obtained from raw, cow's, tank and pasteurized milk, antibiotic resistance profiles and the virulence gene profiles of *S. aureus* and *S. agalactiae* strains. This study reports poor microbiological quality characterized by the occurrence of high microbial counts, which may reduce shelf life and can be a reservoir of pathogenic bacteria that potentially pose public health risks. The findings of the study also revealed the presence of virulence genes of *S. aureus* and *S. agalactiae* and antibiotic resistance profiles of *S. aureus*, *S. agalactiae* and *E. coli*. In this study, we concluded that genetic diversity of *S. aureus*, *S. agalactiae* and *E. coli* strains causes mastitis in a province of North-West, South Africa. Safe milk requires that proper hygiene is enforced personnel and ensuring that the

milking equipment and environment are kept clean at all times as this encourages consumer & brand loyalty.

6.2 FUTURE PERSPECTIVE

Accurate (*S. aureus*, *S. agalactiae* and *E. coli*) species identification is essential to enable rapid intervention and the use of appropriate treatment for the control of mastitis disease. Similarly, accurate species identification is also essential for mastitis pathogen research. A regulatory law implementation in milk and dairy industries, training of personnel, good manufacturing practices and monitoring by regular examination with economic penalties about chemical residues is required to do milk safety. One other area of possible future research that may yield novel approaches to the control of bovine mastitis. safety of milk continues to be a topic of concern in the dairy industry and public health communities. Attention must be paid to save the raw milk as well as the pasteurized milk from the thread of milk quality related problems and public health concern. Further research is needed to make the strategies of eliminating the problems related to raw milk as well as pasteurized milk.

6.2 LIMITATIONS OF THE STUDY

The limitations of the study of included the following;

1. Lack of access to samples from the target sites as this impacted the design or methodology used
2. The methods used to analyse the samples was modified due to limited access to funds and thus metagenomic analysis was not conducted. This had a significant influenced on the results obtain in the study and thus the interpretation of the research findings.

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APPENDICES

APPENDIX A

Appendix 1. A: Details of all the materials; culture media, chemicals enzymes and reagents used in this study.

Culture media

Triple sugar iron (TSI)	g/L
Meat extract	3.0
Yeast extract	3.0
Tryptone	18.0
Meat peptone	2.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferric ammonium sulphate	0.2
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	10.5

63 g of the above components were dissolved in 1 L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121°C for 15 minutes. TSI agar (Biolab, Merck

Diagnostic, South Africa) was used as a medium for preliminary identification of (*S. aureus*, *S. agalactiae* and *E. coli*) based on its biochemical profile.

Blood agar base supplemented with 5% blood g/L

Special peptone	23.0
Starch	1.0
Sodium chloride	5.0
Agar	10.0

Final pH 7.3 ± 0.2 @ 25°C

39 g was suspended in 1 L of distilled water. The medium was dissolved completely by boiling. Thereafter, was sterilized by autoclaving at 121°C for 15 minutes. After cooling to 50°C, it was mixed well with sterile ox blood and poured into sterile petri dishes.

Buffered peptone water **g/L**

Peptone	10.0
Sodium chloride	5.0
Di-sodium hydrogen phosphate	3.5
Potassium dihydrogen phosphate	1.5

20 g of these materials were added in 1 L of distilled water and dissolved. It was autoclaved at 121°C for 15 minutes, then after cooling off it was poured into sterile conical centrifuge tubes.

Buffered peptone water (Biolab, Merck Diagnostic, South Africa) was used for serial dilution when plating isolates on Nutrient agar for colony count.

Nutrient broth	g/L
Meat extracts	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	8.0

16 g of these materials were added in 1 L of distilled water and dissolved. It was autoclaved at 121°C for 15 minutes, then after cooling off it was poured into sterile falcon tubes. Nutrient broth (Biolab, Merck Diagnostic, South Africa) was used as a pre-enrichment medium for DNA extraction overnight cultures.

Nutrient agar	g/L
Gelatin peptone	5.0
Beef extract	3.0
Sodium chloride	8.0
Agar no.2	12.0

28 g of powder was added to 1 L of deionized water and dissolved. The solution was allowed to soaked for 10 min and swirl to mix. It was sterilized by autoclaving for 15 min at 121°C. The

medium solution was allowed to cool to 47°C. The mixture was mixed well then and poured onto sterile petri dishes.

Muller-Hinton agar	g/L
Meat infusion	5.0
Casein hydrolysate	17.5
Soluble starch	1.5
Agar	14.0

38 g of these materials were added in 1 L of distilled water and dissolved. The media was autoclaved at 121°C for 15 minutes, then after cooling off it was poured into sterile petri-dishes. The medium was used to determine the antimicrobial susceptibility profiles of (*S. aureus*, *S. agalactiae* and *E. coli*) isolates.

CHEMICALS

Buffers (50X TAE)

Thermo scientific 50 X TAE-Electrophoresis-buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) stock solutions was supplied to by Thermo Scientific, Johannesburg, South Africa. Resolving DNA or Amplified PCR products, a 1X TAE buffer was prepared for gel electrophoresis.

Sodium hypochlorite

10% (v/v) sodium hypochlorite working solution was prepared by aliquoting 10 ml of stock solution sodium hypochlorite into 1 L Duran bottle with 900 ml of distilled water. The solution was kept at room temperature and used as a disinfectant of the working station.

70% Ethanol

Absolute ethanol (90% v/v) which was supplied for by Merck, Diagnostics, South Africa was used to prepare a 70% (v/v) working solution by; aliquoting 750 ml of absolute ethanol into 1 L Duran bottle with 300 ml of distilled water. The solution was kept at room temperature and used as a disinfectant of the working station.

Ethidium Bromide

In a 5 ml Duran bottle a stock solution of 10 mg/ml was prepared by dissolving the powder into distilled water and a masking tape was used to protect the solution from light, and then stored at room temperature. A final concentration of 1 μ l was used for staining the gel for electrophoresis for a clear visual of DNA or PCR products amplicons.

DNA Loading Dye (6X)

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

30% (w/v) glycerol

The above contents were mixed into a 50 ml Duran bottle. The solution was filter sterilized using 0.45 µm filter and stored at room temperature. The solution was used for agarose gel electrophoresis of extracted DNA.

DNA Ladder/Marker

The standard DNA ladders GeneRuler 100 base pairs and O'GeneRuler 1 Kilo base pairs were used to determine the relative amplicon sizes after the gel electrophoresis. The ladders were supplied for by Thermo Scientific, Johannesburg, South Africa.

Oligonucleotide Primers

The primer (forward and reverse) sets used in this study to amplify the (*Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli*) specific genes, and virulence genes were synthesized and supplied for by the Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa. All primers were supplied in a lyophilized state and provided a synthesis report on how to reconstitute a 100µM stock solution. A 1.5 µl sterile eppendorf tube was used to create and prepare a working solution by aliquoting the required volume of both the forward and reverse primers sets. The primer sets were stored in their separate tubes at -4°C for future use.

ENZYMES

Master Mix (2X DreamTag Green)

2X Dream tag master mix (0.4mM dATP, 0.4mM dCTP, 0.4mM dGTP and 0.4mM dTTP, 4mM MgCl₂ and loading buffer) was used for PCR amplification of target genes and stored at -20 °C

for future use. The enzyme was produced by Fermentas, USA and supplied for by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa.