Characterization of some virulence and antibiotic resistance genes of *Staphylococcus aureus* isolated from cases of Bovine Mastitis in Nkonkobe Municipality, Eastern Cape Province, RSA.

University of Fort Hare
Together in Excellence

By

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DECLARATION

I, the undersigned, declare that the dissertation hereby submitted to the University of Fort Hare for the degree MSc (Microbiology) and the work contained therein is my own original work and has not previously, in its entirely or in part, been submitted to any university for a degree.

Signed.....this.....day of.....2015

Supervisor.....

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LIST ABBREVIATIONS

2D gel	2-Dimensional gel
AFLP	Amplified Fragment Length Polymorphism
AME	Aminoglycoside-modifying enzymes
bDNA	Branched Deoxyribonucleic Acid
blaZ	Penicillin resistance gene
CA-MRSA	Community Acquired Methicillin-Resistant *Staphylococcus aureus*
CLSI	Clinical Laboratory Standard Institute
CMT	California Mastitis Test
CNS	Coagulase negatie staphylococcus
CPS	Coagulase positive staphylococcus
DCT	Dry Cow Therapy
DHI	Dairy Herd Improvement program
DNA	Deoxyribonucleic Acid
EC	Electrical Conductivity
ELISA	Enzyme-Linked Immunosorbent Assay
EMRSA	Epidemic Methicillin-resistant *Staphylococcus aureus*
FISH	Fluorescence In Situ Hybridization
HA-MRSA	Human Acquired Methicillin-Resistant *Staphylococcus aureus*

hVISA	heterogeneous Vancomycin-Intermediate *Staphylococcus aureus*
IgG	Immunoglobulin G
IRT	Infra-Red Themography
MALDI-TOF-MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
*mec*A	Methicillin resistant gene
*mec*I	Methicillin resistance regulatory protein
*mec*R1	Methicillin resistance protein MecR1
MLST	Multilocus sequencing typing
mRNA	Messanger Ribonucleic Acid
MRSA	Methicillin-Resistant Staphylococcus aureus
MRSP	Methicillin *Staphylococcus pseudintermedius*
MSA	Mannitol Salt Agar
PBP2a	penicillin-binding protein 2a
PCR	Polymerase Chain Reaction
PCR-SSCP	Polymerase Chain Reaction single-stranded conformation polymorphism
PFGE	Pulsed-Field Gel Electrophoresis
pH	Potential of Hydrogen
PNA FISH	peptide nucleic acid fluorescence In Situ Hybridization

RFLP	Restriction Fragment Length Polymorphism
RFLP-PCR	Restriction Fragment Length Polymorphism Polymorase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
SCCs	Somatic Cell Counts
spa	Staphylococcal protein A
ssDNA	Single-stranded Deoxyribonucleic acid
Taq DNA	*Thermus aquatics* Deoxyribonucleic acid
VISA	Vancomycin-Intermediate *Staphylococcus aureus*
VRSA	Vancomycin-Resistant *Staphylococcus aureus*
HPA	Health Protection Agency
API Staph kit	Analytical Profile Index Staph kit
ATCC 29213	American Type Culture Collection
µl	Microlitre
bp	Base pairs
CDC	Center for Disease Control and Protection
ETA	Exfoliative A gene
ETB	Exfoliative B gene
IgG	Immunoglobulin G
NaCl	Sodium Chloride
RAPD	Random Amplified Polymorphic DNA

SCC*mec*	Staphylococcal cassette chromosome
TSS	toxic shock syndrome

SUMMARY

Staphylococcus aureus is one of the predominant causative agents of mastitis disease in dairy herds. Mastitis disease has a negative impact in the economic losses in the dairy sector across the globe. The aim of this study is to detect some of the virulence genes in the *S. aureus* isolated from 400 milk samples of subclinical and clinical mastitis dairy cows in Fort Hare dairy farm and Middle Drift dairy farm in Alice in the Eastern Cape province of South Africa. In addition antibiotic resistance pattern and antibiotic resistance genes were investigated. Gram-staining, oxidase test, catalase test and API Staph kit were preliminary biochemical tests used for the identification of *S. aureus* isolates. The MALDI-TOF-MS was also used for further identification. Polymerase chain reaction was performed of genes encoding antibiotic resistance as well as clumping (*clf*A), coagulase (*coa*) gene, toxic shock syndrome (*tsst*), exfoliative toxin A and B (*eta* and *etb*), and the gene segment encoding the immunoglobulin G binding region and X region of protein gene *spa*. A total of 20 (5%) *S. aureus* strains obtained from 400 milk samples from the two farms were subjected to 16 antibiotics for antibiotic susceptibility testing. In Middle Drift dairy farm 11 (5.5%) isolates were obtained from 200 samples and 9 (4.5%) isolates were obtained in Fort Hare dairy farm from 200 samples. A large percent of the isolates were resistant to penicillin G (60%), followed by trimethoprim (60%) and tetracycline (60%), trimethoprim-sulfamethaxazole (55%), telithroprim (55%) and doxycycline (45%). Most of the isolates were sensitive to several (50-85%) antibiotics. Of the twenty isolates tested 12 samples contained the penicillin antibiotic resistance gene (*bla*Z gene), 8 samples contained at least one aminoglycoside-modifying enzyme gene (AME gene); the (*aac(6')/aph(2'')* gene and no amplification occurred for *aph(3')-IIIa* and *ant(4')-Ia*) genes. In the case of the tetracycline antibiotic resistance gene (*tet*K and *tet*M), 2 samples contained *tet*M and a single sample contained *tet*K gene. No amplification was observed for the

erythromycin antibiotic resistance genes (*ermA*, *ermB*, *ermC*, *Mef* and *msrA*). All the samples tested were negative for the expression of toxic syndrome gene (*tsst*), *etb*, and Immunoglobulin G binding region. However, amplification of the clumping factor was observed in 7 (35%) isolates of *S. aureus*, exfoliative toxin (*eta*) expressed 4(20%) isolates; coagulase gene (*coa*) yielded six DNA bands of six differences sizes from 16 (80%) isolates. A total of four different bands size were expressed for the spa X region from 12 (60%) isolates. The data obtained in this study suggests that poor hygienic practices and inadequate management practices are responsible for the increase in *Staphylococcus aureus* isolation. The high resistance of *S. aureus* to antibiotics and the distribution of virulence genes contribute in bovine mastitis in these farms may cause health problems in the community consuming raw milk purchased from these farms.

CHAPTER 1

INTRODUCTION

1.1Raw milk and mastitis

The general health and human welfare often depends on meeting primary nutritional needs. Milk and milk derived products have formed an essential part of daily nutrition and as the modern food processing technologies have slightly developed a variety of milk derived products have elevated rapidly (Javaid *et al.,* 2009). Furthermore, the world's population growth has put tremendous demand in population of milk by dairy farms as it is both economic food and industrial raw milk product (Ratafia, 1987).

Raw milk is regarded as a good liquid environment for the propagation of a huge number of microorganisms. When the milk is drawn from the udder of a health cow, it possesses microbes that have somehow embedded themselves inside the teat through the teat canal and are flushed out during milking. Several hundred to several thousands of microorganisms per milliliter are highly likely to be found during the milking process (Kumar *et al.,* 2010).

In dairy herds mastitis has an overwhelming negative impact as it results in physical, chemical and bacteriological alterations in milk of dairy cattle resulting in reduced quality and quality of milk yield (Sharma *et al.,* 2007). Thus, public interest in the welfare of animal production of this threatening disease as a predominant source of pain and stress for the affected cows give substantial focus to mastitis as a major problem (Fitzpatrick *et al*., 1998).

Mastitis is the inflammatory response of the mammary gland tissue and has been implicated as the major factor of huge economic struggles in dairies. Various species of microorganisms of about 150, mostly bacteria; exhibit a massive potential to cause this disease and are classified as: harmful and environmental udder pathogens. The detrimental udder pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae* and Mycoplasma spp. Microorganisms that are mostly involved in environmental mastitis in dairy herds are *S. aureus*, *Streptococcus* spp, *Escherichia coli* (*E. coli*), *Salmonella*, *Klebsiella*, *Coryne bacterium* and *Mycoplasma* spp. These pathogens propagate between cattle during milking by tools that are utilized such as teat cups, wiping towels, and more often by milker's hands (Fox *et al.,* 1993; Benic *et al.,* 2011).

Several microorganisms are capable of causing mastitis in dairy cows, however; *S. aureus* is the most common disease causing agent of bovine mastitis. Although different management practices for decreasing the prevalence of *S. aureus* have been employed under current dairying, a lot of dairy farms still encounter some level of infection with this microorganism. *E. coli* is found in environmental reservoirs which are likely to result in teat end contamination, where the infection is typically of rapid onset and acute (Hill *et al*., 1984). Consequently, it is quickly observed and

prolongs for a short period of time. Its opportunity to transmit a new infection is low, under the conducive circumstances, but the ability of the gland to get a new infection from other sourced is significantly high (Leigh *et al.,* 2008). Prevalence of mycoplasma mastitis seems to be significantly high in several regions throughout the globe. Amongst 12 species of mycoplasma and Acheloplasma that have been found in milk samples, Mycoplasma bovigenitalium was the first to be recognized as it was implicated that calves had a high risk of potential to be infected by the organism via contaminated milk, colostrum, and sometimes the organism introduced through the teat of the cow (Fox *et al.,* 2005; Gonzalez *et al*., 2003).

Usually the extent of the inflammatory is categorized as subclinical, clinical and chronic forms, and its rate of propagation is lies on the causative pathogen, the age, breed, and of course the health and lactation stage of the animal. Mild mastitis poses extreme challenges as it does not demonstrate any obvious visible signs and symptoms, and it has huge economic cost implications. On the other hand, clinical mastitis shows visible indications such as redness, swelling and cuts on the teats of cows. Chronic form of mastitis is infrequent but then results in prolonged swelling of the mammary gland. Presently, somatic cell counts (SCCs) are key in payments of milk quality, the more SCCs present in milk, the less payment there is and vice versa. (Yalcin *et al*., 2000; Viguier *et al*., 2009).

Staphylococcal mastitis is the frequent disease and poses the greatest economic concern worldwide. The main reservoir of this bacterium is an infected udder. The organism usually situates itself in the udder and establishes mild subclinical infection for a long period of time

(Tsegaye, 1988). During the process of milking transmission of the bacteria takes place through contaminated milking machines, clothes and milkers hands (Radostitis *et al*., 1994). The extreme levels of contamination can be accomplished rapidly under conducive conditions. Its presence in foods can have detrimental effects to human health, extending a public health problem, as these bacteria yield toxins that can produce toxic food infections (Pieterse and Todorov, 2010).

Massive losses of milk production can be prevented by the maintenance of the healthy udder and decreasing the incidence of udder infection and inflammation. This will therefore, will increase milk production as large economic losses are directly and indirectly incurred through loss of milk during treatment phases, culling of cows and death of clinical mastitis infected cattle (Pieterse and Todorov, 2010). Mastitis control programmes have implicated many components of dairy farming, which include feeding scheme, animal husbandry, hygiene and general health care can play a role in reducing the incidence of udder infections. Necessary efforts to decrease the incidence of mastitis infection within a dairy herd can be performed through the usage of antimicrobial agents in combination with excellent farming practices (Pieterse and Todorov, 2010). Research advocated that clinical mastitis is caused by contaminated dry-off preparation (Nicholls *et al*., 1981), teat wipes (Power, 2003) and wash water used to clean udders prior to milking (Erskine *et al*., 1987) and the potent potential of microbe to rapidly grow in soil and water and its resistance to chemical disinfectants (Bannerman *et al*., 2005).

1.2. S. aureus in raw milk

Staphylococcus aureus in raw milk basically comes from cows infected with mastitis disease, milkers and unhygienic conditions. This bacterium in foods can be a risk to human wellbeing, causing an outbreak, as it has the capacity to cause toxic food infections (Quitana and Cameiro, 2006). The ability of *S. aureus* to clot the plasma is highly attributed to the capacity to yield enterotoxins detrimental to the tissues of the host. The availability of *S. aureus* demonstrates unwholesome conditions in the cattle herd and counts beyond 10^3UFC in milk increase the risk of staphylococcal toxin production less susceptible to the heat processes of pasteurization (Tortora *et al.,* 2005). Under normal circumstances the generation of enterotoxin is found at 40ºC to 45ºC, however; Smith et al (1982) spotted production of toxins at 10ºC to 46ºC. The mammary gland can be infected with *S. aureus* by unhygienic conditions, including coughing or sneezing and not washing hands when handling milk storage equipment, during and after milking (Murray *et al.,* 2006; Fagundes and Oliveira, 2004).

The research conducted by Jay (2005) showed microorganisms found on the hands and on the uniforms of food handlers, especially that of milk; is linked with unhygienic practices as the sole critical factor in the contamination of milk. Human interference and food contamination cannot be entirely avoided, however; suitable refrigeration at 5ºC is one of the ways of inhibiting *S.aureus* contamination and formation of staphylococcal toxin (Tortora *et al.,* 2005 de Oliveira *et al.,* 2011).

The infection caused by *S. aureus* in the mammary gland is often a difficult problem or even impossible to eradicate it completely because it has the capacity to produce a wide range of

defensive enzymes and contagious toxins which destroy the udder tissue and allow the bacteria to find its way in the tissue with ease. This organism can also situate itself in the keratin layer of the teat canal which usually plays a role of inhibitory mechanism of entry of bacteria. Some strains of *S. aureus* have protein A to inhibit phagocytosis, it attaches itself to the antibody and the pathogen remains unrecognizable to the neutrophylic cells. Approximately 50% these strains found in bovine mastitis generate β-lactamase which induces the production of abscesses and fibrosis in the vicinity of the infection. Antimicrobial agents are unable to get through the membranes which cover the infected area hence *S. aureus* has an invading mechanism against the antibiotics (Green and Bradley, 2004).

1.3. Economic implications due to mastitis

Many researchers have documented plenty of work on direct economic losses pertaining mastitis. This disease results in reduced milk production and elevated number of clinical treatments. This leaves farmers with no other choice but to slaughter the infected cows (Beaudeau *et al*., 1993; Gill *et al*; 1990; Pretorius, 2008; Shook, 1989).

The world at large has tried by all means possible to control bovine mastitis because of the greatest negative effect it has on public wellbeing and on the reduction of the composition of milk from animals with mastitis. This has undesirable impact on the safety of milk for the quality of the manufactured food derived from the milk as their shelf-life will be reduced. Milk companies base their payments for the farmers by analyzing the number of SCC. The higher the number of SCC; the lesser the payment and the lower the number SCC and then payment increases. Extra economic

losses result from extensive work, costs of feed, consumables costs, antimicrobial agents, disinfectants, laboratory and veterinary costs (Giesecke, 1978; Pretorius; 2008).

In South Africa present information concerning economic losses reveal that out of every 10 cows in a herd, 4 cows are mastitis free, 1 is clinical mastitis infected and the remaining numbers of cows are infected with subclinical mastitis. The extreme increased prevalence of subclinical mastitis in relatively 75% of herds has some shocking implications for the yield and economy of dairy farming, dairy processing, public health implications and control and prevention of clinical mastitis (Giesecke, 1990; Pretorius, 2008).

Economic estimation losses from mastitis during the year of 1989 in South Africa were nearly R414 per cow per year. However, this amount is now obvious much higher than back then, and will continue to increase unless and only if a dairy farmer adapts some strategic plan in the maintenance and reduction of mastitis (Pretorius, 2008). Many losses in the dairy farms are mostly due to subclinical mastitis (82%) than clinical mastitis (18%). Statistically, a rand lost due to clinical mastitis, about four rands sixty cents is lost due to subclinical mastitis (Giesecke, 1990; Pretorius, 2008). The milk yield and SCC in dairy cattle are collectively utilized to assess losses in milk generation because of subclinical mastitis. This is because management ultimate decisions are based on this marriage pertaining cost effective preventative and control measures of mastitis (Bartlett *et al*., 1990; Pretorius, 2008). The work done by Harmon (1994) suggests that the mastitis or increased SCC is commonly in correspondence with lactose reduction, α-lacto albumin, and content of the fat of the milk, because of decreased synthetic activity in the tissue of the mammary.

1.4. Statement of Problem

Bovine mastitis causes a great burden on public health; it reduces milk production, quality and has extreme economic losses in industry. These factors pose negative impact on the milk value for processing and the value of the processed food stuff made from it.

Economic losses results from increased labour, costs of feed, consumable products costs, drugs used of cow therapy, antiseptics and laboratory and veterinary needs (Giesecke, 1978; Pretorious, 2008). Presently, there are no published studies conducted in Eastern Cape Province concerning the harmful effects of mastitis in dairy cattle and human welfare in the consumption of raw milk. To the best of our knowledge no similar work has been done in this area on the virulence genes and antibiotic resistant genes in *Staphylococcus aureus*. Therefore, the outcomes of the study might elucidate the distribution of the prevalent *S. aureus* in bovine mastitis; help improve control measures of *S. aureus* infections in dairy herds (de Oliveira *et al.*, 2011) in Nkonkobe region, Eastern Cape (RSA).

1.5. Hypothesis

Staphylococcus aureus in bovine mastitis possess some antibiotic resistance and multiple virulence genes.

1.6. Overall objective

Evaluate the incidence of some antibiotic resistance and virulence genes of *Staphylococcus aureus* in bovine mastitis.

1.7. Specific objectives

- To isolate and identify *S. aureus* in cases of Bovine Mastitis
- To determine the antibiotic profiles of the identified of *S. aureus*.
- To ascertain the antibiotic resistance genes.
- To characterize the virulence genes.

CHAPTER 2

2.1. Genus Staphylococcus

Staphylococcus is a genus of bacteria which is broadly distributed across the globe and linked to various kinds of infections of different sites in humans and animals. *Staphylococcus* species are profoundly known of their pathogenecity and ability to inhibit antimicrobial impact. The group *Staphylococcus* is in the bacterial ancestor Staphyloccaceae (Ludwig, 2009). These appear as grape-like clusters and Gram-positive spherical shaped organisms that remain intact to one another following each successive division.

The genotypic paradigm for incorporating an organism to the group of *Staphylococcus* undergoes through the determination of guanine-cytosine content of 30-39 mol% and phylogenetic trees compiled by comparison of 16S rRNA or 23S rRNA sequences (Takahashi *et al*., 1999). The physical characteristics is carried out in accordance with ultrastructure, chemical properties of the cell wall, Gram positive bacteria and catalase reaction positive for all organisms, inspite of *S. aureus* subsp anaerobius and *S. saccharolyticus,* which live without oxygen. This group exhibits over 50 species categorized into two groups according to their capability to yield coagulase activity.

2.1.1. Coagulase-positive Staphylococci

Coagulase-positive Staphylococci comprises of the most contagious *Staphylococci*. *S. aureus* is regarded as the most disease causative microorganism due to its capability to generate a large variety of virulence factors that allows it to settle in different tissues of various species. The coagulase has a mechanism to alter fribrinogen into fribin threads during clotting process (Palmo *et al*., 1999). The protein is coded by *coa* gee which has a conserved and a recurring polymorphism that can be utilized to determined certain smilarities among *Staphylococcus* coagulase isolates (Roeinoso *et al*., 2006). The *coa* gene contains 81-bp tandem short sequence repeats (SSRs) that vary in number and sequencing as determined by RFLP analysis of Polymerase Chain Reaction products (Goh *et al*., 1992).

The *spa* protein is a membrane-bound exoprotein described and famously known for its capability to attach to the Fc site of immunoglobulins of a number of animal species.It is ecoded by the spa gene that consists of a polymorphic (X) and together with conserved site (Korren *et al*., 2004).

The assortment of the *spa* region is high likely to arise from removal and replication of the same units, point mutation and this deviation can be employed in scientific studies of epidemiology. In a study conducted by Frenay and colleagues (1994) implicated epidemic Methicillin-Resistant *Staphylococcus aureus* strains over seven repeats and later on Montesinos and co-workers (2002) mentioned isolates with 11 repeats as most common type responsible in an epidemic human outbreak resulted by Methicillin-Resistant *Staphylococcus aureus*. The *coa* typing molecular procedures are utilized to add value of the spa protein typing by giving more supported

presumption on strain lineage and clonality among isolates with more or less similar or identical *spa* repeated patterns (Tenover *et al*., 1994). It has been noted that the use of multiple genetic markers has become profoundly important for relating strains because recombination will ultimately broaden up resistant species of staphylococcus in such a way that clonal types within a determined given region cannot be differentiated by a particular locus.

S. aureus is known to generate a wide variety of exoproteins that enhance its high potential to inhabitate itself within slime and hemolysins of the host. The attachment of *S. aureus* on biological surfaces is commonly the initiation of the particular primary phase in the development of infections. Slime yield begins attachment to embedded surfaces which serve as protective matrix thereby making bacteria less accessible to the host's fighting mechanism (Coelho *et al*., 2009). The production of the slime is monitored by the *ica* operon (*icaADBC*) and the co-expression of the *icaA* and *icaD* genes ultimately comes to a specific elevation in such generation (Arciola *et al*., 2001).

In the pathogenesis of Staphylococcal infections the α and β hemolysins are the two most imperative factors that are recognized and β-toxin is an Mg^{2+}-dependent sphingomyelinase C which destroys sphingolipid found in animal cell membranes (Linehan *et al*., 2003).The *accessory gene regulator* operon (*agr*) is known as a quorum-sensing gene cluster that possess an up-regulating production activity of concealed factors as α and β-hemolysins, DNAses and sphingomyelinase and proteases. In *S. aureus accessory gene regulator* also has a down regulating activity that yields cell-associated virulence determinants in a cell density-dependent pattern (Lyon

et al., 2000). Two devergent transcriptional units within the *agr* region are maintained and monitored by the P2 RNA II and P3 RNA III promoters. The P3 transcript is responsible for the up-regulation of secreted virulence factors as well as the down-regulation of surface proteins (Novick, 2000).

In a research performed by Coelho and co-workers (2011) examined the existence of some *S. aureus* virulence factors which encompass *coa, spa, hla, ehlb* in an attempt to comprehend the prevalence of *S. aureus* strains at Rio de Janeiro dairy farm in Brazil, and play a significant role in the discovery of preventative strategies to retard the infection rate. Devriese and co-workers (2005) proposed a reclassification of other CPSs which are implicated as important pathogens; *Staphylococcus intermedius* is an example of one. They created the *Staphylococcus intermedius* group (SIG) including *Staphylococcus intermedius*, and *Staphylococcus pseudointermedius* and *Staphylococcus delphini* being new specie. The leukotoxin, enterotoxin, and hemolysins, together with elements essential for biofilm formation were a series of virulence factors found in *S. intermedius* strains isolated from animals (Futagawa-Saito *et al*., 2006).

2.1.2. Coagulase-negative Staphylococci

Coagulase-negative *Staphylococci* group constitutes a wide variety of *Staphylococcus* species and these were initially regarded as less harmful pathogens (Huxley *et al*., 2002). Quite a number of CNS have been isolated from animal clinical specimen which include some of the following: *Staphylococcus simulans, Staphylococcus chromogenes, Staphylococcus warneri,*

Staphylococccus haemolyticus, and *Staphylococccus sciuri* (Lilenbaum *et al*., 2000; Pereira *et al*., 2009; Pyöräla *et al*., 2009; Soares *et al*., 2008).

Bes *et al*. (2000) advocated that the conventional procedures for identification of CNS were firstly designed for identification of human strains and that their limitations for precise identification animal derived strains seems is possibly restricted to volume of information stored in databases. Moreover, the method designed by Bannerman (2003) cannot be used due to its costly expenses and requires plenty of time in clinical laboratory. A series of molecular targets such as the *groEL* gene have been adapted for the identification of Staphylococcus species (Goh *et al*., 1996). The *groEL* gene is known as a general phylogenic marker because of its found almost everywhere and has a conservative nature (Segal and Ron, 1996). It is also a profoundly proven universal DNA target for detection of CNS species by RFLP-PCR.

2.2. Staphylococcal infections in animals

Several studies have a significant increase in animal infections as a result of *Staphylococcus species*. As it stands, *Staphylococcus aureus* is the predominant bacteria which consequently result in mastitis and other harmful infectious diseases. This bacterium is widely distributed in cattle leading to retarded production and quality of milk. For many decades, *S. intermedius* was famously known as a causative pathogen for pyoderma in dogs. More diseases which include the following pyometra, otitis externa and purulent infections of the joints, eyelids and conjunctiva have been associated with this bacterium (Werkenthin *et al*., 2001) Most recently, work done by Devriese and co-workers (2005) proposed a new classification, they came up with the *S. intermedius* group (SIG) including *S. intermedius*, a newly discovered specie *S. pseuintermedius* and *S. delphini*.

Sasaki *et al*. (2007) motivated out that *S. pseudintermedius* is the prominent specie that contributes in this pathogenicity. Again, *S. intermedius* has been implicated that is restricted to feral pigons and *S. delphini* was commonly regarded as a result of suppurative skin lesions in dolphins is currently considered to occupy a larger scale of infectious animal species. *S. aureus* subspecies *anaerobius* has been reported in lymphadenitis in sheep and *Staphylococcus chleferi* subspecies coagulans in external otitis in dogs.

Research concerning CNS was mainly focused on its virulence for animals and man. In veterinary medicine these organisms are of paramount importance as they are regarded as emerging pathogens of mastitis. Neverthless, Coagulase-Negative Staphylococcus are less harmful than most mastitis diseases and infection is often not visible initial stages, they result in prolonged infections, which lead in elevated milk SCCs and reduced quality of milk (Pyöräla and Taponen, 2009). The prevalence of CNS mastitis is higher in cows that are giving birth for the first time than in older cows. This pathogen has been seen in contagious diseases in household pets (Pereira et al., 2009). CNS species differ based on the environmental region under analysis and origin of the sample. Soares and colleagues (2008) discovered the prevalence of *Staphylococcus xylosus* in milk samples infected with mastitis, except *Staphylococcus chromogenes, Staphylococccus simulans* and *Staphylococcus epidermidis* which seem to be most isolated CNS globally from mammary secretion samples (De Vliegher *et al*., 2003; Taponen *et al*., 2006).

Nowadays, antibiotic resistance brings out perplexing remarks of public health and this confusion seems to be alarming due to its short generation period and competent gene recombination

mechanisms. *Staphylococcus aureus* is perfect pathogens that prominently demonstrate the seriousness of antibiotic resistance threat across the world. However, antimicrobial agents fail to act successfully towards all other coagulase-positive staphylococci.

2.3. Diagnosis of Bovine Mastitis

Early diagnosis is very imperative as a result of increased economic costs of mastitis. Some of the assays that are frequently employed to examine the milk quality through detection of the swelling of mammary gland and diagnosis of the infection and its causative pathogens include the analysis of SCCs, enzymatic analysis, California Mastitis Test, Bromo Thymol Blue, whiteside test, trypsin inhibition test, milk pH, and electric conductivity (Pyorala, 2003, Joshi *et al.,* 2006). Colourimetric and fluorometric assays are someof the laboratory enzyme techniques which have been designed for quantifying the concentration of enzymes (NAGase or LDH) elevated during this infectious disease. Mastitis-causing pathogens usually are identified by culturing techniques, but their disadvantage lies with prolonged periods of laboratory work and cost effective materials (Awale *et al.,* 2012).

2.3.1. Somatic Cell Count (SCC)

Milk SCC is often carried out as an indirect indicator of subclinical mastitis (Hamann, 2002). The SCC analysis of milk is currently processed through two systems: The first one is the direct method which is performed by colouring the nuclei of somatic cells and enumerating them routinely from a photograph. Samples of milk are obtained from the cow complex milk. The second method is called indirect method and is conducted through splitting the Deoxyribonucleic acid of the nuclei of somatic cells and quantifying the thickness of the compound. The samples of milk are withdrawn from the primary portion of milk after expulsion has commenced and this procedure is

carried out at quarter level. Mollenhorst and co-workers (2008) suggested that when first milk samples are taken immediately after milk discharge, at least with SCC 50 000-300 00 cells/ml should be quite representative of the quarter complex milk, (Wellnitz *et al*., 2009).

2.3.2. Delaval Cell Counter

The optical fluorescence is the key factor in this counter and propidium iodide is utilized to mark nuclear DNA to assess the SCC in milk. It is a very quick reaction, expensive and the instrument is moveable from one place to another (Viguier et al., 2009).

2.3.3. California Mastitis Test (CMT)

The California Mastitis Test is a cow-side test that is performed on dairy farms using a detergent to identify subclinical mastitis by an indirect estimation of the SCC in milk. The detergent (bromocresol purple) breaks down the cell membrane of somatic cells and followed by the liberation and combination of nucleic acid which then generates a gel-like medium with thickness that is relatively equal to the number of leukocytes (Viguier *et al.,* 2009).

2.3.4. Milk Colour

The colour change in milk is noticed in clinical mastitis and visible damage of the udder as components of blood leak out of the vessels. A quantifying sensor reflecting light intensity can be utilized to evaluate the colour of milk and identify unusual components and blood in milk (Outweltjies and Hogeveen, 2001; Espada and Vijverberg, 2002). A minimum concentration as low as 0.1% can be analysed by constant flow of milk in automatic milking and consequently identified in milk. The traditional method using a black strip cup spotted only 2.0 % of blood in the milk (Rasmussen and Bjerring, 2005). So far, spectacular indicators for abnormal milk and

clinical mastitis are the Green and Blue colours, where as they did not correspond with appearance of the milk (Kamphuis *et al.,* 2008).

2.3.5. Portacheck

This technique is performed using an esterase-catalysed enzymatic based reaction to evaluate the milk Somatic Cell Counts. It is cheaper, rapid and easy to use. It is capable of detecting low SCCs having low sensitivity (Viguier *et al.,* 2009).

2.3.6. Fossomatic SCC

This assay uses the fluorescent signal created to assess the SCC in milk through optical fluorescence, ethidium bromide gets through and intercalates with nuclear DNA, and it is very quick, automated, expensive and sophisticated device to operate (Viguier *et al.*, 2009).

2.3.7. Electrical Conductivity (EC) Test

It has been exploited for the detection of bovine mastitis on phenotypic level (Norberg, 2005). The elevation of ions such as sodium, potassium, calcium, magnesium and chloride are detected by electrical conductance of the milk during soreness. EC can be used during milking time and diagnosis of mastitis-free related differences in EC can be problematic (Viguier *et al.*, 2009).

2.3.8. Culture Tests

Laboratory-performed test employ selective culture to identify a wide range of bacterial species resulting in mastitis and it detects definite microbes that result to mastitis. It requires long periods of time to obtain outcomes and is laboratory-based (Viguier *et al.,* 2009).

2.3.9. pH Test

The elevation of pH milk because of mastitis is detected by bromothymol blue. It is easy to operate, cheap and very quick and it has a low sensitivity (Viguier *et al.,* 2009).

2.3.10. Biomarkers

Biomarkers uses Serum Amyloid A (SAA), Haptoglobin (Hp), Lactate Dehydrogenase (LDH), N-acetyl-ß-d-glucosaminidase and Alkaline Phosphate (AP) as integral parameters to detect alterations in the milk demonstrating the presence of mastitis. (Akerstedt *et al.,* 2011).

2.3.11. Proteomic Techniques

Identification of numerous proteins taking part in mastitis has been identified using 2D-gel electrophoresis and mass spectroscopy (Lippolis and Reinhardt, 2005; Van Leeuwen *et al.,* 2005; Smolenski *et al.,* 2007). These techniques are employed as markers for detection of reduced neutrophil function associated with mastitis (Lippolis and Reinhardt, 2005). The outcomes demonstrated a bypass regulation of k-casein and cytochrome C oxidase and annexin V in animal tissues that are affected with the disease (Yang *et al.,* 2009).

2.3.11.1. Immunoassays

Immunoassays have been designed for the detection of mastitis causative pathogens in milk (Arimi *et al.,* 2005; Arora *et al.,* 2006; Barbuddbe *et al*., 2002; Vaidya *et al.,* 2010) and are readily used for examining milk quality. The ELISA is one of the immunoassays that have solely been designed to detect some of the common pathogens, such as *S. aureus, E. coli*, and *Listeria monoctyogenes*.

Real-time PCR and Multiplex assays are able to concurrently sense different mastitis-causing organisms in milk have been described and most recently developed assays have the ability of detecting eleven of the major mastitis-associated pathogens, including, *Streptococcus agalactiae*, *S. aureus*, *E. coli* and *Streptococcus uberis* (Koskinen *et al.,* 2009).

2.3.12. Infra-Red Thermography (IRT)

IRT shows subcutaneous circulation and metabolism that is quantified by radiated heat liberated by the skin (Jones and Plassmann, 2002). IRT can detect radiated heat released by the udder during clinical mastitis. This technique gives possibly results for identifying mastitis in dry period and first calving.

Berry *et al.* (2003) designed an analytical model the udder temperature surfaced on sequential quantification of normal cows and ambient temperature. They advocated that Infra-Red Thermography demonstrated credibility for early identification of mastitis. This technique, however, was not an excellent method for identification of subclinical mastitis (Barth, 2000). Pilots of research on the identification of visible mastitis using IRT have demonstrated tremendous outcomes (Scott *et al.,* 2000, Kemp *et al.,* 2008).

2.4. Laboratory Diagnosis Staphylococcus species

Many commercial identification equipment that enhance in more quick diagnosis of CNS including all staphylococcus species in human clinical samples have been developed; namely the API Staph Idenification and Staph-Zym system. These system utilize a number of differential

biochemical tests and perform relatively well in identifying human staphylococcal isolates and in animals (Kloos and Bannerman, 1994; Piessens, 2011). However, boundaries in their accuracy have been established (Bannerman *et al*., 1993; Cunha *et al*., 2004; Heikens *et al.,* 2005; Renneberg *et al*., 1995; Piessens, 2011). The API Staph ID test has even been approved for CNS differentiation by the National Mastitis Council but latest findings thoroughly investigated the capability of the system to accurately identify isolates from cows and it showed that the commercial system performed weak in identifying animal accurately (Capurro., 2009; Thorberg and Brändström, 2000, Taponen *et al*., 2006; Taponen *et al*., 2008; Onni *et al.*, 2010; Park *et al.*, 2011a, Piessens, 2011; Sampimon *et al.*, 2009c).

The agreement between API Staph ID and conventional methods in identifying mastitis isolates was 77 percent (Thorberg and Brändström, 2000). However, the level of agreement of 4 percent was indicated when using *rpo*B sequencing as a reference procedure (Sampimon *et al.*, 2009c). Another practical experiment by the API Staph ID test 24 percent of bovine isolates were incorrectly identified using 16SrRNA gene sequencing as a reference, the rest were identified with high confidence levels (Park *et al.*, 2011a). For the API Staph ID system identification of *Staphylococcus chromogenes, Staphylococcus haemolyticus, Staphylococcus simulans, Staphylococcus warneri* and *Staphylococcus epidermidis* animals isolates specifically demonstrated to cause some difficulties (Capurro *et al*., 2009; Thorberg and Brändström, 2000, Taponen *et al*., 2006; Taponen *et al*., 2008;; Onnic *et al.*, 2010; Park *et al.*, 2011a, Piessens, 2011; Sampimon *et al.*, 2009c). These organisms are often linked with intramammary infection and this system is therefore rendered very poor to study CNS species from cow's milk (Park *et al.*, 2011a).

As for the Staph-Zym test, good agreement of about 94 percent has been illustrated with conventional methods, although some additive tests were necessary to identify 45 percent of strains, elevating time and cost (Thorberg and Brändström, 2000). Using *tuf* sequence the Staph-Zym system yielded 61 percent of milk isolates an accurate species name (Capurro *et al*., 2009). In another study, only 3 percent milk isolates were rightly spotted by the Staph-Zym system, and for 59 percent of the isolates no concrete identification could be retrieved because of an ambiguous or lack of typing results (Sampimon *et al.*, 2009c). This low sensitivity seems to be predominant for extramammary CNS than for mastitis-associated isolates. For swab samples from extramammary regions including perineum and udder skin, teat apices, teat canals, hands of staff, and teat cup liners only 57 percent of CNS isolates could be identified with greater than 90 percent of probability using API Staph ID, compare to 83 percent of CNS mastitis isolates (Taponen *et al*., 2008).

These systems have been manufactured for the analysis of human pathogens and contain limited or few animal strains in their database that is a reason they show poor outcomes. The quantity of the CNS differs substantially from cows, humans and other niches. Furthermore, strains differences can take place between isolates of the same group but originating from diverse host species (Zadoks and Watts, 2009; Supré *et al.,* 2009). The main drawback of species identification based on a few number of phenotypic traits is that expression can be highly variable among unique strains within species, jeopardizing precise interpretation and reproducibility of tests (Watts *et al.*, 1984; Zadoks and Watts, 2009). Having more animal strains and species in the database of commercial system should upgrade their accurateness and sensitivity, although the interpretation of weak or reactions remains a challenge and subjective (Piessens, 2011).

2.5. Antiobiotic Resistance Trends in Staphylococcus

S. aureus are often the result of bacteraemia in man (Livermore, 2000). Methicillin-resistant *S. aureus* and vancomycin-resistant *S. aureus* (VRSA) are predominant strains that pose great threat in humans. Around 1961 MRSA strains emerged and became a foremost concern for hospital outbreak in numerous nations. In 1996 VRSA strain became prevalent from MRSA strains a few years after initiating human therapy using vancomycin (Hiramatsu *et al*, 2001 and Livermore, 2000). MRSA strain usually demonstrates some another resistance gene known as *emr* which basically trigger erythromycin resistance. This gene has been studied in several isolates but never from bovine sources but derived from poultry (Teuber, 2001). Research shows that transmission of MRSA is initiated by person-to-person and no work has been reported concerning the transmission of MRSA from bovine to humans. In a study performed in Belgian in the 1990s 1-3 percent of MRSA resistance types were discovered but these resistance types were not found in 1980s through 1990s. These were also of human origin (Devriese *et al*, 1997) and according to present scientific data; the Methicillin-Resistant *S. aureus* strains originating from the use of lactating or dry cow therapy is very unusual.

A semisynthetic penicillin known as Methicillin was introduced in the United Kingdom in 1961. It was used to destroy penicillin resistant *S. aureus* in nosocomial community which was a huge problem around 1950s. However, it was soon discovered that methicillin resistance in *S. aureus* has increased, that was during the early years of 1970s. Its resistance became prevalent yearly and during 1990, 2 percent of *Staphylococcus aureus* isolates implicated in bacteremia in the United Kingdom was resistant to methicillin. The proportion reached 43 percent between 1997 and 2002 in England. In the United Kindom; the Health Protection Agency was the first of identify and

number epidemic strains EMRSA-1 to17 and in the country the EMRSA-15 and-16 the most common strains, which now account for the majority of all MRSA bacteremia. They consist of unique genetic constituent to various toxins rather than a broad spectrum of antibiotic resistance traits. It is still perplexing that EMRSA strains have been so dodgy. It may be caused by their inherent characteristics or alterations in new medical practice both within and between healthcare facilities (Casey *et al*., 2007).

The type II or III Staphylococcus cassette chromosome *mec* (SCC*mec*) from Hospital-acquired-MRSA initiates resistance, whereas Community-Associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) isolates exhibit type IV SCC*mec*. The toxin Panton Valentine Leukocidin of CA-MRSA is thought to be in part responsible for their increased virulence. The HA-MRSA strains, EMRSA-15 and-16 possess unique antibiotic defencelessness patterns to Community-Acquired Methicillin-Resistant *Staphylococcus aureus* strains, with the former tending to be susceptible to gentamicin although resistant to ciprofloxacin (Casey *et al*., 2007).

Community-Acquired Methicillin-Resistant *Staphylococcus aureus* strains commonly withstand susceptibility to antibiotics that consists of Beta-lactam. In 1996 the first case of decreased sensitivity to vancomycin was reported, vancomycin-intermediate *S. aureus*. Since that time hundred vancomycin-intermediate *S. aureus* isolates have been reported across the globe. Susceptibility to methicillin differs between hospital and non-nosocomial *Staphylococcus epidermidis* isolates, this implies that a high fraction of nosocomial isolates are defiant, whilst commensal and infective community isolates are normally susceptible (Casey *et al*., 2007).

Methicillin-resistant *S. epidermidis* isolates are mostly defiant to all β-lactam in vivo, besides a number of isolates demonstrate apparent susceptibility during in vitro testing. Moreover, CNS species may form part of the genetically related group for future antibiotic resistance development in *S. aureus*. However, largely methicillin-resistance CNS species are more mostly resistance to aminoglycosides and tetracyclines than are the predominant United Kingdom clones of Methicillin-Resistant *Staphylococcus aureus*. Fluoroquinolones initially established susceptible effect against Methicillin-Resistant *Staphylococcus epidermidis* isolate but resistance has become known and became extensive due to heavy use in hospitals and the community. In England between 1997 and 2002, the majority clinical isolates of CNS species are responsive to vancomycin, with less than 1 percent among those from hospital-acquired bacteremia were resistant (Casey *et al*., 2007).

2.5.1. Methicillin resistance

These antimicrobial agents are usually used in other infections other than staphylococcus species. Bacteria have develop some defiant mechanisms against this set of antibiotics which involves generation of β-lactamases and low affinity penicillin-binding protein 2a (PBP2a) determined by the existence of the chromosomal *bla* and *mecA* genes. Four decades ago, Devriese and colleagues (1972) were the first researchers to advocate that MRSA can be found from cows with mastitis.

Latest research conducted has demonstrated a little occurrence of MRSA mastitis and low pervasiveness of methicillin resistance between bovine *S. aureus* isolates (Juhász-Kaszanytky *et al*., 2007; Lee, 2003; Moon *et al*., 2007), therefore this implies that MRSA is relatively not an extreme bovine mastitis threatening agent. However, its emergence and rapid in animals is rational and seeks for accurate and prompt study so as to comprehend epidemiology. The prolonged low

incidence of MRSA mastitis over a number of years the since the first identification brings about some concerns taking into a close contact of humans with udder of dairy cattle. However, plenty of information published present MRSA infections in dogs, chicken, cats, cattle, rabbits and horse (Goni *et al*., 2004; Hartmann *et al*., 1997; Lee, 2003; O' Mahony *et al*., 2005; Rich and Roberts, 2004; Weese, 2005; Weese *et al*., 2006).

In a study performed by Weese and Van Duikeren .(2010) indicated that in some occasions animals are a supply of man Methicillin-resistant *Staphylococcus aureus* disease nevertheless; man may also supply infection in animals. The contact of domestic animals to MRSA was merely anticipated due to its significant popularity in man. Epidemiological alterations of MRSA in animals and its effect on human health are currently on the surface and requires extensive study to provide more understanding and effective response.

Research conducted by Garcia-Alvarez et al. (2011) using a DNA microarray screening technique confirmed a novel MRSA. This strain was analysed genome sequencing and it was noticeably dissimilar to previously known MRSA strain. Apparently the strain consists of SCC*mec* that encodes vastly divergent genes so as to vary to whichever described SCC*mec* in MRSA or in every other microorganism. Studies enlightened that was situated in the genetic lineage clonal complex 130, which has been recently linked with MRSA from cows and other animals, except man.

Katayama et al. (2000) indicated that SCC*mec* is transportable inherent element that exhibit *mec* and *ccr* genes complex, encoding for methicillin resistance and recombinases to facilitate its relocation activity. The *mecR1* and *mecI* are regulator elements situated alongside the *mecA* on the chromosome which control the PBP-2a expression. The *mec* regulator gene mutation is regarded to be associated with constitutive PBP-2a generation. The *mecRI*, *mecI* and *mecA* carried by *Staphylococcus* spp are phenotypically methicillin susceptible because of the suppression of PBP-2a production by *mec* regulator elements. The alterations of genome in *mec* regulator genes are considered to eliminate their repressor action and *mecA* gene transcription, which may ultimately result to PBP-2a production. There are four classes of *mec* gene complex and there are three allotypes *ccr* gene complex. Diverse arrangement of *mec* and *ccr* gene complex types consists of six kinds of SCC*mec* elements (Ito *et al*., 2004). Witte *et al*. (2007) mentioned that the during 1960s in routine clinical laboratories the identification of methicillin resistance has been an obstacle ever since the emergence of MRSA mainly with heterogeneous expression of resistance in most staphylococcal strains currently prevalent. Misidentification of methicillin resistance has severe clinical problem. Treatment failure may due to false-susceptibility and the dissemination of resistant *Staphylococcus spp* making it hard to employ control measures and resulting to the elevation of health costs and glycopeptides overuse (Velasco *et al*., 2005).

2.5.2. Vancomycin resistance

In 1958 the glycopeptides vancomycin was discovered and is known as the inhibitor of cell wall synthesis in *S. aureus* and other Gram-positive organisms. Beta-lactam antibiotics prevent cell wall synthesis by attaching to the active site of PBP-2a, vancomycin acts with an entirely mechanism and so it has been a highly recommended treatment for severe infections caused by

Methicillin-resistant *Staphylococcus aureus* (Howden *et al*., 2010), however; the rise in vancomycin utility has resulted to the emergence of two types of glycopeptide-resistant *S. aureus.* The glycopeptides *S. aureus* is vancomycin intermediate resistant *S. aureus* (VISA) and the secondary one is called vancomycin-resistant *S. aureus* (VRSA).

Hiramatsu *et al*. (1997) reported reduced vancomycin susceptibility (VISA) on clinical *S. aureus* isolate and in the medical community this incident was a cause of concern. From that time on, heterogeneous VISA (hVISA) strains of *S. aureus* have now been reported for several countries including the United States, Japan, Australia, Scotland, Brazil, Hong Kong, South Africa and others (Beirbaum *et al*., 1999; Chang *et al*., 2003; Denis *et al*., 2002; Ferraz *et al*., 2000, Gemmell, 2004; Howden *et al*., 2010; Kim *et al*., 200; Perichon and Courvalin, 2006). It has become apparent that the phenotype of VISA is associated with the thickening of the bacterial cell wall, a submissive resistance action that lessen vancomycin entry to its active site, which is situated in the cytoplasmic in division septum (Howden et al., 2010). Consequently, the acyl-D-alanyl-D-alanine targets accumulate in the periphery that sequesters glycopeptides (Cui *et al*., 2003).

2.5.3. Other antimicrobial resistance

clindamycin has been a treatment of choice for the infections in domestic animals such as cats and dogs caused by MRSA and methicillin *Staphylococcus pseudintermedius* (MRSP) in veterinary medicine (Walth (er *et al*., 2008; Weese *et al*., 2006; Schwarz *et al*., 2008; Wettstein *et al*., 2008). On the other hand, some *staphylococci* may carry an inducible form of clindamycin-resistanc and these staphylococcal strains demonstrate susceptibility patterns on routine antimicrobial

susceptibility testing, however; resistance can be encourage for the duration of treatment causing treatment failure (Swenson et al., 2007; Yilmaz *et al*., 2007).

Gram-positive and negative bacteria species, intracellular pathogens and protozoan parasites have been attacked by azithromycin which has been extensively used to its remarkable pharmacokinetics features and broad range activity. It was prescribed because it can be utilized orally and potential routes by a single dose in relatively a very short period. Yet, most data available that recommends its value in veterinary treatment was because of its credible therapeutic efficiency in infections that were found in human. The rise of antimicrobial resistance prevalence in pet animals is a result of experimental antimicrobial chemotherapy lacking previous achievement of bacterial detection and antimicrobial susceptibility assays (Guardabassi *et al*., 2004; Morgan *et al*., 2008).

2.6. Nucleic acid-based techniques for the identification antibiotic-resistant of Staphylococcus species

Staphylococci species identification in clinical laboratories has increased significantly. On the other hand, the CNS identification is still a challenge, because the biochemical behaviour of the genus is more likely the same and several clinical isolates demonstrate intermediate traits. Moreover, the kits employed for the detection of staphylococci excludes many other species from *Staphylococcus* genus, and they inconsistency for particular species identification (Miliane Moreira Soares de Souza *et al.*, 2012).

The methods of molecular identification are hallmark to complete spaces, as gene definite markers are being recognized. Similarly, molecular systems have been developed to enhance the recognition of resistant bacterial strains to antibiotics. Therefore, gene-based detection systems give fast and highly responsive methods to detect the incidence of resistance genes and have a significant role in the resistance mechanisms clarification (Miliane Moreira Soares de Souza *et al.*, 2012).

The benefit of genotypic identification of antibiotic resistance and categorization of bacteria encompasses the following: (1) To investigate for a definite resistant determinant; (2) liberty upon phenotypic classifications such as susceptibility, intermediate susceptibility and resistance for which breakpoints may be at variance among nations; (3) recognition of significantly low-level resistance which is not easy to sense with phenotypic techniques; (4) decrease of the period of detection via its performance with clinical specimens and this is principally imperative for organisms which are often a huge challenge to culture; (5) detection time of slow growth of the organisms should be retarded (6) More exact and speedy remedial calculation; (7) trivial biohazard danger once it is not required to proliferate by culture of a microbes (Sundsfjord *et al*., 2004).

However, the genotypic methods possess certain constraints and difficulties: (a) It deals with the screening of resistance determinants whereas antimicrobial therapy detects susceptibility; (b) One can merely screen for what is previously known so it exploits novel resistance systems; (c) high potential of obtaining incorrect results as a result of silent genes and pseudogenes; (d) Clinically relevant resistance genotypes maybe misidentified; (e) false-negative results can generate

alterations in primer binding sites; (f) Mixed microbial samples possess inhibition of nucleic acid which causes low clinical sensitivity; (g) quantitative measurement of the specific mRNA need to be targeted in order to detect regulatory mutations that have an impact in expression of genes; (h) no standard available for performing genetic testing as it is done for conventional culture-based susceptibility test techniques, (Sundsfjord *et al*; 2004).

Hybridization and amplification methods are well known genetically based technology systems, even though the majority amplification technologies are somewhat based hybridization technology. The DNA in a model is rendered single-stranded in hybridization then permitted to bind with a probe with a single strand. Years ago a nitrocellulose membrane was used as a stationary form for hybridizations with target DNA, but in modern days a variety of solid support are being employed. The probe will immediately hybridize subsequent to binding of the target. The radioactive isotopes, antigenic substrates, enzymes or chemiluminescent compound are often used to tag probes. Antimicrobial resistance in *Staphylococcus* spp is presently detected by Southern and Northern Blotting, FISH, microarray and Branched DNA (bDNA) (Miliane Moreira Soares de Souza *et al.*, 2012).

In Southern blotting, DNA acts as a substrate for hybridization analysis with labelled DNA or RNA probes that directly target specific DNA sequences in the blotted DNA (Southern, 1975). Conversely, Northern blotting immobilized RNA in the membrane rather than DNA. The Southern blotting technique was performed to sense mecA gene in *S. aureus* and to assess the efficiency of the systems as Polymerae Chain Reaction (Bignandi *et al.*, 1996; Lan Mo and Qi-nan Wang, 1997).

Fluorescence In Situ Hybridization (FISH) system was in the beginning designed for clinical analysis (Levsky and Singer, 2003). This technique also uses hybridization connecting the infiltration of a fluorescent labelled specific DNA probe into fixed cells, subsequently specific binding occurs to the corresponding sequence of the target specific DNA sequence. FISH gives rapid instantaneous identification of integral gene whilst linked with the organism (Bottari *et al*., 2006).In FISH amplification of the DNA of the target sequence is no carried out and FISH detects specific bacterial community and antibiotic genes (Rahube and Yost, 2010). *S aureus* was quickly detected by the technique known as a peptide nucleic acid fluorescence in situ hybridization (PNA FISH) (Forrest et al., 2006; Lawson *et al*., 2011).

PNA probes possess hybridization features such as high specificities strong affinities, and swift kinetics, causing enhanced hybridization to vastly structured targets and this is due to their unchanged, neutral backbones. Furthermore, the relatively hydrophobic feature causes PNA probes to penetrate the hydrophobic bacterial cell wall with subsequent mild fixation conditions that will not end up distorting cell characteristics (Stefano and Hyldig-Nielsen, 1997).

DNA microarrays permit the mass screening of sequences by hybridization. This technique works with gene-specific probes placed on a silicon chip. The sample Deoxyriboucleic acid is isolated, tagged, hybridized and a reporter system is used to identify target-probe duplexes. To determine several of nucleic acid in the target the probe-target hybridization is usually identified and quantified by detection of fluorophore-, silver-, or chemiluminescence-labelled targets (Schena *et*

al., 1995). This method allows recognition of an enormous figure of resistance genes in a single test and has the capability for noteworthy computerization in a microchip system. There are several other models of the utility of Deoxyribonucleic acid microarray for finding of antibiotic resistance genes in Staphylococcal (Cui *et al*., 2005; Frye *et al*., 2006; Garneau *et al*., 2010; Monecke *et al*., 2007; Zhu *et al*., 2007a). Research performed by Shore *et al*., (2011) discovered a new MRSA strain is not detected as MRSA by mostly used traditional and real-time PCR technique usually employed to monitor patients for MRSA.

Branched DNA (bDNA) system was designed by Chiron Corp. and exploits numerous hybridization sites for enzyme-coupled probes (Nolte, 1998). The target-specific probes come together to solid planes are allowed to arrest target single-stranded DNA. Another probe is permitted to hybridize with the target consists of a 5' end that cannot hybridize with a bDNA probe. The probe is bristle-like formed, about 15 bristles are intact to each and every probe, and each bristle is bounded to by three alkaline phosphatise reporter molecules. An indication is created by the incorporation of chemilunescent substrate. This technique has been extensively utilized to identify *mec*A gene in Staphylococcus spp. culture and from blood (Kolbert *et al*., 1998; Zheng *et al*., 1999).

Mulllis and colleagues (1987) proposed the use of PCR and it was the first problem-solving technique published by Saiki and co-workers (1988). The PCR assay became famous when the thermo stable DNA polymerase from *Thermus aquaticus* greatly known as the Taq DNA polymerase was introduced (Saiki *et al*., 1988). PCR deals with heating cycles of the samples for

denaturing and annealing of the primers. This remarkable essay has been frequently employed for DNA amplification in order to identify antimicrobial genes and *S. aureus* (Simeoni *et al*., 2008).

Multiplex PCR is a modified version of Polymerase Chain Reaction that has been used detect antimicrobial genes in *Staphylococcus aureus* (Amghalia *et al*.,2009; Braoios *et al*.,2009; Zhang *et al*., 2005) which exhibits numerous primer sets within a one mixture of PCR components to yield amplicons of different magnitudes that are definite to different nucleic acid sequences by targeting several genes in a single experiment, additional data may be retrieved from a one test run that would need quite a lot of the reagent and often tiring.

RFLP is a technique that exploits differences in homologous DNA sequences. This implies that, it deals with a variation between samples of homologous DNA molecules that are from various positions of restriction enzymes sites. In analysis of PCR-RFLP, the Polymerase Chain Reaction product is digested by restriction enzymes and the subsequent restriction fragments are split by agarose gel electrophoresis based on their lengths (Santos *et al*., 2007).

In PCR-single-stranded conformation polymorphism (PCR-SSCP), the amplified product of PCR is denatured into two single-stranded molecules and subjected to non-denaturing polyacrylamide gel electrophoresis. A secondary structure of the single-stranded DNA (ssDNA) molecule is then determined by the nucleotide sequence, buffer conditions, and temperature. PCR-SSCP has a great

potential of identifying an abundance of over 90% of all single-nucleotide alterations found in a 200 long nucleotide sequence (Hayashi, 1992).

The sequencing of DNA is performed by dideoxysequencing and is one of the globally adopted and famous techniques (Sanger *et al*., 1977). Technology advancement brought sequencing of DNA within the means of a number of diagnostic laboratories. Latest developments in DNA sequence techniques have resulted in quick analysis mutational resistance with ease (Ronaghi *et al*., 1998). The recognition of linezolid-resistance in enterococci and of linezolid-resistance *Staphylococcus aureus* and *Staphylococcus epidermidis* have been detected through DNA sequencing assay (Sinclair *et al*., 2003; Zhu *et al*., 2007b).

Microbiology laboratory has developed several routine diagnostic methods through the real-time PCR techniques (Espy *et al*., 2006; Mackay, 2004). Quite a number of research studies have demonstrated the employment of such systems for identification of resistance determinants and scrutiny of antimicrobial-resistant *Staphylococcus* species. (Fang and Hedin, 2003; Huletsky *et al*., 2004; Palladino *et al*., 2003; Paule *et al*., 2005; Thomas *et al*., 2007; Volkmann *et al*., 2004). The assay mintors the propagation of amplicon in realtime with branded primers, oligonucleotide probes and/or fluorescing amplicons generating a noticeable measurablee signal in relationto the quantity and amplicon specificity. Quite a lot of developments have been initiated which include decreased PCR product size, temperature condtions reduced, elimination of distinct post-PCR detection system have permitted computerization, reduced detection period, and ultimately diminished the potential hazards of contamination (Miliane Moreira Soares de Souza *et al.*, 2012).

Additional essential systems encompasses multiplex PCR techniques with a use of several sets of primers for the detection of several antimicrobial resistance genes at the same time (Depardieu et al., 2004; Martineau et al., 2000a; Sabet *et al*., 2006; Seputiene *et al*., 2010; Suhaili *et al*., 2009).

2.6.1. Staphylococcus species molecular typing techniques

On the taxonomy of Staphylococcus species retrospective research suggested that on DNA-DNA re-association shows that in the genus there were nine different groups of species, which are basically led by *S. epidermidis*, , *S. simulans*, *S. intermedius*, *S. hyicus*, *S. sciuri*, *S. auricularis*, and *S. aureus* (Klosos and George, 1991). The 16S rRNA gene (De Buyser *et al*., 1992), the tRNA gene intergenic spacer (Maes *et al*., 1997), the heat shock protein 60 (HSP60) gene (Kwok *et al*., 1999), and the *femA* gene (Vannuffel *et al*., 1999) are some of many other molecular targets which have been taken advantage of; for the identification of *Staphylococcus* species by molecular systems.

The technology of molecular probe hybridization has utilized these targets and they have been beneficial in laboratories that have a panel of probes and then exclusively used for *Staphylococcus* species identification. The *nuc* gene is one of the molecular targets that have been detected in *S. aureus* (Brasktad *et al.*, 1992).

Pulsed-field Gel Electrophoresis (PFGE) is the principally globally utilized technique in studying the epidemiological pattern of *S. aureus* (Chung *et al*., 2000). This assay is operated by incorporating organisms in agarose, situ lysing of the organism, and cutting the chromosomal DNA with restriction endonucleases (Finey, 1993; Goering and Winters, 1992). The portions of agarose which contain the DNA fragment are then incorporated into the wells of an agarose gel, and the restriction fragments are then determined into a pattern of separate bands in the gel by an equipment that switches the direction of current based on a programmed pattern. The patterns of DNA of the isolates are then matched up with one another to resolve their relationship. Research studies of multicentre using PFGE can be performed due to the newest improvement in the standardization of electrophoresis condition (Chung *et al*., 2000; Oliveira *et al*., 2001) and advance of normalization and analysis software (Duck *et al*., 2003).

Multilocus sequencing typing (MLST) has been extensively adopted and employed in typing of microorganisms and largely utilized as a research system in numerous works of *S. aureus* evolution and epidemiology (Maiden *et al*., 1998; Aires de Sousa *et al*., 2003; Coombs *et al*., 2004; Enright *et al*., 2002; Mato *et al*., 2004). It distinguishes bacterial isolates based on polymorphism within internal fragment translated into a different allele, and every isolate is categorized as a sequence type by the binding of alleles of the seven housekeeping loci (Enright *et al*., 2002). MLST particularly is more advanced than PFGE because of its definite nature of DNA sequences which are stored easily with the analogous clinical data on each isolate in the database.

In order to fight and defeat the challenge posed by phenotypic species identification, several molecular methods have been employed for CNS differentiation. DNA sequencing of genes has been frequently put in practice in phylogenetic studies and is now a generally utilized method for species identification of staphylococci and several other microbes (Takahashi *et al.,* 1999; Zadoks and Watts, 2009). Quite a number of target genes have been use for genotypic species identification of Staphylococcus species, including the 16rRNA gene,*hsp*60/*cpr*60/*gro*L (heat shock protein 60), *rpo*B (beta subunit of RNA polymerase), *sod*A(superoxide dismutase A) *gap* (glyceraldehydes-3-phosphate dehydrogenase, and *tuf* (elongation Tu) (Goh *et al.,* 1996; Takahashi *et al.,* 1999; Kwok *et al.,* 1999; Martineau *et al.,* 2000; Drancourt and Raoult, 2002a; Heikens *et al*., 2005; Capurro *et al.,* 2009). A variety of CNS species gene sequences are stored in GenBank for reference, and have been used in many latest studies for the identification of CNS isolates from bovine milk samples (Capurro *et al.,* 2009; Piessens, 2011).

Gel electrophoresis has been used for the comparison of DNA fragments, restriction fragments, amplified sections which are segregated according to the molecular size generating a bacterial fingerprint. The elementary principle is to compare fingerprints of isolates to these of reference strains to come to final species identification. Polymerase Chain Reaction based methods using primers targeting specific sites of the bacterial genome have been optimized for rapid identification of Coagulase Negative Species isolates from man and animals such as transfer-RNA intergenic spacer (Maes *et al.,* 1997), PCR 16S-23S rDNA gene internal transcribed spacer PCR (Bes *et al.,* 2000). PCR-RFLP analysis is used to increase the resolution of PCR fingerprinting. RFLP-PCR analysis of *rr*, *gap,* and *hsp*60 has also been adopted to speciate CNS isolates from animals (Santos *et al.,* 2008a, Onni *et al.,* 2010; Park *et al.,* 2011a). Non-PCR based methods, ribotyping, in which

genome restriction is followed by hybridization with a probe complementary to ribosomal DNA and it can be automated and performs very accurately in differentiating CNS species (Bes *et al.,* 2000; Taponen *et al.,* 2008). In a nutshell the fingerprints yielded with these methods exhibit a restricted number of fragments and are highly species-specific. Again, their main advantage is that they are quick, easy to use and inexpensive, however, due to limitations in their differentiation, they sometimes fail to discriminate closely relate CNS species (Onni *et al.,* 2010; Park *et al.,* 2011a; Piessens, 2011).

Polymorphism in definite restriction sites and nucleotides adjacent to these sites are scanned using Amplified Fragment Length Polymorphism (AFLP) typing method (Vos *et al*., 1995). (AFLP) has been also adopted for CNS species detection. The technique entails the following (1) two restriction enzymes within the restriction of genomic DNA (2) binding of oligonucleotide adapters to fragmented restriction sequences and (3) the discriminatory magnification of subset of the fragments with adapter-specific primers extended with (a) selective nucleotide(s) (Vos *et al.,* 1995; Piessens, 2011). Usually a primer is fluorescently labelled and fragment separation is done by capillary electrophoresis permitting for high-resolution and high-throughout genotyping of huge numbers of isolates. Conversely to other employed methods, AFLP has whole-genome coverage and maps polymorphisms in restriction sites and adjacent sequences, and insertions and deletions in amplified fragments, dispersed over the entire bacterial genome. Due to its superior differentiating power and wide taxonomic range, it has been widely used in phylogenetic and taxonomic studies, and has been described as a suitable method for delineation of species within various bacteria genera The actual gold standard for species determination, total genomic DNA-DNA hybridization, is fairly laborious and unfeasible in many laboratories. Nevertheless, AFLP

analysis generally demonstrates good agreement with DNA-DNA hybridization results and is considered a good alternative (Piesssens, 2011).

Another advanced molecular identification method that generates fingerprints by fragmentation of the entire cell instead of the genomic DNA is Matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis (MALDI-TOF). Both AFLP and MALDI-TOF have been proven useful for bovine CNS species identification, whereas the MALDI-TOF identification method has been well validated utilizing a huge number of CNS reference strains previously identified with gene sequencing. AFLP method has not been validated by comparison with reference method (Taponen *et al.,* 2006; Taponen *et al.,* 2007; Piessens, 2011).

2.7. Prevention and Control

2.7.1. Hygienic procedures

It is highly emphasized that the milking team should always put on gloves whenever they are milking cows and remove them after use. And again, it is essential to fore strip at least a few squirts of milk from all the teats and observe unusual or abnormalities in milk. The filth on the teats must often be wiped up with a dry, single-use towel. It is imperative not to use water as a cleaning medium of any milking methods, even if a sanitizing reagent is mixed with water because the activity of the hygienic reagents is not constant all through the milking process, and water has a high potential of introducing harmful microbes which are relatively very challenging to eradicate (Petersson-Wolfe *et al*., 2010).

A predip that is usually purchased commercially should be utilized with a dipper or cup and permitted for contact for a period of 30 seconds. Sprayers used as disinfectants should be put in use, however; sometimes it is difficult to spray teats that are away from the milking personnel. Soaps are put in use for cleaning the cows' teats, but it as to be done with carefulness to ensure that adequate components of the active constituent do contact the teat and teat skin. The teats should be dried up every time with a fresh disposable paper towel or cloth towel (Rasmussen *et al.* 1992; Petersson-Wolfe *et al.*, 2010).

Visible chapping, cracks, or lesions must be checked which may be habitats of pathogenic microbes. It is very important to perform postmilking teat dipping after milking so as to ensure that the tubs of all the teats are enclosed. It is also recommended that at each end of milking any remaining teat dip should be thrown away and cups have to be dried up after being rinsed with water (Petersson-Wolfe *et al.*, 2010).

2.7.2. Cows infected with S. aureus must be milked separately and last

Clinical mastitis infected cows, and/or cows which have been administered with antimicrobial agents must be the last ones to be milked. There has been a reduction of *S. aureus* mastitis and bulk-tank SCC due to the segregation of infected cows during milking process (Wilson *et al.* 1995; Petersson-Wolfe *et al.*, 2010). In most cases, if the animals cannot be slaughtered, it is highly suggested to separate them from the main herd to avoid the perpetuation of infectious pathogens and a quick and harmful effect on SCC of bulk-tank milk. In rare instances whereby the animals cannot be segregated, thus it is essential to put leg tags in order to recognize those cows and make

sure that hygienic procedures are exercised by the milking personnel. Mastitis-free, primiparous cattle ought to be milked before subclinical mastitis infected adult cows (Petersson-Wolfe *et al.*, 2010).

2.7.3. Milking equipment

The route of Staphylococcus infections is encouraged during the milking process as the pathogens break through the teat duct. Vacuum suction pump fluctuations generated by liner slips and floaded lines result up and down movement of milk against the end of the teat. Therefore, it is of paramount importance to ensure adequate functioning equipment to inhibit new infections as the force caused by the vacuum can propel up the bacteria into the teat canal and teat cistern. The situation that endangers the teat end must be reduced significantly; these include liner slips, extreme impermanent vacuum losses, short vacuum reserve, insufficient vacuum regulation and sudden milking clusters detachment (Petersson-Wolfe *et al*, 2010).

The vacuum has been turned off followed by the removal of the teat clusters. Investigations have demonstrated that about 10-15 percent of new infections have been caused by slipping teat cup liners. Consequently, a short vacuum level blocks air ventilators, or a limit in the small milk tube is experienced. The slippage of the liner is often cause by poor cluster arrangement, irregular weight allocation in the cluster, or bad condition of the liner. Improper milking can be caused by bad condition of liner, incorrect pairing of the claw inlet and short milk tube, lightness of the clusters, unbalanced clusters under the udder; or high vacuum levels (Halleron, 1997; Petersson-Wolfe *et al*, 2010).

Frequent, maintenance is very useful for milk quality and for the prevention of mastitis disease. Every 4 weeks the vacuum regulators, pulsators, and air filters should to be dirt-free. The component of rubber that is damaged should be replaced even if its service period is yet to come. It is suggested that evaluation of the milking system should be conducted every three months of operation. It is recommended to carry out these tests during the milking session and not between milking (Petersson-Wolfe *et al*, 2010).

2.7.4. Antibiotic therapy of cattle infected with S. aureus

It should be noted that antibiotic action does not inevitably control mastitis but it may reduce the infection period. Antibiotics become ineffective as the cow matures and even as the initial lactation carry on. In 10 Dutch herds it was found that the cure rates were at least 34 percent out of 89 cows that were treated for subclinical mastitis (Sol *et al.* 1997; Petersson-Wolfe *et al*, 2010). The outcomes relieved that the probability of heal was lesser in adult cows with elevated Somatic Cell Count and in cattle infected in backward quarters for the period of initial and within the lactation phase. In Finnish herds the *S. aureus* infections were found in 36 percent of clinical mastitis cases (Pyorala and Pyorala. 1997; Petersson-Wolfe *et al*, 2010). Cattle that possessed a Somatic Cell Count of less than a million were high likely to eradicate an infectivity composed with those beyond the stop point. Good and effective antibiotic action is greater for the period of lactation and treated in premature phases whereas the action is subordinate when treating persistent infections. Immature clinical infections must be healed thoroughly, especially in cows giving birth

for the first time. When cows are monitored and treated throughout the early on phases of illness the tissue damage can be reduced significantly. In instances of off-label treatment options it is advisable to seek a veterinarian for further information. The use of DHI program Somatic Cell Count records together with visual examination of first strips milk sample outcomes will give efficacy of the treatment. A lot of work has been done to observe the worthness of pirlimycin action both in heifers prior calving and in cattle as a comprehensive therapy treatment for the duration of lactation (Petersson-Wolfe *et al*, 2010).

The pirlimycin treatment is highly recommended by manufacturers because its characteristics of chemical design that permits it to penetrate mammary tissues. It has been observed that, a tube of pirlimycin treatment in each quarter of the heifers' decreases *S. aureus* infections at calving between six to twelve days before calving (Belschner *et al.* 1996; Petersson-Wolfe *et al*, 2010). Moreover, mastitis information forwarded to the Food Drug Administration demonstrates that 2 tubes, administered a day separately to infected quarters of cows for the period of lactation, resulted in an alleviation rate of 36.6%, whilst only 1.1% of untreated controls improved naturally. The rate of cattle healed all through lactation stage increased to 49.9 % in field cases.

Single-quarter, extended therapy with repeated label Same level doses of pirlimycin has been studied per quarter in an attempt to supply drug levels further than the usual life of the leukocytes that naturally destroy this infection. The procedure is extensively used globally for novel intramammary infections with *S. aureus*, as it elevates cure rates and it was established that cure

rate of 50% in one month after treatment was discovered over 100 cows that underwent treatment therapy (Belschner *et al*, 1996; Petersson-Wolfe *et al*, 2010).

2.7.5. The application of Dry Cow Therapy (DCT)

DCT has been implicated to be effective in eradicating infection than lactating treatment, but Dry Cow Therapy is not effective if the infection have become chronic when lactation cease (Kirk *et al.* 1997; Petersson-Wolfe *et al*, 2010). It is suggested to treat all quarters with a commercially available DCT when a cow is subjected to the dry off period. Virtually, these are the phases to follow when performing Dry Cow Therapy: (1) The milk must be entirely sucked out of the cows, every time teats must be dipped in post milking teat dip and blot dried out after half a minute, (2) alcohol pads should be utilized to scrub the tips of the teats before slightly placing in the antibiotic tube into the teat. Even though interior teat sealants do not inhibit the increase of *S. aureus* infections, the commercially accessible manufactured products does enhance retard new-fangled ecological infections. For the first fourteen days interior teat sealants are useful and throughout the last seven to 10 days of the dry period when cattle are most prone to pathogens caused mastitis disease. This rise in vulnerability is partially caused by the stress related with drying and pre-calving time, (3) Teats should be dipped again after treatment, prior to turning the cows out into a clean, dry environment (Petersson-Wolfe *et al*, 2010).

2.7.6. Impregnated heifers

In preliminary period of calving or lactation fresh infections are frequently observed in heifers and are mainly generated by *S. aureus*. If not managed they become strongly visible and harmful and

emerge though the first lactation and the following episode of lactation. Again, these infections elevate the rate of detrimental perpetuation to other cows (Petersson-Wolfe *et al*, 2010).

In studies carried out in Louisiana demonstrated the evaluation of administration of dry cow therapy to heifers. A penicillin and dihydrostreptomycin product was dispensed for the duration of the primary, second, or third trimester of pregnancy in thirty-five bred heifers from four dairy cattle herds. Three groups of heifers showed a remarkable reduction of infection and Somatic Cell Count; however, Heifers which were subjected to dry-treatment during the second trimester of pregnancy illustrated a significant decrease in mastitis and Somatic Cell Count at calving (Nickerson *et al.* 1995; Petersson-Wolfe *et al*, 2010). Studies advocate the dry cow treatment for heifers at least 2 months prior the usual the period of calving. Teat tips must be dirt-free and germ-free prior to and subsequent to treatment. The Antibiotic remainder must be checked in the milk between three to five days subsequent to calving (Petersson-Wolfe *et al*, 2010).

In a study survey conducted in Tennessee, an antibiotic treatment administere for lactating cows with either cloxacillin or cephapirin was given to heifers at a week earlier than likely calving period (Oliver *et al.* 1992; Petersson-Wolfe *et al*, 2010). Cephapirin showed promising treatment outcomes as compared to cloxacillin, although subsequently; resulted in antibiotic remains in milk at three days subsequent to calving. Administering heifers with cephapirin 2 weeks ahead of the estimated calving reduced the antibiotic remainder obstacle. Thus, it is highly suggested that heifers be administered with antibiotics action at least 2 weeks sooner than the estimated

conceiving period, by means of safety measures demonstrated under the previous section antibiotic therapy (Petersson-Wolfe *et al*, 2010).

2.7.7. Precautions at calving

Several mastitis infections, besides the *S. aureus* mastitis infections, begin before or after giving birth. A pasture that is well-drained is recommended for cows that are about to give birth. A clover-grass sod is required, conversely to mud-covered lots. Calving sites have to be well cared for to avoid mud-spattered sites where cattle would rest in. Untidy, wet pens and lots are inhabited by several pathogens. Pens need to be well equipped, tidy up, dry and restful. At 14 to 21 days prior the calving period the Selenium-vitamin E supplementation or injections are for reduction of mastitis after birth (Petersson-Wolfe *et al*, 2010).

The National Research Council stipulated that every a single day for the period of the dry period 100 International Units of Vitamin E and 500 International Units per day during lactation period. Vitamin A (beta-carotene), copper, and zinc are amongst the minerals and vitamins that have demonstrated to decrease the incidence of mastitis. It is said that suitable diet can be managed and sustained to retard occurrence of mastitis by examining animals to identify micronutrients deficiencies, supplying an even share, avoiding badly fermented silages and incorporating nutritional supplementation of vitamin E and selenium (Petersson-Wolfe *et al*, 2010).

The aim of this study was to evaluate the incidence of some virulence genes of *S. aureus* from raw milk collected from May 2013 to July 2013 in cattle herds from Fort Hare Dairy Farm and Middle

Drift Dairy Farm. Furthermore, the study was aimed to characterise these isolates using susceptibility profiles to sixteen selected antibiotics and detect their antibiotic resistance genes.

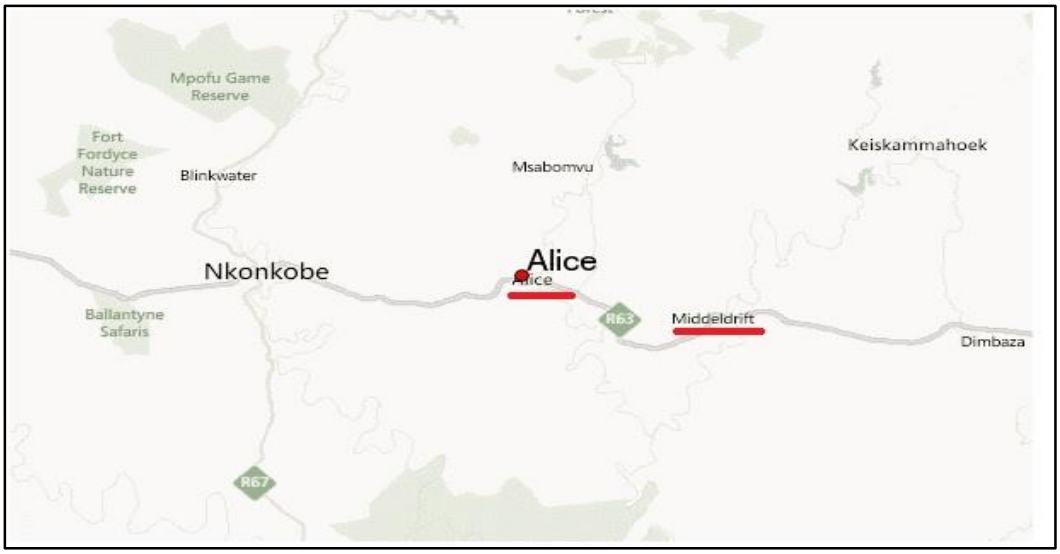
CHAPTER 3

3.1. METHODOLOGY

3.1.1. Area of the study and collection of the samples

Alice is rural based setting that is estimated to harbour a population of about 11, 000 and 14,000 people. These two Dairy Farms are 20 kilometres away from one another. Many households around this area and families of the employees of these Dairy farms often feed from the unpasteurized milk purchased from these Dairy farms. The permission to perform the study was kindly granted by the farm managers.

Four hundred milk samples were collected from two commercial Dairy Farms. Middle Drift Dairy farm and University of Fort Hare Dairy Farms situated in Amathole District under Nkonkobe Municipality in Alice, in the Eastern Cape Province of South Africa. Only lactating cows were used in this study. Physical examinations were conducted on all lactating cows for evidence and signs of clinical mastitis. Cows were categorised as clinical if they exhibited clinical geatures of mastitis, or sub-clinical if no apparent signs were present but they had indicated positive for California mastitis test (DeLaval, Kwa-Zulu Natal, South Africa). Two hundred milk samples were collected from each Dairy farm from May 2013-July 2013. Prior sampling, the teat ends were scrubbed with cotton soaked in 70 percent ethanol and the first squirts of milk were discarded. About 50 ml of milk were taken from all quarters of each cow into sterile capped conical centrifuge tubes to make one milk sample and the pH of each sample of milk was then determined on arrival in the Laboratory using a pH-meter.



http://w0.fast-meteo.com/locationmaps/Alice-2.10.gif

3.1.2. Isolation of presumptive S. aureus from milk samples

Ten microlitre of each milk sample were spread plated onto Mannitol Salt Agar (MSA) (Merck, Johannesburg, South Africa). The plates were then incubated at 37°C 24-48 hours. After the incubation period plates that demonstrated yellow colonies which indicated fermentation of mannitol by an organism were sub-cultured onto Nutrient agar plates and kept in 4˚C refrigerator for further bacterial identification.

3.1.3. Identification of Bacteria

Gram-staining was performed according to the method of Health Protection Agency, (2007) with some minor modifications and the microscopic appearances of grape clusters were subjected to preliminary biochemical test (the oxidase and catalase test). For the Gram staining a smear was prepared on the microscopic slide and gently heat fixed with fire on a Bunsen burner. The fixed smear was then flooded with 0.5% methylcrystal violet and left for 30 seconds. The microscopic

slide was then rinsed with tap water. After that, the slide was poured with 1% Iodine solution and allowed to contact for 30 seconds. Water was applied on the slide to wash off excess Iodine solution. Immediately the smear was washed away with 95-100% ethanol until the colour disappeared. The tap water was allowed to contact with the slide and 0.1% counterstain (safranin) was poured and left to contact for about 2 minutes. Thereafter, the slide was washed with water, blot dried and observed under the miscroscope at 100X magnification for Gram-positive cocci.

3.1.4. Impregnated Oxidase test strip method

Some fresh growth from the culture plate (nutrient agar plate) was scrapped with an inoculating loop and rubbed on the filter paper. A reaction of a blue colour was observed within a short period of 30 seconds (Health Protection Agency, 2010).

3.1.5. Catalase test

The catalase test was performed on the microscopic slide by taking a single colony onto a nutrient agar plate. A drop of 3% hydrogen peroxide solution was poured on top the colony placed on the microscopic slide and vigorous bubbling occurring within 10 seconds indicated a positive catalase reaction (Health Protection Agency, 2010).

3.1.6. API Staph kit

The API Staph kit (BioMerieux, Marcy I'Etiole, France) was also used for the identification of the microorganisms and the protocol was conducted according to the manufacturer's instructions. The API Staph Strip system consists of 20 miniaturized biochemical tests and some are modifications of the conventional procedures (Wesley and Jana, 1982). API system has preformed strips

containing the substrates and it is usually interpreted after the incubation period of 24 hours at 37 ˚C (Bran *et al.,* 1978).

3.1.7. MALDI-TOF MS

For further bacterial identification the glycerol stocks of the presumptive *Staphylococcus* species organisms were sent to the University of Pretoria in the Gauteng Province of South Africa for further analysis with Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

3.1.8. Antimicrobial Susceptibility Testing

All *S. aureus* from the 52 Staphylococcus species isolated, were subjected to antibiotic susceptibility test in order to determine their antibiotic-resistant profiles (Kirby *et al,* 1966). Fresh 18-24 hours old cultures were prepared and utilized for antibiotic sensitivity tests. Colonies were then suspended into 5 ml of the normal saline in a test tube and then adjusted to meet 0.5 McFarland turbidity standards (~ 1.5×10^8 cfu/ml). Thereafter, bacterial suspensions sterile cotton swabs were soaked into each tube and swabbed right around on the surface of a Mueller-Hinton agar plate (Merck, Johannesburg, South Africa). Susceptibilities of the isolates to a set of sixteen different antibiotic discs were examined. The antibiotics tested were randomly selected and some were selected based on the studies conducted in Mafikeng, North West, South Africa for comparison purposes. The antibiotic discs were carefully placed and gently pressed onto the inoculated Mueller-Hinton agar (Merck, Johannesburg, South Africa) and the plates were incubated aerobically at 37 ˚C of 18-24 hours. The zones of inhibition diameters were measured in millimetres and the values obtained from the Clinical Laboratory Standard (2013) were used to

interpret the outcomes. *S. aureus* isolates were then categorized as resistant, intermediate resistant or susceptible to a certain antibiotic.

3.2. MOLECULAR CHARACTERIZATION

3.2.1. Isolation of DNA

DNA extraction was performed based on the procedure of Maugeri *et al*. (2004) and Lopez Saucedo *et al*. (2003). A loop full 24-hour culture of *S. aureus* colonies grown onto Nutrient Agar plates were suspended into 200 µl of sterile nuclease free water and vortexed for 2 minutes using MS2 Minishaker (Digisystem Laboratory instrumnets Inc, New Taipei City, Taiwan). The cells were then centrifuged at 13 00 rpm for 10 minutes (ThermoFisher Scientific, S chwarte, Germany) Five hundred microlitres of distilled water was pippetted into the 1.5 µl eppendorf tubes, vortexed and the cells were lysed using a heat Dri-Block DB.2A (Techne, Johannesburg South Africa) for 15 min at 100ºC. The pellet was removed by centrifugation at 10 000 rpm for 5 min using a MiniSpin microcentrifuge (ThermoFisher Scientific) kept at 4ºC. The supernatant was transferred in new eppendorf tubes and used for PCR reactions.

3.2.2. Polymerase chain reaction (PCR) to determine the amplicon sizes of antibiotic resistance genes.

PCR amplifications were carried out with a pair of specific primers as outlined in Table 2. The reaction mixture (30 µl) contained 1 µl of primer F, 1 µl primer R, and 1 µl DNA sample, 12 µl of PCR master mix (Fermentas, South Africa) and 15 µl of nuclease-free water (Fermentas, South Africa) in a PCR tube. The tubes were subjected to thermal cycling with the program show in Table 2. One positive control containing *S. aureus* reference strain ATCC 29213 South African Bureau of Standards (SABS), No. ESC 20) and one negative control containing water were

included in each reaction. The 50X (1L) reaction buffer was diluted to make 1X working solution (Fermentas, South Africa). The PCR products were separated on gel electrophoresis on a 1.5% agarose gel containing 10 µl of ethidium bromide. The gel was thereafter visualized under Alliance 4.7 transilluminator (UVITEC Limited, UK). The sizes of amplicons were determined by comparison to a 100 bp molecular weight marker (Fermentas, South Africa).

3.2.3. Polymerase chain reaction (PCR) to determine the amplicon sizes of virulence genes.

PCR amplifications were carried out with a pair of specific primers as shown in Table 3. The reaction mixture (30 µl) contained 1 µl of primer F, 1 µl primer R, and 1 µl DNA sample, 12 µl of PCR master mix (Fermentas, South Africa) and 15 µl of nuclease-free water (Fermentas, South Africa) in a PCR tube. The tubes were subjected to thermal cycling with the program show in Table 3. One positive control containing *S. aureus* reference strain ATCC 29213 (South African Bureau of Standards (SABS), No. ESC 20) and one negative control containing water were included in each reaction. The PCR products were separated on gel electrophesis in a 1.5% agarose gel containing 10 µl of ethidium bromide. The gel was then visualized under Alliance 4.7 transimullinator (UVITEC Limited, Cambridge, UK). The sizes of amplicons were determined by comparison to a 100 bp molecular weight marker (Fermentas, South Africa

Table 1: Details of the antibiotics that were used in the study to test for antibiotic resistance

Group	Antibiotic	Generally accepted antibiotic disc concentration (µg)	Inhibition zone(mm)		
			Resistant	Intermediate	Susceptible
	Penicillin G	10 units	≤28	-	≥29
Aminoglycosides	Gentamicin	10	≤12	-	≥15
	Amikacin	30	≤14	-	≥17
	Kanamycin	30	≤16	-	≥18
	Tobramycin	30	≤12	-	≥15
Tetracyclines	Tetracycline	30	≤ 14	-	≥19
	Doxycycline	30	≤12	-	≥16
	Minocycline	30	≤14	-	≥19
Fluoroquinolones	Ciprofloxacin	5	≤15	-	≥21
	Moxifloxacin	5	≤20	-	≥24
	Gatifloxacin	5	≤19	-	≥23
Folate Pathway Inhibitors	Trimethoprim-sulfamethoxazole	1.25/23.75	≤10	-	≥16
	Trimethoprim	5	≤10	-	≥16
	Linezolid	30	≤20	-	≥21
Macrolides	Erythromycin	15	≤13		≥23
	Telithromycin	15	≤18	-	≥22

Eleven set of primers were used to target antibiotic resistance genes of *S. aureus* isolates. The antibiotic resistance genes targeted were for Penicillin (*blaZ*), Gentamicin (*aac(6')/aph(2'')*, *aph(3')-IIIa* and *ant(4')-Ia*), Tetracycline (*tet(K)*, *tet(M)*), and Erythromycin(*ermA*, *ermB*, *ermC*, *Mef* and *msrA*). All the 20 *S. aureus* isolates were tested for antibiotic resistance genes regardless for their antibiotic susceptibility profile results (Table 2).

Table 2: Primers for PCR for the identification of S. aureus and detection of the antibiotic resistant genes. (Table 2 continues from page 56-58)

Target gene	Primer sequence (5'-3')	PCR Program	Reference	(bp)
aac(6')-aph(2'')	TTG GGA AGA TGA AGT TTT TAG A CCT TTA CTCCAA TAA TTT GGCT	94ºC, 5min; 10 times (94ºC, 30 sec; 64ºC, 30 sec; 72ºC, 45ºsec) 25 times (94ºC, 45 sec; 50ºC, 45 sec, 72ºC for 1 min); 72ºC; 10 min	Amghalia *et al*., 2009	174
*erm*A	TAT CTT ATC GTT GAG AAG GGA TT CTA CAC TTG GCT TAG GAT GAA A	94ºC, 5min; 10 times (94ºC, 30 sec; 64ºC, 30 sec; 72ºC, 45ºsec) 25 times (94ºC, 45 sec;	Amghalia *et al*., 2009	139

		50ºC, 45 sec, 72ºC for 1 min); 72ºC; 10 min		
ermB	CTA TCT GAT TGT TGA AGA AGG ATT GTT TAC TCT TGG TTT AGG ATG AAA	94ºC, 5min; 10 times (94ºC, 30 sec; 64ºC, 30 sec; 72ºC, 45ºsec) 25 times (94ºC, 45 sec; 50ºC, 45 sec, 72ºC for 1 min); 72ºC; 10 min	Amghalia *et al*., 2009	142
ermC	CTT GTT GAT CAC GAT AAT TTC C ATC TTT TAG CAA ACC CGT ATT C	94ºC, 5min; 10 times (94ºC, 30 sec; 64ºC, 30 sec; 72ºC, 45ºsec) 25 times (94ºC, 45 sec; 50ºC, 45 sec, 72ºC for 1 min); 72ºC; 10 min	Amghalia *et al*., 2009	190
msrA	TCC AAT CAT AGC ACA AAA TC AAT TCC CTC TAT TTG GTG GT	94ºC, 5min; 10 times (94ºC, 30 sec; 64ºC, 30 sec; 72ºC, 45ºsec) 25 times (94ºC, 45 sec; 50ºC, 45 sec, 72ºC for 1 min); 72ºC; 10 min	Amghalia *et al*., 2009	163
Mef	AGT ATC ATT AAT CAC TAG TGC TTC TTC TGG TAC AAA AGT GG	94°C, 5 min; 30 times (94°C, 1 min; 55°C,	Lim *et al.* 2002	348

		1min; 72°C, 2 min; 72°C, 10 min)		
Aph (3')-1-IIIa	AAA TAC CGC TGC GTA CAT ACT CTT CCG AGC AA	95°C, 5 min; 30 times (95°C, 2 min; 54°C, 1min; 72°C, 1 min; 72°C, 7 min)	Choi *et al.*, 2003	242
Ant (4')-Ia	AAT CGG TAG AAG CCC AA GCA CCT GCA TTG CTA	95°C, 5 min; 30 times (95°C, 2 min; 54°C, 1min; 72°C, 1 min; 72°C, 7 min)	Choi *et al.*, 2003	135
Tet(K)	GTA GCG ACA ATA GGT AAT AGT GTA GTG ACA ATA AAC CTC CTA	95°C, 3 min; 30 times (95°C, 30 sec; 54°C, 30 sec; 72°C, 30 sec; 72°C, 4 min)	Strommenger *et al.*, 2003	360
Tet(M)	AGT GGA GCG ATT ACA GAA CAT ATG TCC TGG CGT GTC TA	95°C, 3 min; 30 times (95°C, 30 sec; 54°C, 30 sec; 72°C, 30 sec; 72°C, 4 min)	Strommenger *et al.*, 2003	158
blaZ	ACT TCA ACA CCT GCT GCT TTC TGA CCA CTT TTA TCA GCA ACC	95°C, 3 min; 30 times (95°C, 30 sec; 54°C, 30 sec; 72°C, 30 sec; 72°C, 4 min)	Baddour *et al.*, 2007	173

Table 3: Oligonucleotide primers and PCR programs for amplification of the genes encoding virulence determinants in Staphylococcus aureus isolates.

Gene	Primer Sequence (5'-3')	PCR program*	Reference	Size of product (bp)
coa	Coa-1 CGA GAC CAA GAT TCA ACA AG Coa-2 AAA GAA AAC CAC TCA CAT CA	30 times (94°C, 1min; 58°C, 1min; 72°C, 1 min)	Aslantas et al., 2007	730
clfA	ClfA-1 GGC TTC AGT GCT TGT AGG ClfA-2 TTT TCA GGG TCA ATA TAA GC	35 times (94°C, 1 min, 57 °C, 1min, 72°C, 1 min)	Stephan et al., 2001	980
spa (X region)	Spa-III CAA GCA CCA AAA GAG GAA Spa-IV CAC CAG GTT TAA CGA CAT	30 times (94°C, 1 min; 60°C, 1 min; 72°C, 1 min)	Fre'nay et al., 1996	320
spa (IgG binding region)	Spa-1 CAC CTG CTG CAA ATG CTG CG Spa-2 GGC TTG TTG TTG TCT TCC TC	30 times (94°C, 1min; 58°C, 1min; 72°C, 1 min)	Seki et al., 1998	920
tst	TSST-1 ATG GCA GCA TCA GCT TGA TA TSST-2 TTT CCA ATA ACC ACC CGT TT	30 times (94°C, 2 min; 55°C, 2 min; 72°C, 1 min)	Johnson et al., 1991	350
eta	ETA-1 CTA GTG CAT TTG TTA TTC AA ETA-2 TGC ATT GAC ACC ATA GTA CT	30 times (94°C, 2 min; 55°C, 2 min; 72°C, 1 min)	Johnson et al., 1991	119
etb	ETB-1 ACG GCT ATA TAC ATT CAA TT ETB-2 TCC ATC GAT AAT ATA CCT AA	30 times (94°C, 2 min; 55°C, 2 min; 72°C, 1 min)	Johnson et al., 1991	200

Table 4: Stastically analysis of the antibiotic resistance profiles of 20 S. aureus isolates.

		Tetracycline	Gentamicin	Erythromycin	Penicillin	Trimethoprim	Kanamycin	Trimethoprim_sulphamexazole	Telithromycin	Doxycycline	Tobramycin	Moxifloxacin	Linezolid	Gatifloxacin	Ciprofloxacin	Minocycline	Amikacin
N	Valid	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	Missing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean		2.3500	1.3000	1.6500	2.8000	2.3000	1.9500	1.9000	2.0000	2.1000	1.2500	1.2000	1.8000	1.1500	1.2500	1.6500	2.1500
Median		3.0000	1.0000	1.0000	3.0000	3.0000	1.5000	1.0000	2.0000	2.5000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	2.0000
Mode		3.00	1.00	1.00	3.00	3.00	1.00	1.00	1.00[a]	3.00	1.00	1.00	1.00	1.00	1.00	1.00	3.00
Std. Deviation		.87509	.73270	.87509	.61559	.97872	.99868	1.02084	1.02598	.96791	.63867	.61559	1.00525	.48936	.63867	.81273	.87509
Minimum		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Maximum		3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00

1=susceptible, 2=intermediate, 3=resistance. This table illustrates antibiotic resistance profiles of 20 *S. aureus* isolates. This data was generated by SPSS software.

CHAPTER 4

4.1. RESULTS

4.1.1. Prevalence of S. aureus in tested samples of milk

Of the 400 milk samples collected 52 presumptive *S. aureus* isolates were identified. The Middle Drift farm had 12 subclinical cows and 14 clinical cows, Whilst Fort Hare had 18 subclinical and 8 clinical cows. The 52 presumptive *S. aureus* isolates were further identified and by the API Staph kit identification system amd were confirmed by the MALDI-TOF. The 52 presumptive isolates were identified by the MALDI-TOF as: *S. xylosus* (n=7); *S. haemolyticus* (n=4); *S. aureus* (n=20), *S. sciuri* (n=5) *S. cohnii* (n=1); *Enterobacter cloacae* (n=4); *Proteus mirabilis* (n=2); and 9 were not reliable identification.

4.1.2. Antibiotic resistance profile of *S. aureus*

In this study a huge number of the isolates were resistant to penicillin G (85%), some were resistant to trimethoprim (60%) and tetracycline (60%). More resistance was observed in trimethoprim-sulfamethoxazole (55%), Telithroprim (55%), and Doxycycline (45%) antibiotics (Table 4). A small proportion of the isolates from these two farms (5%-20%) were resistant to other antibiotics. Most of the isolates were sensitive (50%-85%) to several antimicrobial agents (Table 4).

Table 5: Resistance of S. aureus isolates to different antimicrobial agents

Antimicrobials	Resistant (No%)	Intermediate (No%)	Susceptible (No%)
Trimethoprim	12(60)	3(15)	5(25)
Kanamycin	2(10)	2(10)	16(80)
Trimethoprim-sulfamethoxazole	11(55)	0(0)	9(45)
Telithroprim	11(55)	0(0)	9(45)
Doxycycline	9(45)	3(15)	8(40)
Tobramycin	4(20)	0(0)	16(80)
Moxifloxacin	3(15)	0(0)	17(85)
Minocycline	5(25)	5(25)	10(50)
Linezolid	9(45)	0(0)	11(55)
Gatifloxacin	4(20)	0(0)	16(80)
Ciprofloxacin	2(10)	1(5)	17(85)
Gentamicin	4(20)	0(0)	16(80)
Erythromycin	3(15)	4(20)	13(65)
Tetracycline	12(60)	2(10)	6(30)
Penicillin G	17(85)	0(0)	3(15)
Amikacin	1(5)	2(10)	17(85)
Mean	6.8 (34)	1.4(6.9)	11.8(59.1)

Table 6: Genotypic characteristics of S. aureus isolates from Fort Hare and Middle Drift dairy farms (antibiotic resistance genes).

Farm					Gene	(bp)					
[No. of											
Isolates]	AME genes			Eryth	genes				tetr	genes	Penc genes
	Acc(6')-aph(2'')	*aph(3')-IIIa*	*ant(4')-Ia)*	*ermA*	*ermB*	*ermC*	*msrA*	*mef*	*tetk*	*tetM*	*blaZ*
	(174)	(242)	(135)	(139)	(142)	(190)	(163)	(348)	(360)	(158)	(173)
FH farm [9]	2	0	0	0	0	0	0	0	0	0	3
MD farm [11]	6	0	0	0	0	0	0	0	1	2	8
Total	8	0	0	0	0	0	0	0	1	2	12

FH=Fort Hare, MD=Middle Drift, AME=Aminogylcoside enzyme, Eryth=Erythromycin, tetr=tetracycline, penc=penicillin

Table 7: Genotypic characteristics of S. aureus isolates from Fort Hare and Middle Drift dairy farms (virulence genes).

Farm							Gene	(bp)							
[No. of															
Isolates]	*coa*						*clfA*	*tsst*	*eta*	*etb*	X region				IgG
	(400)	(500)	(600)	(610)	(700)	(800)	(980)	(350)	(119)	(200)	(190)	(290)	(300)	(310)	(920)
FH [9]	0	1	1	3	4	1	3	0	1	0	2	2	1	1	0
MD [11]	4	0	2	0	0	0	4	0	3	0	0	0	3	0	0
Total	4	1	3	3	4	1	7	0	4	0	2	2	4	1	0

FH=Fort Hare, MD=Middle Drift

4.1.3. Antibiotic resistance genes of S. aureus

Of the 20 isolates tested for penicillin antibiotic resistance gene 12 (60%) were found to possess this gene (Figure 1). Three isolates were from Fort Hare dairy farm and eight isolates were from Middle Drift dairy farm (Table 6).The *blaZ* gene showed 173 bp in all the isolates tested. Of all the antibiotic resistance genes tested, the express for the *blaZ* resistance gene has showed to be the most prevalent in *S. aureus* isolates.

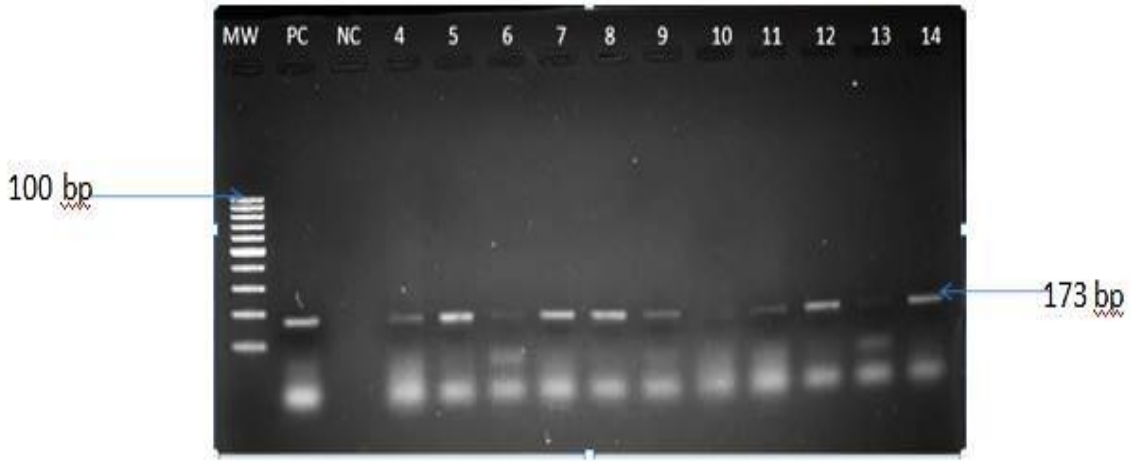


Figure 1: Typical PCR products of the penicillin (blaZ) antibiotic resistance gene. Lane MW: 100 bp, Lane PC: ATCC29123 positive control, Lane NC: negative control, Lane 4-14: are strains of S. aureus.

All the isolates were tested for Gentamicin antibiotic resistance genes and out of the three genes targeted only one gene (*aac(6')/aph(2'')*) was detected by PCR amplification reaction and only eight (40%) isolates were found to possess this antibiotic resistance gene which showed a base pair size of 174 (Figure 2). Two isolates were from Fort Hare dairy farm and six were from Middle Drift dairy farm (Table 6).

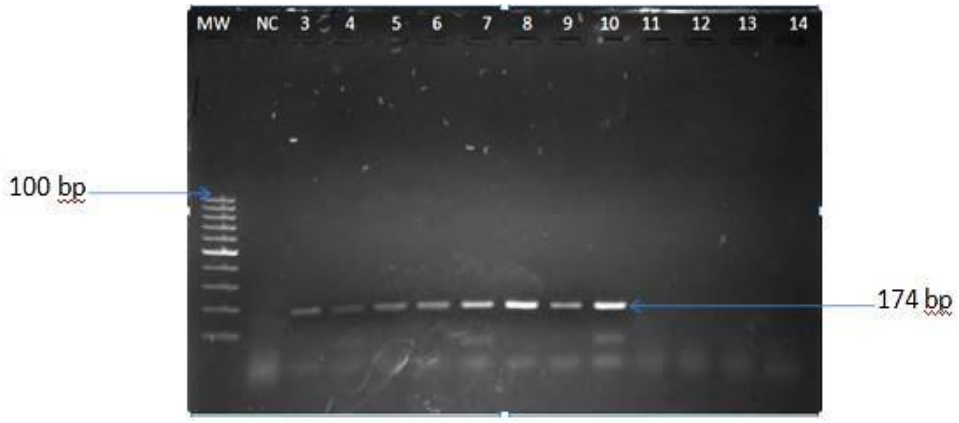


Figure 2: Amplicons of the gentamicin (aac(6')/aph(2'')) antibiotic resistance gene. Lane MW: 100 bp, Lane NC: negative control, Lane 3-10: are strains of S. aureus cultures.

At least three (15%) isolates possessed two different tetracycline antibiotic resistance genes. Two isolates were found to exhibit the same gene (*tetM*) and one isolate (*tetK*) another gene. Two isolates showed 360 bp and one isolate had 158 bp. (Figure 3). All three isolates were from Middle Drift dairy farm and none was detected from Fort Hare farm (Table 6).

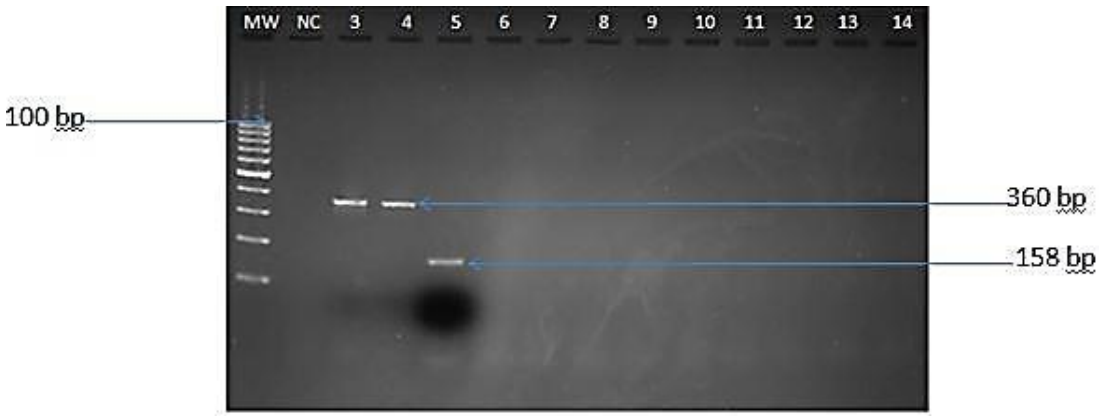


Figure 3: Typical amplicons of the tetracycline (tet (M), tet (K)) antibiotic resistance genes. Lane MW: 100 bp, Lane NC: negative control, Lane 3 and 4: tet(M) and Lane 5: tet(K).

4.1.4. Virulence genes of *S. aureus*

Amplification of the clumping factor (*clfA*) gene showed a single amplicon with size of precisely 980 bp for 7 *S. aureus* strains demonstrating no polymorphisms (Figure 4). Three isolates were from Fort Hare dairy farm and four were from Middle Drift dairy farm (Table 7).

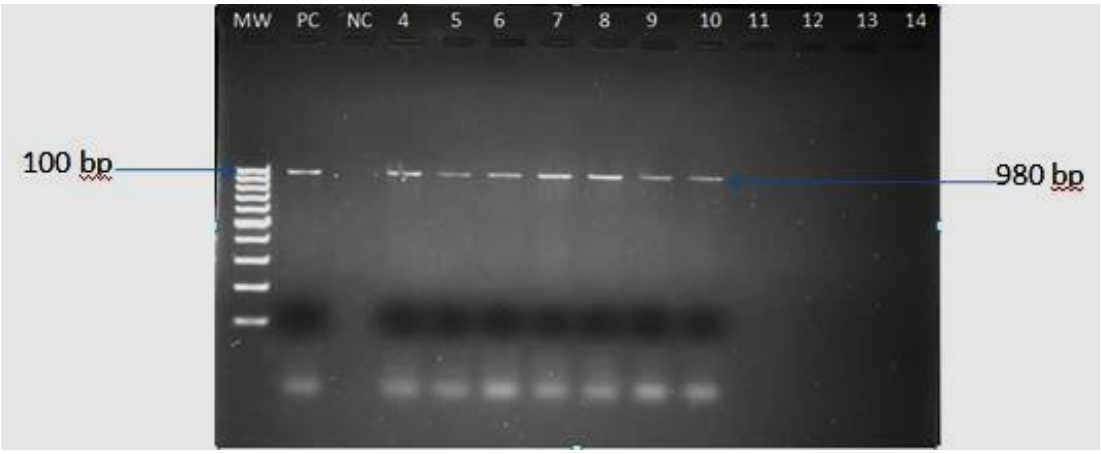


Figure 4: Amplicons of the genes encoding Staphylococcal clumping factor (clfA) genes. Lane MW: 100 bp, Lane PC: ATCC 29213 positive control, Lane NC: negative control, Lane 4-10: are strains of *S. aureus*.

In the case of exfoliative toxin A (*eta*) four amplicons of 119 bp were observed (Figure 5). One isolate was from Fort Hare dairy farm and three isolates were from Middle Drift dairy farm (Table 7).

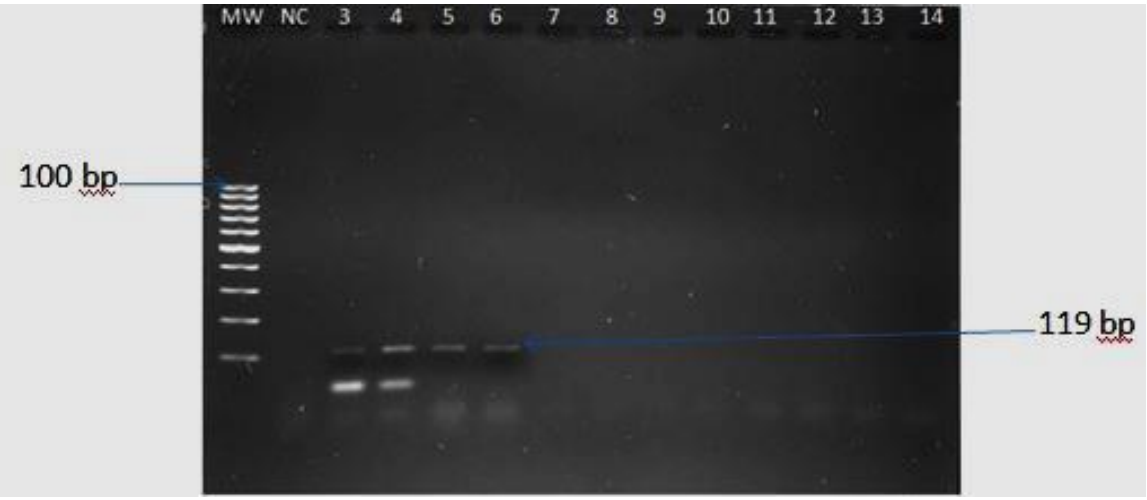


Figure 5: Amplified amplicons of the genes encoding Staphylococcal exfoliative toxin a (eta) genes. Lane MW: 100 bp, Lane NC: negative control, Lane 3-6: are strains of S. aureus.

Polymorphism was found in the *coa* gene. Of the 20 samples 16 specimens contained the *coa* gene (Figure 6). 10 isolates were from Fort Hare farm and Middle Drift dairy had 6 isolates (Table 7).

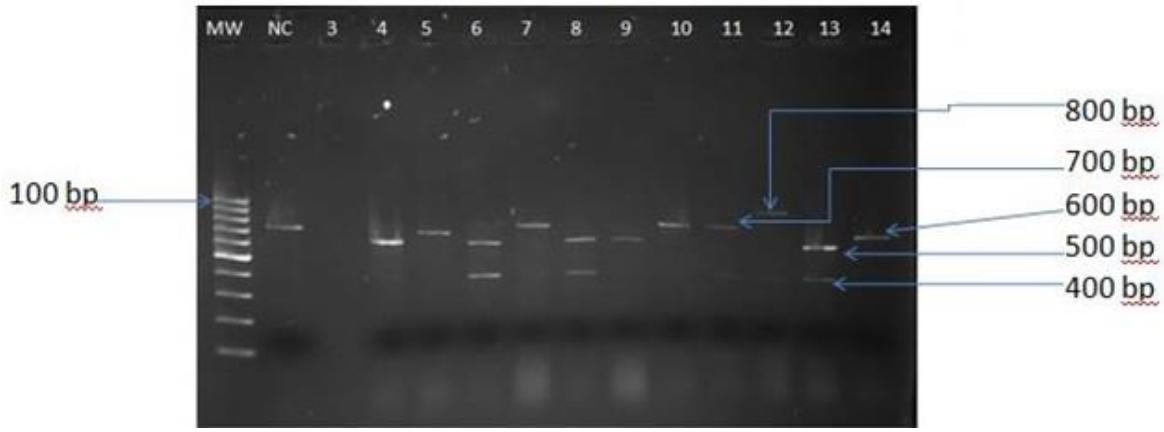


Figure 6: Amplicons of the coagulase (coa) gene. Lane MW: 100 bp, Lane NC: negative control, Lane 4-14: are strains of S. aureus.

Polymorphic expression of 12 specimens of *Staphylococcus aureus* contained *spa* gene (X-region) (Figure 7). Eight isolates were from Fort Hare dairy and four isolates were from Middle Drift dairy farm (Table 7).

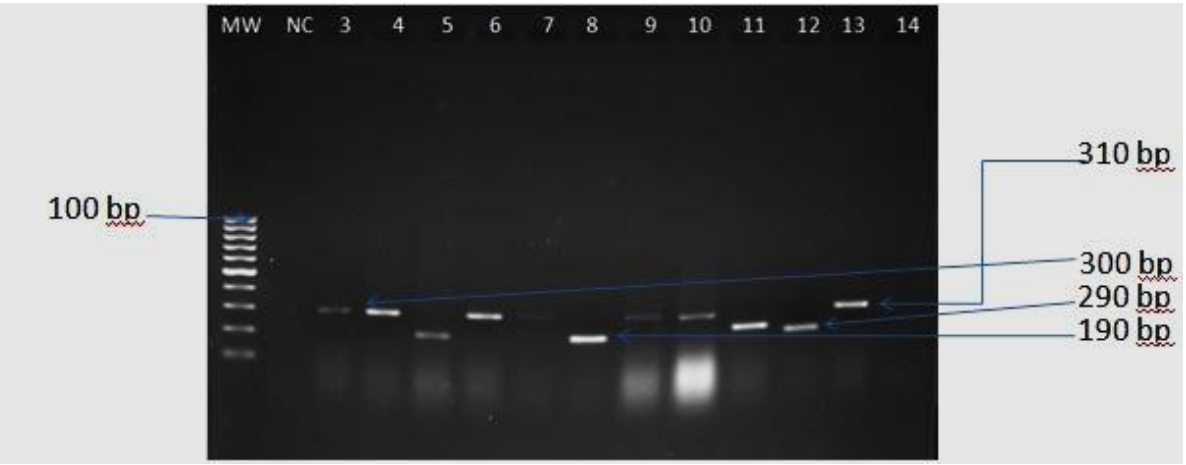


Figure 7: Different PCR products of the repeats of the spa (X region). Lane 1: MW, Lane NC: negative control, Lane 3-13: strains of S. aureus.

CHAPTER 5

5.1. DISCUSSION

Mastitis disease is one of the detrimental diseases associated with huge economic losses in dairy industry. *Staphylococcus aureus* is the predominant causative agent responsible for this disease worldwide. The progression and alterations of antibiotic resistance in this bacterium is a cause of concern in the dairy sector. *S. aureus* has been reported as one of the important reasons of bacterial contamination. Milk and products derived from milk serve as excellent conveyers of Staphylococcal food poisoning (De Buyser *et al.*, 2001; Jorgensen *et al.*, 2005).

Food surveillance for microbial contamination is important for public health protection and consumer interest (Addis *et al*., 2011). Being a transmissible mastitis bacterium, *S. aureus* represents a risk for mastitis outbreaks occurring in a herd (Smith et al., 2005). *S. aureus* may also cause human infections and is associated with both community acquired and nosocomial morbidity and mortality (Zadoks et al., 2000). A range of illnesses in humans from minor skin infections, such as pimples, impetigo, boils, cellulites, furuncles, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases, such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), and septicaemia (Chamber, 2001) have been associated with *S. aureus*. The limitations of the study were the lack of labour during the collection of milk samples and sometimes most of the cows targeted were sent for drying therapy.

This work reports sixteen antibiotics from different classes that were used for susceptibility tests in 20 *S. aureus* isolates. The *S. aureus* bacteria isolated from cases of mastitis were mostly

resistance to penicillin (85%). Similarly, in the study conducted by Sori *et al.* (2011), 87.2% *S. aureus* isolates were found to be resistant to penicillin G antibiotic. In the research performed by Ateba and co-workers *S. aureus* were highly resistant to penicillin (94.4%). This is similar with the work of Gooraninejad and co-workers (2007) where they indicated that cows with mastitis in Tehran had 57% resistance of *S. aureus* to penicillin. Moreover, the research conducted in Argentina by Gentillini et al. (2002) revealed that 40% of *S. aureus* strains were resistant to penicillin G. Myllys and colleagues (1998) also indicated that in Finland 50% of *S. aureus* isolates were resistant to this drug. The resistance of *S. aureus* to penicillin and other β-lactams is attributed to the fact that around 50% of *S. aureus* strains produce lactamase (Green and Bradley, 2004).

In the present study some *S. aureus* isolates showed resistance to tetracycline (60%). Contratry, the report conducted by Momtaz *et al*. (2012) revelead bacterial resistance of 26.1% in some isolates. Tetracyclines including doxycycline which showed 45% resistance in this work inhibit protein synthesis by preventing the attached of aminocyl-tRNA to the ribosomal acceptor site (Roberts, 1996). However, in this work it is evident that the mutations within bacterial genes conferred some resistance to the antibiotics.

Some of the *S. aureus* isolates demonstrated 55% resistance to trimethoprim sulphamethxazole. The trimethoprim antimicrobial agent showed 60% resistance. However, the work done by Momtaz *et al*. (2012), implicated that 17.4% of *S. aureus* isolates were resistant to this antimicrobial agent. Thombson and colleagues (1993), have mentioned that sulphonamides resistance is caused by two genes known as *Sul1* and *sul2* encoding dihydropteroate synthesis. The trimethoprim resistance is usually caused by several mechanisms such the overproduction

of the enzyme dihydrofolate reductase, mutations in the structural gene for dihydrofolate reductase, and acquisition of a gene encoding a resistant dihydrofolate enzyme which is the most resistant in clinical isolates (Thomson, 1993). This is a worrisome situation because farms normally buy antibiotics based on hearsay and administer those antibiotics without finding out what is the specific organism that is causing mastitis and without the help of a veterinary doctor.

The present study has tested eleven different antibiotic resistance genes of *S. aureus* and only three out of eleven were detected by polymerase chain reaction. The penicillin (*blaZ* gene) gene was found to be the most prominent (60%) antibiotic resistance gene. Various studies have reported that penicillin resistance was between 51, 61, 91 and 100 percent (Kantzanou *et al.*, 1999; Sirmatel *et al*., 2004; Savas *et al.*, 2005, Zmantar *et al*., 2008).

The penicillin antibiotic resistance gene was then followed by aminoglycoside-modifying enzyme gene (AME gene) (40%) and the least detected (15%) was the tetracycline antibiotic resistance gene. In our study *S. aureus* isolates were tested for the presence of aminoglycoside-modifying enzyme genes (AME genes), the (*aac(6')/aph(2'')*, *aph(3')-IIIa* and *ant(4')-Ia*) by single PCR reactions. Interestingly, at least one of the AME gene was detected in 8 (40%) of 20 *S. aureus* isolates. This is accordance with the study conducted by Duran and colleagues (2012), the presence of at one of the AME gene ((*aac(6')/aph(2'')*) was expressed in 111 (37.3%) of 298 staphyloccal isolates. Prevoius studies have reported that the (*aac(6')/aph(2'')* gene as the most prevalent AME gene found in staphylococci (Choi *et al*., 2003; Yadegar *et al*., 2009). A similar trend was reported in Europe (Vanhoof *et al*., 1994). Other researchers reported that this gene was found less frequently among clinical isolates of MRSA strains (Ida *et al.*, 2001).

In this study several virulence factors of the pathogen were found and coagulase gene is one of them. The amplification of the *coa* gene expressed six different with relatively 400 bp for four (36%) isolates, 500 bp for one (9.1%) isolate, 600 bp for three (27.3%) isolates, 610 bp for three (27.3%) isolates, 700 bp for four (36%) isolates, 710 bp for one (9.1%) isolate, and 800 bp for one (9.1%) isolate. Our results are in accordance with the study of Shana *et al*. (2009), where by the amplification of the coagulase gene demonstrated four distinct sizes of polymorphisms with precisely 400 bp for a single (4%) strain, 600 bp for twelve (57%) strains, 700 bp for two (9%) strains and 900 bp for six (28%) strains. In Brazil, Cabral *et al.* (2004) advocated that the PCR products of relatively 600 bp are predominant in bovine cases of mastitis. This similar pattern was also observed in this study as 600 bp was relatively in abundance than other band sizes.

In this study it was observed that about four isolates of *S. aureus* expressed more than one amplicon of *coa* and Shana and co-workers (2009) suggested this is attributed to the fact that coagulase gene is formed by more than a single allele (Goh *et al*. 1992, Aslantas *et al*. 2007) observed and seven type of *coa* gene and out of the seven; two accounted for more than 50% of the *S. aureus* strains. Goh and co-workers (1992), implicated that the coagulase gene is one of the most main virulence factors of this pathogen. This gene increases bacterial growth and stimulates infection using the mechanism of the host (Aarestrup *et al*., 1995). Previous studies suggested that the single-banded *coa* PCR amplicons originate from animals whereas double-banded PCR amplicons have been observed in some *S. aureus* isolates of human-origin (Goh *et al*., 1992; Schwarzkopf and Karch, 1994; Lange *et* al., 1999; Raimundo *et al*., 1999).

A further characterization of the *S. aureus* isolates showed amplification of the clumping factor virulence gene of 980 base pairs for 7 (35%) isolates with no size polymorphism in a single reaction. Similary, in the study conducted by Momtaz *et al.* (2010) showed that 50.79% of the *S. aureus* strains contained the clumping factor gene. As with previous studies (Stephan *et al.,* 2001), the amplification of the clumping factor (*clfA*) gene resulted in a single amplicon with a size of approximately 1,000 bp, indicating no size polymorphisms of this gene. The presence of the clumping factor gene is considered as *Staphylococcus* species virulence gene in development and severity of mastitis in cows. Moreover, as with the coagulase gene*;* it is also envolved in the proliferation of bacteria growth and fuels infection by manipulating the mechanism of the host (Aarestrup *et al*., 1995).

The *spa* X region virulence gene was also detected in the isolates. The *spa* X region of the protein A binds to the Fc portion of IgG and protects bacteria during the initial phases of infection. The present study has presented a sum of four different *spa* X region of approximately 290 bp for two strains (16.7%), 190 bp for two strains (16.7%), 300 bp for four strains (33.3%) and 310 bp for one strain. Similarly, the *spa* region produced one amplicon of 250 bp for ten *S. aureus* strains (38%), 280 bp for 9 strains of *S. aureus* (14%) and and 180 bp for eight strains of *S. aureus* 47% (Shana *et al.,* 2009). Quite a number of studies have attempted to distinguish between epidemic and nonepidemic strains and have advocated that strains with more than seven repeats in the X region of the protein A are highly likely to be epidemic, where as the presence of seven or fewer repeats was indicative of nonepidemic strains (Frenay *et al*., 1996). This virulence gene facilitates colonization and skin infection and mammary glands (Frenay *et al*., 1996). Jakubczak *et al*. (2007) have revelead that among strains isolated from cows that were affected by mastitis, a highly variable number of repeats were observed but the frequency of strains with more than seven repeats was higher. However

in the present study fewer repeats of the *spa* X region were observed with implicates that the *S. aureus* strains found in these farms nonepidemic strains.

Kumar *et al.* (2011) suggested that polymorphism in spa X region protein A, IgG binding region, coagulase and clumping factor genes demonstrated important genetic heterogeneity between *S. aureus* isolates. These results are in agreement with those of Sabour *et al.* 2004. Variations of spa have been extensively used to distinguish between epidemic and non-epidemic and MSSA strains (Montensions *et al.,* 2002, Reinoso *et al.,* 2008; Kumar *et al.,* 2010).

None of the isolates expressed the *tsst* gene, *etb* and *spa* (IgG binding region). Contrary to our findings, outcomes obtained by Zschock *et al.* (2004) gave at least three positive isolates (3.3%) in a single PCR reaction of the *tsst* gene. It has been implicated that *tsst* gene causes toxic shock syndrome in humans and has a pathogenecity system of bovine mastitis with its superantigenic features (Ferens *et al.,* 1998; Zschock *et al.,* 2000; Schuberth *et al.,* 2001; Omoe *et al.,* 2003). In an investigation conducted by Matsunanga and colleagues revealed that from peracute bovina mastitis incidences all *S. aureus* isolates yielded TSST-1 and associated the outcomes with clinical cases. Moreover, a highly significant prevalence of the *tst* gene (19.2%, 46.2% and 60.9%) in bovine, goats and sheep was reported (Smyth *et al.,* 2005).

Some strains of this bacterium are known to produce one or both exfoliative toxins (ETs), ETA and ETB. These two immunologically exfoliative toxins have been associated with a succession of impetigious staphylococcal diseases known as staphylococcal scalded skin

syndrome (Iandolo, 1989; Marrack and Kappler, 1990; Johnson *et al.,* 1991; Hayakawa *et al.,* 1998). In the present study no positive results were observed in the PCR amplification of the 20 *S. aureus* isolates for the *etb* gene. However, only four (20%) were obtained for the *eta* gene. Contratry, no positive isolates were obtained for both *eta* and *etb* genes in the study performed by Karahan and co-workers (2009). Retrospective studies suggested that *S. aureus* ET genes are usually of human-origin and are found in some type of skin infection in infants. Having said that, yet some researchers up to this day; have not explicitly agreed that whether the animal and human-origin *S. aureus* isolates exhibit these genes (*eta* and *etb*) and possibly generate these typical toxins (Adesiyun *et al.,* 1991; Hayakawa *et al.,* 1998). Nevertheless, some work has been published advocating that ET genes were obtained in 3.9% of the animal-origin *S. aureus.* Again, *eta* genes were obtained at the rate of 1.2 percent in cows with bovine mastitis and 0.6% from milk tanks (Hayakawa *et al.,* 1998). In Denmark between 2000 and 2002, it was reported that none of the ET genes were prevalent in a sum of 414 *S. aureus* isolates with mastitis cows (Larsen *et al.,* 2000, 2002). Furthermore, none of these genes were found in 128 *S. aureus* in some other countries in cows with mastitis cases. Researchers from Germany, Switzerland and Indonesia stated that no ET genes were obtained from cattle with mastitis (Akineden *et al.,* 2001; Stephan *et al.,* 2001; Salasia, 2004).

During the sample collection it was observed that the milkman use the same gloves throughout the milking session in between cows. They do not wash off their hands at times; they use impure ground water to clean the milking parlour, utensils and containers. These poor farm management practices contribute to the presence of *S. aureus* in the milk and improving the hygienic situations of the milking environment may diminish the prevalence of *S. aureus* and inhibit its transmission to humans.

To the best of our knowledge no study has been conducted in Alice to evaluate the incidence of virulence genes and resistance of *S. aureus* to various antimicrobial agents from raw milk. This suggests that precautionary and control measures need to be adopted in these two farms. The farm owners and milking personnel need to be alert about the harmful implications which may arise as a result of poor management practices and unhygienic conditions in the farms.

CONCLUSION AND RECOMMENDATION

The findings of our study revealed that there was high resistance of *S. aureus* against penicillin phenotypically and genotypically, respectively. It was also observed that *S. aureus* strains possessed several virulence genes including the coagulase gene which was the most prevalent virulence gene amongst others in this study. The high level of phenotypic and genotypic penicillin resistance and implication thereof warrant extensive investigation. Furthermore, the farm management need to take extra precautions to keep and maintain milking utensils hygienically clean.

CHAPTER 6

6.1. REFERENCES

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APPENDIX

Figure 1 Showing a milking personnel handling milking equipment with bare hands.

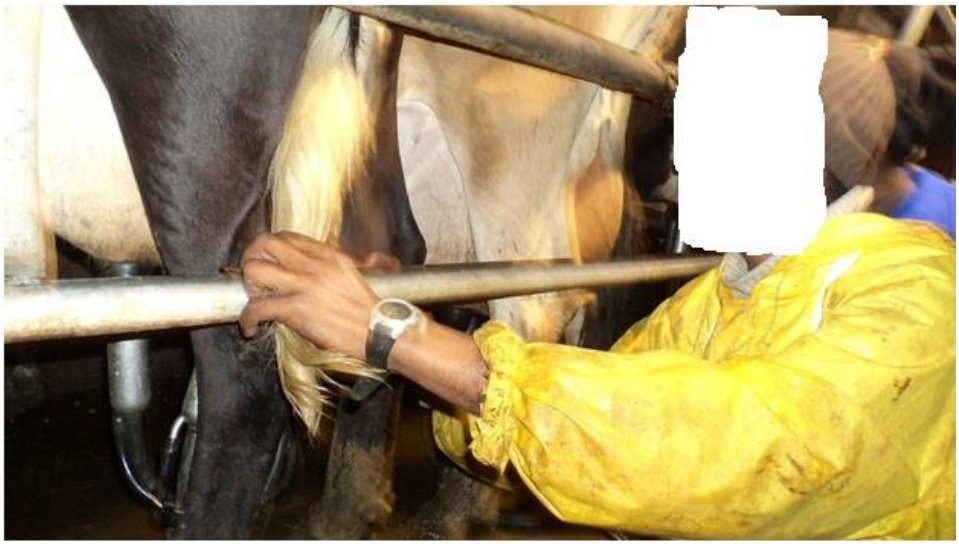

Figure 2 shows a milker in the milking parlour with dirty clothes and without gloves.

Figure 3 Dirty cows from a muddy environment are being milked without being washed off.

Figure 5 Showing fermentation of mannitol caused by presumptive *Staphylococcus aureus* isolates on Mannitol Salt Agar plates.

Figure 4 Observation of the microscopic slide after a Gram-staining procedure by Pekana Abongile.

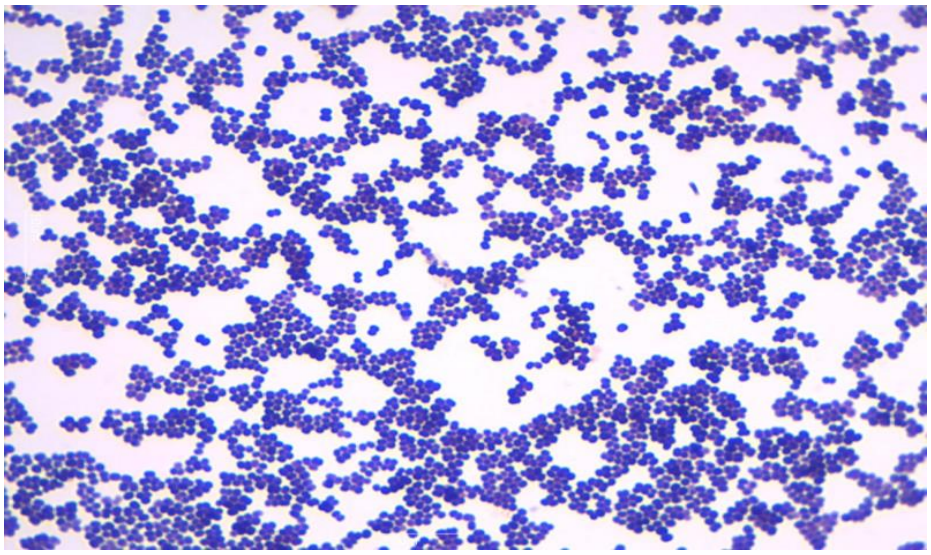

Figure 5 shows microscopic observation gram-positive cocci of presumptive *S. aureus* isolates

Figure 6 shows the preparation of the API Staph kit for the identification of presumptive *S. aureus* isolates.

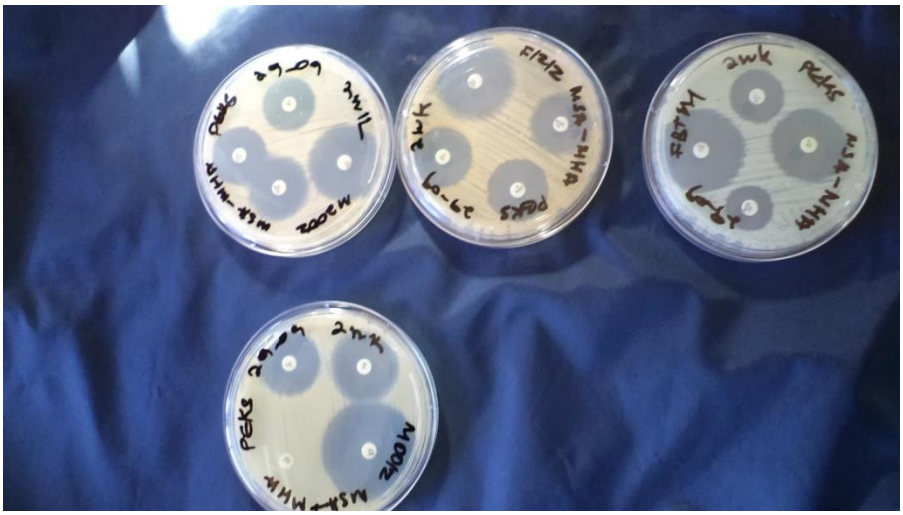

Figure 7 demonstrating disc diffusion antibiotic susceptibility test onto Mueller-Hinton Agar plates.

Table 1 pH results of the milk samples in Middle Drift Dairy farm.

#	Cow tag	pH	#	Cow tag	pH	#	Cow tag	pH	
1.	2404	7.06	32.	2249	6.46	63.	H521	6.69	
2.	2470	6.57	33.	8389	6.45	64.	8401	6.68	
3.	2047	6.60	34.	2253	6.45	65.	0147	6.42	
4.	2313	6.49	35.	8253	6.53	66.	2266	6.43	
5.	2265	6.59	36.	9086	6.66	67.	2572	6.52	
6.	2265	6.64	37.	2316	6.55	68.	2124	6.94	

7.	2394	6.64	38.	2046	6.67	69.	2231	6.53	
8.	9004	6.53	39.	9021	6.64	70.	2308	6.44	
9.	2207	6.96	40.	H246	6.33	71.	2283	6.50	
10.	2334	6.64	41.	0922	6.43	72.	9015	6.48	
11.	9078	6.40	42.	2040	6.46	73.	2041	6.67	
12.	2503	6.50	43.	7172	6.53	74.	2497	7.01	
13.	10028	6.38	44.	2457	6.59	75.	0074	6.51	
14.	0972	6.60	45.	2441	6.42	76.	9104	6.53	
15.	2128	6.49	46.	9083	6.38	77.	0180	6.35	
16.	0090	6.49	47.	2361	6.47	78.	2529	6.48	
17.	8103	6.51	48.	8456	6.51	79.	2351	6.74	
18.	2479	6.62	49.	2099	6.41	80.	2098	6.39	
19.	H816	6.38	50.	9090	6.38	81.	0922	6.83	
20.	10171	6.40	51.	9041	6.60	82.	8111	6.67	
21.	2540	6.49	52.	7048	6.44	83.	8207	6.61	
22.	7005	6.59	53.	0971	6.52	84.	2105	6.41	
23.	2327	6.60	54.	9044	6.63	85.	2627	6.39	
24.	2043	6.70	55.	0976	6.56	86.	2522	6.49	
25.	2352	6.46	56.	2144	6.58	87.	2532	6.57	
26.	9056	6.56	57.	9069	6.48	88.	9030	6.43	
27.	7003	6.46	58.	0988	6.41	89.	0083	6.44	
28.	0002	6.41	59.	2475	6.85	90.	2492	6.50	
29.	9643	6.53	60.	2079	6.85	91.	2503	6.50	
30.	H248	6.44	61.	2430	6.40	92.	0962	6.61	
31.	0026	6.49	62.	H522	6.47	93.	0968	6.77	

94.	8351	6.61	109.	1618	6.82	124.	1172	6.69	
95.	2269	6.67	110.	1899	6.56	125.	6007	6.72	
96.	8178	6.67	111.	1376	6.55	126.	10276	6.59	
97.	8346	6.58	112.	1859	6.60	127.	1172	6.63	
98.	2273	6.47	113.	10040	6.77	128.	7141	6.70	
99.	8122	6.65	114.	1941	7.00	129.	6193	6.02	
100.	0130	6.43	115.	7017	6.57	130.	8088	6.62	
101.	6373	6.62	116.	10158	6.58	131.	1148	6.68	
102.	1612	6.59	117.	1272	6.58	132.	8023	6.61	
103.	1202	6.68	118.	1073	6.58	133.	9173	6.64	
104.	9078	6.57	119.	8048	6.61	134.	1194	6.98	
105.	1524	6.74	120.	1762	6.54	135.	1778	6.69	
106.	7053	6.70	121.	1126	6.58	136.	1464	6.55	
107.	10083	6.53	122.	8122	6.59	137.	1380	6.66	
108.	1034	6.27	123.	1235	6.74	138.	1416	6.94	

139.	1051	6.70	160.	1783	6.71	179.	8012	6.76	
140.	6145	6.74	161.	1906	6.62	180.	1240	6.71	
141.	1422	6.64	162.	1260	7.13	181.	8123	6.65	
142.	1636	6.80	163.	1418	6.83	182.	6246	6.78	
143.	1965	6.58	164.	8100	6.61	183.	1708	6.72	
144.	1800	6.86	165.	1237	6.51	184.	7029	6.77	
145.	1651	6.60	166.	1231	6.79	185.	1185	6.78	
146.	1455	6.77	167.	6172	6.96	186.	8047	6.74	

147.	1119	7.08	168.	1586	6.70	187.	1890	6.70	
148.	8211	6.69	169.	9010	6.94	188.	9030	6.74	
149.	7014	6.74	170.	8133	6.98	189.	1785	6.77	
150.	1167	6.75	171.	1965	6.67	190.	9208	6.68	
151.	1887	6.64	172.	1751	6.60	191.	1041	6.62	
152.	1671	6.70	173.	1400	6.63	192.	1273	6.71	
153.	1067	6.77	174.	1309	6.75	193.	1631	6.74	
155.	1308	6.73	175.	6346	6.65	194.	9022	6.61	
156.	1638	7.05	176.	1175	6.63	195.	1170	6.65	
157.	1900	6.55	177.	1815	6.69	196.	1749	6.75	
158.	1779	6.64	178.	10388	6.64	197.	1544	6.74	
						198.	1254	6.72	
						199.	1271	6.61	
						200.	292	6.75	

Table 2 pH results of the milk samples in Fort Hare Dairy farm

#	Cow tag	pH	#	Cow tag	pH	#	Cow tag	pH
1.	6373	6.62	32.	8023	6.61	63.	1237	6.51
2.	1612	6.59	33.	9173	6.64	64.	1231	6.79
3.	1202	6.68	34.	1194	6.98	65.	1586	6.70
4.	9078	6.57	35.	1778	6.69	66.	9179	6.74
5.	1524	6.74	36.	1464	6.55	67.	1300	6.66
6.	7053	6.70	37.	1380	6.66	68.	1876	6.71
7.	10083	6.53	38.	1416	6.94	69.	9010	6.94
8.	1034	6.27	39.	1051	6.70	70.	8133	6.98
9.	1618	6.82	40.	6145	6.74	71.	1965	6.67
10.	1899	6.56	41.	1422	6.64	72.	1751	6.60
11.	1376	6.55	42.	1636	6.80	73.	1400	6.63
12.	1859	6.60	43.	1965	6.58	75.	6346	6.65
13.	10040	6.77	44.	1800	6.86	76.	1175	6.63
14.	1941	7.00	45.	1651	6.60	77.	1815	6.69
15.	7017	6.59	46.	1455	6.77	78.	10388	6.64
16.	10158	6.58	47.	1119	7.08	79.	8012	6.76
17.	1272	6.58	48.	8211	6.69	80.	1240	6.71
18.	1073	6.58	49.	7014	6.74	81.	8123	6.65
19.	8048	6.61	50.	1167	6.75	82.	6246	6.78
20.	1762	6.54	51.	1887	6.64	83.	1708	6.72
21.	1126	6.58	52.	1671	6.70	84.	7029	6.77
22.	8122	6.59	53.	1067	6.77	85.	1185	6.78
23.	1235	6.74	54.	1308	6.73	86.	8047	6.74
24.	1172	6.69	55.	1638	7.05	87.	1890	6.70
25.	6007	6.72	56.	1900	6.55	88.	9030	6.74

26.	10276	6.59	57.	1797	6.64	89.	1785	6.77
27.	1772	6.63	58.	1783	6.71	90.	9208	6.68
28.	7141	6.70	59.	1906	6.62	91.	1041	6.62
29.	6193	6.02	60.	1260	7.13	92.	1273	6.71
30.	8088	6.62	61.	1418	6.83	93.	1631	6.74
31.	1148	6.68	62.	8100	6.61	94.	9022	6.61

95.	1170	6.65	97.	1544	6.74	99.	1271	6.61
96.	1749	6.75	98.	1254	6.72	100.	292	6.75

101.	096	6.94	120.	2280	6.50	142.	9095	6.77
102.	2580	6.17	121.	0011	6.60	143.	0139	6.79
103.	2475	6.68	122.	H176	6.40	144.	2396	7.00
104.	2070	6.90	123.	2099	6.90	145.	9047	6.85
105.	0832	6.30	124.	9042	6.59	146.	08133	6.82
106.	2524	7.00	125.	0118	7.00	147.	2606	6.63
107.	0130	6.65	130.	8382	6.73	148.	8040	6.33
108.	9020	6.87	131.	2688	6.76	149.	9039	6.79
109.	6178	6.64	132.	0841	7.00	150.	2394	6.90
110.	8122	6.63	133.	2585	6.64	151.	0129	6.68
112.	2104	6.62	134.	244	7.22	152.	2047	6.58
113.	0025	6.78	135.	2390	6.74	153.	0837	6.80
114.	8121	7.00	136.	8148	6.84	154.	2231	6.88
115.	2212	6.71	137.	09226	6.66	155.	8156	6.87
116.	8007	6.51	138.	11036	6.48	156.	0132	6.83
117.	2406	6.68	139.	2520	6.68	157.	2599	6.89
118.	2547	6.58	140.	2264	6.63	158.	0157	6.76
119.	09103	6.80	141.	0154	6.84	159.	0162	6.73

160.	0701	6.80	171.	2249	6.44	182.	2019	6.79
161.	2437	7.00	172.	0180	6.76	183.	0054	6.69
162.	2374	7.09	173.	2427	6.62	184.	9112	7.02
163.	2074	6.44	174.	09114	6.79	185.	51145	6.78
164.	0111	6.76	175.	0996	6.96	186.	9027	6.73
165.	6010	6.59	176.	8279	6.84	187.	0083	6.60
166.	9048	6.82	177.	0055	6.76	188.	7001	7.03
167.	0942	6.78	178.	2598	6.16	189.	8029	6.38
168.	6173	6.85	179.	2357	6.77	190.	0190	6.52
169.	2294	6.70	180.	2271	6.82	191.	2197	7.10
170.	2055	6.94	181.	6541	6.82	192.	8346	6.80

193.	8050	6.93	196.	7006	6.86	199.	2352	6.81
194.	0938	6.39	197.	2073	6.72	200.	2558	7.00
195.	0912	6.84	198.	9026	6.57			