EVALUATION OF GENES ASSOCIATED WITH MASTITIS IN CROSSBRED DAIRY CATTLE

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2020

DECLARATION AND APPROVAL

I hereby declare that this thesis is my original work and has never been submitted in this or any other university for the award of a degree.

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APPROVAL

This thesis has been submitted for examination with our approval as the University of Nairobi supervisors.

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DEDICATION

I dedicate this thesis to my father Victor E. E. Tarbal (V.E.E.T), Mother Erominia Atim and the rest of my family members.

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LISTS OF ABBERIVATIONS AND ACRONYMS

μl	Microliter
g/L	gram per litre
ml	millilitre
A	Adenine
ANOVA	Analysis of Variances
BLAST	Basic Local Alignment Search Tool
Blastn	Basic Local Alignment Tool against known nucleotides
C	Cytosine
CM	Clinical Mastitis
CMT	California Mastitis Test
DCT	Dry Cow Therapy
DF	Degree of freedom
DNA	Deoxyribonucleic Acid
DMSCC	Microscopic Somatic Cell Count
EDTA	Ethylene diamine tetra acetic Acid
FAO	Food and Agricultural Organization
G	Guanine
G-C	Guanine - Cytosine
GDP	Gross Domestic Products
HSD	Honest Significant Differences
MEGA	Molecular Evolutionary Genetic Analysis

NCBI	National Centre for Biotechnology Information
LSCS	Lactation Somatic Cell Scores
LF	Left Fore
LGB	Beta-lactoglobulin gene
LH	Left Hind
LTF	Lacto-transferrin/Lactoferrin
LSD	Least Significant Difference
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reactions
RF	Right Front
RH	Right Hind
SC	Somatic Cells
SCC	Somatic Cell Counts
SCS	Somatic Cell Scores
SPSS	Statistical Package for Social Science
T	Thymine
Ta	Annealing Temperature
Tm	Melting Temperature
U	Uracil
UoJ	University of Juba
USAID	United States Agency for International Development
UV	Ultra- Violet

ABSTRACT

Mastitis is a detrimental disease that economically affects dairy farming by reducing milk quality and quantity produced, the sudden death of dairy cows, premature culling of dairy cows before attaining their maximum production ages, and the cost of disease prevention and treatments. In Sub-Sahara Africa, mastitis prevalence among smallholders' dairy farmers have been recorded to exceed 50 percent, and it has continued to cause significant threats. In East Africa, subclinical mastitis prevalence varies between 16 and 80 percent among smallholders' dairy farmers. Full udder quarters of 38 lactating crossbred dairy cows were cleaned with 75% alcohol and screened with the California mastitis test (CMT) kit for subclinical mastitis and somatic cell count (SCC). A total of 152 milk samples were collected aseptically from each quarter of 38 lactating dairy cows into screw-capped bottles `for direct microscopic somatic cell count analyses in the laboratory. Ninety-six blood samples from both lactating and non-lactating crossbred cattle were obtained for genomic DNA extractions. The genomic DNA was later run in polymerase chain reactions with two oligo primers of 252 bp (LGB) and 301bp (LTF) for beta-lactoglobulin lactoferrin genes, respectively. Results CMT scores carried out in the farm revealed that 55.01% of udder quarters were negative for subclinical mastitic, 43.99 % trace, and 1.32 % were positive for subclinical mastitis. The Least Square Difference (LSD) for pairwise comparison between CMT scores and lactation stage were significantly different between First and second lactation at 0.25±0.11, the second and third at 0.27±0.0118 at $P \geq 0.05$. The means of SCC among the breeds were significantly other at $P \geq 0.05$, for Ayshire and Friesian (68.055±18.82 cells/ml); Ayshire and Guernsey (71.976±23.844 cells/ml); Friesian and Jerseys (64.863±21.429 cells/ml); and Guernsey and Jersey (68.78±25.952 cells/ml). Results of PCR-DNA sequenced showed that there were several genetic variations in the nucleotide sequences. These were identified as Single Nucleotide Polymorphisms

(SNPs) associated with mastitis resistance or tolerance. The genetic variations found among nucleotide sequences of both breeds positive for subclinical mastitis and those free from subclinical were oriented in either transitions or transversions models due to tautomeric shifts in the nucleotide sequences. They were either substituted or inserted, which eventually created variability among nucleotide base sequences. They could also be a result of point shift mutation in the nucleotide sequences. In conclusion, the study also found out that milk quality and somatic cell count were closely associated with the presence of beta-lactoglobulin and lactoferrin genes. The nucleotide sequences of genes have shown significant associations with milk quality and somatic cell counts.

Keyword; Crossbred, Gene, Somatic Cell Count, Mastitis resistant

CHAPTER ONE: NTRODUCTION

1.1 Background information

Mastitis affects dairy production economically through its associated costs and production effects (Gupta *et al*., 2016; Martin *et al.,* 2018). These costs may include: the price of drugs, reduced milk productions, and household income through the discarding of milk in times of treatment, sudden death, and premature dairy cows culling (Jingar *et al.,* 2017). Mastitis is associated with declines in the quantity of milk produced due to high bacterial loads (Sharma *et al*., 2011; Jadhav *et al.,* 2016) and the increase in milk somatic cells. These cells include; polymorphonuclear neutrophils, macrophages, lymphocytes, and mammary epithelial cells (Jadhav *et al.,* 2016). The numbers of these cells in milk are used as an indicator to monitor mammary gland health, and their presence can be used to signal mastitic conditions in the udders of dairy cattle (Li *et al.,* 2014; Jadhav *et al*., 2016).

In sub-Sahara Africa, the prevalence of mastitis among smallholder dairy farmers have been recorded to exceed 50 percent and continues to raise significant threats to dairy producers. In the great lakes region and western parts of Africa, milk production is mostly by smallholder dairy farmers (FAO, 2014). In East Africa, the prevalence of subclinical mastitis varies between 16 and 80 percent. To date, mastitis remains one of the significant factors limiting milk's maximum production by smallholder dairy farmers (FAO, 2014).

Mastitis prevalence has continued to increase despite practices adopted to manage it, such as continuous monitoring for subclinical mastitis, dry cow therapy, and culling of chronically infected cows. Udder health is a valuable trait in dairy cows that need to be maintained for high production of milk and better quality milk that meets consumers' demand in the market (Abebe *et al.,* 2016). The udder health status of a dairy cow is a fundamental factor that boosts production outputs and

increases the economic viability of a farm through income earned from the sales of dairy products and less money spent on disease control and prevention (Edwards *et al.,* 2015). Immunity against mastitis is fundamental in addressing the economic challenges faced by smallholder dairy farmers (Fang *et al.,* 2017). Traits associated with mastitis resistance may play a key role in improving milk quality and quantity. Information on systems that underlie these attributes is essential for smallholder dairy farmers' profitability and can be applied in designing selection criteria for dairy cows against mastitis (Peters *et al.,* 2015; Ismail *et al*., 2018).

Generally, the presence of somatic cells in milk signifies deterioration in milk quality, and the SCCs are internationally used to evaluate milk quality (Sorensen *et al.,* 2016). Breeds of dairy cattle with robust immune systems always produce significant amounts of somatic cells in the milk (Teresiah *et al.,* 2016; Jadhav *et al.,* 2016). However, in some instances, a high milk-producing breed of dairy cattle can produce many somatic cells due to inadequate milking and poor management (Gupta *et al*., 2016).

Beta-lactoglobulin and lactoferrin genes play significant roles concerning mastitis resistance and tolerance (Fang *et al*., 2017). The genes have a bactericidal and bacteriostatic action on pathogenic agents of mastitis. As compared to both SCC and SCS, mastitis has a low heritability making it a challenge to select mastitis resistance. Due to this, selective breeding can lower mastitis incidences in crossbred dairy cattle, where suitable candidate genes associated with mastitis resistance are identified (Cai *et al.,* 2018).

Mastitis is a polygenic trait related to milk quality and production traits. These make it complicated for molecular markers to be used in selective breeding for mastitis resistance in dairy breeds of cattle (Tiezzil *et al.,* 2015).

1.2 Problem statement

The low heritability of mastitis poses a significant challenge in selecting dairy cattle against the disease. Although traditional animal breeding and selection are broadly employed in the section for mastitis resistant dairy breeds, there has been no significant result (Curone *et al.,* 2018). Smallholder dairy farmers make massive losses due to subclinical mastitis on their farms. Making dairy enterprises unprofitable (Fonseca *et al.,* 2009; Cameron *et al.,* 2015; Peters *et al.,* 2015). Most of the losses are incurred due to the associated effects of the disease on the farm due to the challenge of diagnosing disease on farms. Inadequate awareness about the impact caused by the subclinical mastitis in dairy production is still a significant obstacle in smallholder dairy enterprise profitability

1.3 Justifications

Mastitis is a disease of economic importance in dairy through its associated effects on dairy production. The condition causes enormous production challenges like the death of dairy cows before attaining their maximum production ages, increased production cost, and declined in production, which in turn affects the returns earned. The frequent use of antibiotic drugs in mastitis treatment and prevention results in their accumulation as residues in animal bodies. These residues find their way into milk, making it unfit for human consumption raising public health concerns (Jadhav *et al*., 2016). This study was undertaken to evaluate genes associated with mastitis in crossbred dairy cattle, with a view of determining the relationship between California mastitis test scores and somatic cell scores in a sampling population, associations between somatic cell count and lactation stages, and to evaluate the associations between genes and milk quality in different crossbred genotypes of dairy cows.

1.4 Objectives

1.4.1 Overall objective

i. To determine genes associated with mastitis and Somatic Cell Counts in crossbred dairy cattle.

1.4.2 Specific objectives

i. To assess the relationship between California Mastitis Test scores (CMT) and Somatic Cell Counts in different crossbred dairy genotypes.

ii. To evaluate the relationship between genes associated with milk quality and Somatic Cell Counts in crossbred dairy cattle.

1.5 Hypotheses of the study

i. The presence of somatic cell count and California mastitis scores results are highly associated with the presence of subclinical mastitis in udder quarters of crossbred dairy genotypes.

ii. Mastitis is not only a factor that determines the level of somatic cell count present in milk and quality.

CHAPTER TWO: LITERATURE REVIEW

2.1 Mastitis in dairy cattle

Mastitis is an inflammatory of udder tissues due to physical trauma (injuries) or microorganisms' infections (Kibebew, 2017). The disease is identified as abnormalities in the udder such as swelling, heat, redness, hardness, or pain (for clinical). Other mastitis indicators can be irregularities in milk, such as a watery appearance, flakes, or clots (Iraguha *et al.,* 2017). Based on causal factors, mastitis can be classified into four groups: Bacterial, viral, protozoa, and fungal. The primary cause of mastitis on smallholder dairy farms are bacteria (Harjanti *et al*., 2018). The three significant bacterial pathogens implicated include; *Escherichia Coli (E. coli), Staphylococcus spp,* and *Streptococcus spp* (Fonseca *et al*., 2011). Other bacteria associated with mastitis include *Klebsiella spp, Enterobacter spp, and Citrobacter spp* (Ferronatto *et al.,* 2018). *Escherichia coli* causes an acute and severe form of mastitis on dairy farms and is a potential cause of clinical mastitis in smallholder dairy farms (Jiahui *et al.,* 2015). *Streptococcus spp* causes clinical mastitis, but the complex epidemiology of *Streptococcus Uberis* makes it challenging to control mastitis (Tomazi *et al.,* 2019). *Staphylococcus spp* causes up to 50% of mastitis infections in smallholder dairy herds, and it is contagious (Davies *et al.,* 2016).

The low heritability of clinical mastitis is attributable to an unstable environment and poor management in the dairy herd. A well-managed herd reduces environmental differences, which are predisposing factors causing mastitis. The dairy herd with a good management strategy results in a stable environment with reduced predisposing factors and improved heritability (Abebe *et al.,* 2016; Wellnitz *et al.,* 2018). This strategy improves the chances of selection against mastitis resistance and tolerance in the dairy herd, genetically, creating a stable environment enhances a

significant improvement in the flock (Boas *et al.,* 2017). Therefore, heritability for milk production traits is usually moderate to high due to management (Koeck *et al.,* 2010).

2.1.1 Classification of mastitis based on clinical signs

Mastitis is categorized into two major types as clinical and subclinical (Hoekstra *et al*., 2018). Clinical mastitis exhibits signs such as sudden death, change in milk composition and appearances, swelling, and redness of the udder (Abebe *et al.,* 2016; Zavadilova *et al.,* 2017). Unlike clinical mastitis, subclinical mastitis does not show overt signs in the affected animal and is therefore very difficult to diagnose (Cai *et al.,* 2018; Hussein *et al.,* 2018). Due to its presentation, subclinical mastitis severely affects smallholder dairy farmers and results in significant losses due to low quality and reduced quantities of milk (Holland *et al.,* 2015; Peters *et al.,* 2015). It is because it takes a long time before detection as compared to the clinical type. The prevalence of subclinical mastitis in older lactating dairy cows is higher than the first lactating heifers (Santman-Berends *et al*., 2012). Continued reduction in milk production without clinical signs of disease over more extended periods is one indicator of subclinical mastitis. (Abebe *et al*., 2016; Balaji *et al.,* 2016). The chances of curing mastitis decrease with increasing cows' ages (Singh *et al*., 2018). The presence of somatic cell counts exacerbates infection in all udder quarters, although subclinical mastitis curing rate varies among quarters. The subclinical mastitis caused by *Staphylococcus aureus* in forequarter cures faster than one in hindquarter (Barkema *et al.,* 2006; Günther *et al.,* 2017).

Mastitis causing agents have become resistant to antibiotic drugs (Yuan *et al.,* 2017; Ameen *et al*., 2019). For instance, *Staphylococcus aureus* is resistant to methicillin and is one of the significant concerns in the dairy industry (Sporer et al., 2016; Hischebeth *et al.,* 2019). Its association with

subclinical mastitis makes it very difficult to detect and cause significant dairy farms losses (Shraf *et al*., 2018). In public health, antibiotic accumulation in animal bodies, which results in residues in milk consumed, is another primary concern (Wang *et al*., 2015; Singh *et al.*, 2018).

2.1.2 Predisposing factors causing Mastitis

Mastitis predisposing factors can be broadly divided into two groups, namely: Managerial and herd factors. (Peter *et al.,* 2015). Management factors include inappropriate milking procedures, poor sanitary practices, inadequate bedding, and inconsistent and irregular hygiene practices on the dairy farm (Hogenboom *et al.,* 2019). The non-management (herd) factors include age, parity, lactation stage, milk yield, teat conformation, and hormone cycle stage of the lactating animal. Seasonal changes have also been associated with herd factors that predispose dairy cattle to mastitis (Eggins, 2019; Friman *et al*., 2019). The prevalence of mastitis has been shown to increase with the cow's age and parity (Jingar *et al.,* 2017). Based on the lactation stage, mastitis prevalence is higher initially, moderate at the mid and higher again at the peak (Abebe *et al*., 2016). Continuous prevalence of mastitis infections in a dairy herd escalates the spreads to other unexposed dairy cows on the same farm, further complicating the situation (Oliveira *et al*., 2015).

2.1.3 Mastitis based on the Mode of infections

Based on infection, mastitis can be classified into two major groups: contagious and environmental mastitis. Infectious mastitis is caused by pathogens that reside mainly in the surrounding environment and on the animal's body (Friman *et al*., 2019). The pathogens get access to the animal via teat canals from the climate, resulting in mammary gland infections (Iraguha *et al.,* 2015; Curone *et al.,* 2018). Routine disinfection of teats and udder quarters before and after

milking and Dry Cow Therapy (DCT) are some of the control measures applied to reduce the rate of multiplication of pathogens and their spread on the dairy farm (Derakshani *et al*., 2018).

2.2 California Mastitis Test (CMT)

The CMT is the simplest method used in screening dairy cattle for subclinical mastitis and intramammary infections (Godden *et al*., 2017). It is faster, cheaper, and a reliable field diagnostic tool used in assessing the mammary gland health status of the udder quarters (Kandeel *et al*., 2018). An equal volume of the mixture of milk samples and the California mastitis test reagent (3% sodium lauryl sulphate and bromocresol) are mixed. Milk samples from each quarter is added in a clean chamber of CMT paddle. The paddle comprises four separate wells, and each sample of milk from each quarter is placed into each well (Bhutto *et al.*, 2012; Reddy *et al.*, 2015). The paddle is then shaken gently in circular motions, and the thick gel formed is interpreted, as shown in Table: 1.

Table 1. The interpretation of California Mastitis Test in regards to somatic cell count

CMT Score	**Reaction observed**	**Equivalent milk Somatic cell count per millilitre**	**Interpretation**
Negative	No gel formed or Faint cloudy disappears in about a second	0 - 200,000; 0 - 25 percent neutrophils 150,000 to 200,000 30 to 40% neutrophils	Healthy quarter
Trace	Viscous formation happens instantly after mixing, but no disposition to gel Viscous disappears over a short time,	200,000 to 300,000 40 to 60 % neutrophil	Susceptible Resistance
1^+	Distinctive viscous matter formed Straight away After formation of a slight gel.	310,000 to 500,000 60 to 70 percent neutrophil	Subclinical Mastitis
2^+	Distinguishable viscous matter formation occurs instantly after admixture distinctive gel formed on the surface of the mixture is raised like fried egg	> 5,000,000; 70 to 80 percent of Neutrophil	Subclinical Mastitis

Modified after Radostitis *et al*., (2000)

The neutrophil percentage is adapted from Quinn *et al*., (2000)

2.3. Somatic Cell Count

Somatic cell count includes macrophages, polymorphonuclear neutrophils, lymphocytes, and epithelial cells. The number of cells is usually reported in milk per milliliter (Division, 2018) and used internationally to scrutinize/screen milk quality and udder health status in the dairy herds (Li *et al.,* 2014). They are composed of leucocytes 85 % and epithelial cells 15 to 25 %. The composition of cells varies between a healthy udder and a postnatal but stable udder (Division, 2018; Malik *et al.,* 2018).

Healthy cows have a somatic cell count within a range of 250,000 cells/ml to 300,000 cells/ml on average (Sadeghi & Amer, 2015). SCCs are parts of the immune response in udder quarters that take part in the udder's inflammation response. The increase in milk SCC is directly proportional to the udder quarter infection (Litwinezuk *et al*., 2011; Division, 2018; Vissio *et al*., 2018). The SCC above 310,000 cells/ml is the recommended value for monitoring udder health status (Zecconi *et al.,* 2019). However, the threshold level of SCC for assessing and tracking dairy herds is often 2.0 x 10^5 cells/ml and below (Division, 2018). The SCC is typically used to monitor quarters' health status. The increase in SCCs can be caused by breed, parity, milk production level, lactation stage, poor management practices, and environmental factors.

The elevated levels of SCC result in an equal decrease in milk quality, milk yield, and milk quantity produced (Thomas *et al.,* 2015). The increase is also positively linked to changes in the amount of average microbial loads and physicochemical elements of milk and milk quality (Jadhav *et al.,* 2016). Somatic Cells play a critical role in the immune response of infected udder quarters of cows (Li *et al*., 2014; Azmi *et al*., 2017). The increased somatic cell count in the milk causes inflammation of udder tissues, which subsequently results in lower quality and quantity of milk produced (Division, 2018; Malik *et al*., 2018).

2.3.1 Factors that affect the number of SCC

Many factors influence the number of SCCs in milk at both individual cattle and the herd level. These factors include the age of the animals, breed of cattle, the number of parity, lactation stage, and frequency of milking, udder injury, and production level (Division, 2018). The udder health status is the most valuable factor in milk quality, yield, and quantity (Shraf *et al*., 2018)). Malik et al. (2018) reported that the higher the milking frequency with the automated apparatus, the higher the somatic cell count in cow milk. The older dairy cows produce a large number of somatic cell counts. They are a result of exposure to several cases of intra-mammary infections (Bhakat *et al*., 2019). The level of somatic count varies from one breed to another (Anon, 2016). The higher amount of milk a cow produces, the higher the somatic cell count in milk. SCC in milk increases with an increase in parity of the dam (Thomas *et al*., 2015; Hadrich *et al*., 2018; Ruegg and Pantoja, 2019). Factors related to an animal's environment, such as climatic variations, can also cause a change in the SCC level (Alhussein & Dang, 2018). For instance, during the wet season, the number of SCC is usually higher than the dry season (Malik *et al.*, 2018).

2.3.2 Association between Somatic cell counts and Mastitis infections

An increase in SCC is directly related to bovine mastitis incidence amongst dairy cows (Davis & South, 2015; Wall *et al*., 2018). Somatic Cell Counts of above 2.0 x 10^5 cells/ml are used to confirm mammary and udder infection status in lactating dairy cows (Morin *et al.*, 2018; Malik *et al.*, 2018; Musayeva *et al.*, 2018). Cattle that possesses a low somatic cell count are considered to have an incapacitated immune response to intra-mammary infections (Maye *et al*., 2017). Thus, those that produce more somatic cell counts are deemed to have an excellent immune response (Nyman *et al*., 2014). The SCC is often higher at the onset of the udder infections in dairy cows (Hemati Doust *et al.*, 2014). The presence of SCC in bovine milk can

be used to detect the occurrence of subclinical mastitis (Hemati Doust *et al.*, 2014). The mastitic condition changes milk constituents that eventually lower milk quality (Division, 2018; Malik *et al*., 2018).

2.4 Somatic cell score

Somatic Cell Score (SCS) is a log-transformed of somatic cell count in milk. It is a pseudo phenotypic trait for selections against mastitis resistance in dairy herds (Wang *et al.*, 2015). Unlike mastitis, somatic cell scores have relatively moderate heritability, which can be easily applied in selecting resistance (Meredith *et al*., 2012; Wang *et al.*, 2015; Republic, 2017). Selection for reduced SCS is one of the crucial factors that should be included in the breeding objectives. The SCS can help address mastitis problems in dairy herd selection (Meredith *et al.*, 2012; Rainard *et al.*, 2018). Heritability estimates of Lactation Somatic Cell Scores vary between 0.10 and 0.16, and it is higher in early lactation periods than late lactation (Alam *et al.*, 2015; Rainard *et al.*, 2018).

Genetically, associations between Lactation Somatic Cell Scores (LSCS) are usually in the range of 0.62 to 0.99 (Alam *et al.*, 2015). The selection for mastitis resistance (SCS) is typically successful when the low estimated breeding values are considered (Rainard *et al.*, 2018). The least values of Somatic Cell Scores indicate the low incidence of mastitis and intra-mammary gland infections of the udders (Mayel *et al*., 2017). Several lactation traits model analyses regarding somatic cell scores can result in stronger mastitis-resistant dairy cows (Alam *et al*., 2015; Rainard *et al*., 2018).

2.5. Genes involved in Immune response

2.5.1 Beta-lactoglobulin gene

The beta-lactoglobulin gene is an acid-stable, amphiphilic, whey protein commonly present in milk at normal pH (Singh & Gallier, 2018). It occurs in three different forms: Tetrameric, octameric, and multimeric (Al Shabib *et al*., 2020). The gene comprises 10 -15% milk protein and is a single chain polypeptide of 18kbp. It has a 3g/L concentration and contains about 162 amino acids (Alim *et al.*, 2015). It is one of the whey proteins and lipocalin present in cow milk. The lipocalin protein adheres easily to several hydrophobic molecules. The Beta-lactoglobulin gene can bind iron through siderophores, thus playing a critical role in fighting pathogens in milk. Therefore, it affects milk quantity and quality in bovines (Alim *et al*., 2015).

Dairy traits such as milk quality and somatic cells are closely associated with the Beta-lactoglobulin gene (Singh *et al*., 2015). The increased SCC results in a decline in the amount of the beta-lactoglobulin gene in milk (Litwi *et al.,* 2011). The gene works in an antagonistic manner to the lactoferrin gene, but both possess antibacterial effects. Therefore, the two genes can complement the mammary gland immune mechanisms against bacterial infections (Chaneton *et al.,* 2010; Singh *et al.,* 2014).

Beta-lactoglobulin gene inhibits the growth of common causative agents of mastitis such as *Staphylococcus aureus* and *Streptococcus spp*. The action reduces the spreading rate of infections, which helps improve the quality of milk (Chaneton *et al.,* 2010; Ateya *et al*., 2016). Due to its ability to influence the number of somatic cells in milk, the lactoferrin gene is an appropriate novel candidate gene for selecting mastitis resistance in dairy herds (Martin *et al.,* 2018). SCCs monitor the general udder health, including subclinical mastitis in dairy cows (Singh *et al.,* 2014). Early detection by the farmer is essential because it can help them maximize their profits by reducing the cost of milk production and disease control.

2.5.2 Lactoferrin gene

The bovine lactoferrin gene belongs to the transferrin family (Firyal *et al.*, 2017). The lactoferrin gene is a glycoprotein found in most biological fluids such as saliva, bile, blood, milk mucous secretions, and tears (Chopra *et al.*, 2013; Asadollahpour *et al.*, 2016). The genomic DNA of this gene has a molecular size of 34.5kb (Dinesh *et al.*, 2015). The gene is secreted in milk during lactation by polymorph nuclear Neutrophils (Nanaei *et al.*, 2016).

In cow milk, the lactoferrin gene plays biological and physiological roles and is strongly correlated to Somatic Cell Count (Cheng *et al.*, 2008; Arnould *et al.*, 2009; Pawlik *et al.*, 2014). The concentration of protein in milk is determined by a number of many factors, including parturition, lactation stage, days of involution, subclinical and clinical mastitis (Sharma *et al.*, 2015). In bovines, the concentration varies between 0.02 and 17.8 mg/ml. The concentration can rise sharply with udder infection (Zabolewicz *et al.*, 2012; Sharma *et al.*, 2015). The higher concentrations of the lactoferrin gene in milk can be used to indicate milk quality and subclinical mastitis (Musayeva *et al*., 2018). Higher concentrations of the gene are usually recorded in drying-off cows, and those undergoing mammary involution (Zabolewicz *et al.*, 2014). Due to its bacteriostatic properties, this gene plays a significant role in maintaining a sound immune system and is generally associated with low incidences of Bovine Mastitis (Sharma *et al*., 2015). It has many functions, including protecting the udder from microbial invasions, homeostasis, and control of inflammations in the mammary glands of dairy cows (Kochanowska *et al.*, 2018).

In ovine (sheep), lactoferrin concentrations increase at the start of the lactation (0.74mg/ml) and suddenly drop within the first three days (Navarro *et al.*, 2018). In the third week of lactation, the concentration then settles between 0.07 and 0.5 mg/ml (Navarro *et al.*, 2018). The lactoferrin gene's density in milk is influenced by several factors: age, lactation stage, parity,

and colostrum level (Musayeva *et al.,* 2018). In sheep, the gene's volume is directly proportional to the amount of SCC in milk (Sharma *et al.,* 2015).

2.6. Optimization of Polymerase Chain Reactions for amplification

The optimization process relies on balancing the amplifications and prevention of universal PCR outcomes (Kalender *et al.,* 2019). Care should be taken in all steps in the process, right from DNA template production to the series of cycles (Tabibnejad *et al,* 2016). Generally, the steps undertaken in this program are Initialization, denaturation, annealing, extension, and PCR cycling. The reagent concentrations used are critical in PCR amplification. Such as the primers, magnesium chloride, deoxyribonucleoside triphosphate, Taq polymerase, and DNA template (Azam *et al.,* 2017).

2.6.1 Primers' Design

Developing correct primers is crucial to the success of a PCR reaction experiment. A set of primers is designed for a specific targeted region of DNA amplification (Baker *et al.,* 2017). Conventionally, a primer is oriented in the direction of 5'→3', which allows annealing to the sense or non-template strand (plus strand) in the direction of 3'→5' (Kalender *et al*., 2017; Thomsen *et al*., 2017). They result in primer dimers caused by primers' self-annealing to each other (Ye *et al*., 2018; Kalender *et al*., 2019).The differences in these primers' melting points' make it challenging to predict annealing temperatures that would allow efficient binding to the appropriate sequences during thermal cycling (Ye et al., 2012; Piepenburg *et al*., 2019).

A primer must range between 15-30 nucleotide bases in length. The G-C bonding should be within the range of 40% to 60% of the whole primer. The 3' prime end of the primers must have a G or C to allow clamping of primers and block "breathing" of the ends. This improves

the efficiency with which primers bind. DNA breathing happens when ends do not anneal but instead split apart (Ye et al., 2012; Clarke *et al*., 2017).

The hydrogen bonding in G-C base pairs enhances breathing of DNA and increases the melting point of the primers. The 3' end of primers contain both sense strand and antisense strand primers which are not complementary to other sequences of the primer. Therefore, the formation of primer dimers should be avoided. Most primers have excellent melting points within the range of 52℃ and 58℃ (Smith, 2018). In some instances, this may extend from 45℃ to 65℃ depending on the required melting point of a primer. The final melting temperature of the two primers should not differ by 5℃. The primer melting temperature is an estimate of the DNA to DNA hybrid stability and is a critical determinant of the annealing temperature (Ye et al., 2012). An increase in annealing temperature leads to insufficient primer-template hybridization. This results in low-quality PCR outcomes. The decrease in annealing temperature results in formation of nonspecific outcomes characterized by huge mismatched base pairs (Azam *et al*., 2017).

2.6.2 Formation of DNA Template

This process searches for perfectly matching primer annealing sites that can generate a PCR product, the resulting products must be sorted according to size (Thomsen *et al*., 2017). They are usually assigned specific lengths and positions in the original sequences. The primers that produced the PCR products can be used as linear or circular molecular templates besides determining the size of anticipated PCR outcomes (Kalendar *et al*., 2017).

The initialization and denaturation of DNA templates are carried out by heating usually, between a range of 92℃ and 98℃ for 30 seconds to 15minutes. However, this values vary depending on the nature of the template DNA and the buffer salt concentration (Zhang *et al*., 2019). The denaturation dissociates the two-stranded of DNA nucleotides to form two separate

single strands of DNA. These are later used as DNA templates for PCR amplification by thermos-stable DNA Taq polymerase (Javier *et al*., 2018). Temperatures of between 52℃ and 58℃ make stable annealed templates to denaturize the specific DNA sequences and functional primers for the Taq DNA polymerase. This stage takes about 30-60 seconds.

The duration of the final extension depends on the amplicon length and composition. This should be optimized to ensure full-length polymerization and better yield of the specific DNA sequence. The formation of a new DNA starts as the reaction temperature is elevated to the optimal level of 70℃ to 80℃ for thermos-stable DNA Taq polymerase. This process takes 60 to 120 seconds (Ye et al., 2012). The following cycle starts with a return to 95℃ for the denaturation process. Every stage of the cycle must be optimized for every template and primer combinations.

However, processes of annealing and extension can be combined into a single step in the situation where the temperatures are the same. The cycles of the process of amplification vary from 30 to 35 cycles of reaction (Ye *et al.*, 2018).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Area

The study was done in Kanyariri Veterinary Teaching Farm, which is a learning and research facility for the University of Nairobi. The farm sit on a 375acre piece of land in Lower Kabete located 2 kilometres west of the College of Agriculture and Veterinary Sciences and 15 kilometres from the Nairobi city centre. Kanyariri farm is on an elevation of 5,600 feet (1,700 meters) above sea level with an average temperature of 18.7˚C. It receives rainfall amount of about 869 mm per annual.

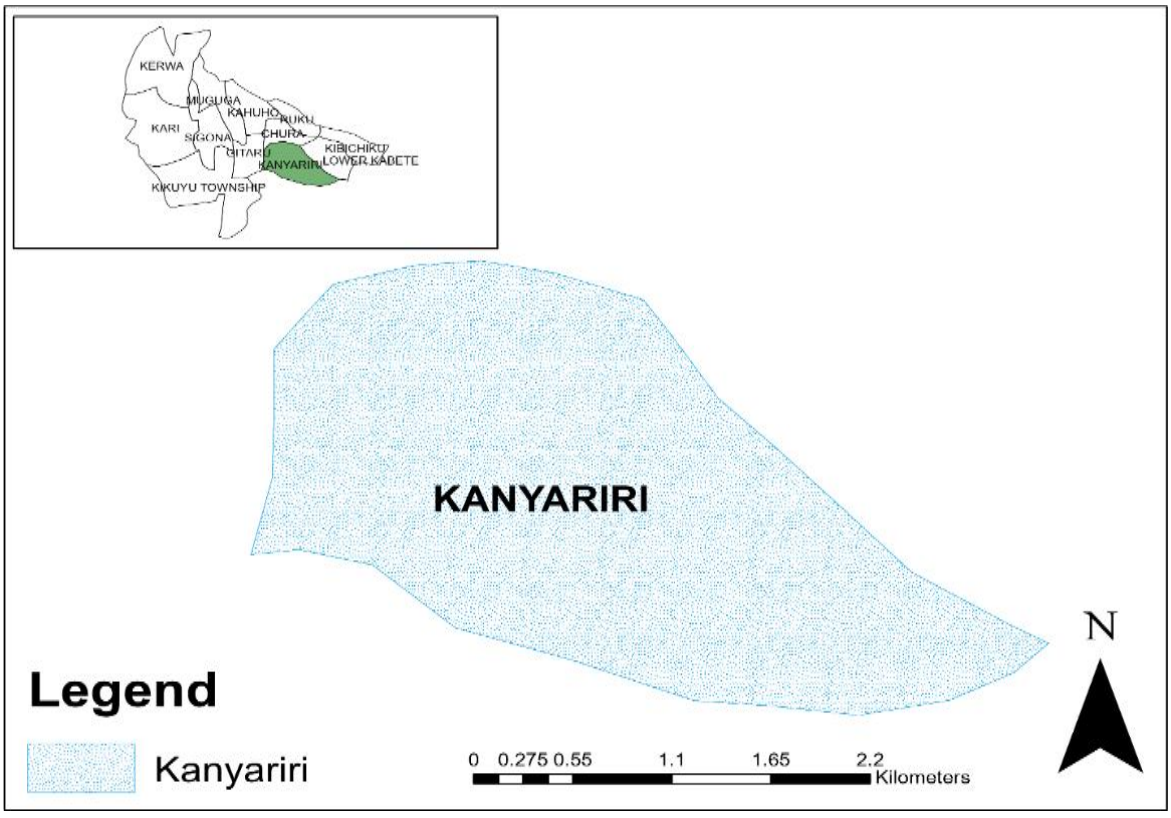


Figure 1: A Map showing Kanyariri Veterinary Farm – University of Nairobi

3.2 Sample collections

3.2.1 Milk samples' collections

A total of 38 crossbred dairy cows were selected based on lactation, each of their udder quarters were screened for mastitis by California Mastitis Test kit (Schalm, 1957). Each of the udder quarters were cleaned using 75 percent ethanol before collecting 5ml of milk samples aseptically into 10ml screw cupped tubes. These were then transported at -20℃ to the Microbiology Laboratory in the Department of Food Science and Nutrition, Faculty of Agriculture, University of Nairobi. Milk samples were stored in a freezer at -21℃ before they could be analyzed for Somatic Cell Counts.

3.2.2 Blood samples' collections

About 5ml of blood samples were obtained aseptically from the external jugular vein of each of the 96 crossbred dairy cattle at Kanyariri Veterinary Farm for DNA analysis. The samples were drawn into vacutainer tubes containing EDTA as an anticoagulant. The tubes were shaken gently to allow thorough mixing of the blood sample with anticoagulant (EDTA). They were then placed in vertical position in a cool box at (-20℃) and transported to a Molecular Genetics Laboratory at the Department of Animal Production (CAVS). The vacutainers were transferred to the freezer for storage at -21℃ before DNA isolation. 152 milk samples from each quarter of the 38 lactating cows were also examined microscopically for counts of somatic cells.

3.3 Microscopic Analysis of SCC

A thin layer of milk samples were spread on slides, stained with an iodine solution as a basic dye and allowed to dry. These slides were labelled to match samples' containers identification numbers, each of these slides were placed on microscopic stage and examined with a magnification of 100x independently, where leucocytes were appearing as dark spotted dots.

These leucocyte cells were counted directly as somatic cells and multiplied by thousand (Prescott and Breed, 1910; Ferronatto *et al.*, 2018). To obtain Somatic Cell Scores, SCC were log transformed using the formula (Rupp *et al.,* 1999; Yuan *et al.,* 2013).

$$\text{SCS} = Log_2(\frac{SCC}{100{,}000} + 3)$$

3.4 Statistical analysis

The SCC data was analyzed using SPSS version 21.0. To calculate correlation coefficient, Tukey test was used for mean separation of somatic cell count among crosses of dairy cattle, LSD and to test if there was a significant difference of breed effect on mastitis resistant traits; CMT Scores were analyzed using one-way ANOVA (SPSS) at P≤0.05 level of significance. The models below were used in testing the effect of breed on SCC.

(i) $\boldsymbol{y_{ij} = \mu + G_i + e_{ij}}$

Where $\boldsymbol{y_{ij}}$ the effect of the SCS is in the udder quarters**,** $\boldsymbol{\mu}$ is the average for traits, $\boldsymbol{G_i}$ is the breed's effects, $\boldsymbol{e_{ij}}$ represent the residual error. The analysis of the association among the breeds with SCC that reflect mastitis susceptibility traits was solved using the equation below;

(ii) $\boldsymbol{y_{ij} = \mu + G_i + p_t + e_{ij}}$

Where y_{ij} is the observation of somatic cell count, μ is the overall mean of SCC, G_i and, p_t were the fixed effect of breed and the fixed effect of SCC respectively, and e_{ij} residual error About 5ml of blood samples were obtained aseptically from the external jugular vein of each of the 96 crossbred dairy cattle at Kanyariri Veterinary Farm for DNA analysis. The samples were drawn from jugular vein of each cows into vacutainers containing EDTA as an anticoagulant (Clayton *et al.*, 2012). The tubes were shaken gently to allow thorough mixing of the blood sample with anticoagulant (Lima-Oliveira *et al*., 2017). They were then placed in vertical position in a cool box at (-20℃) and transported to a Molecular Genetics Laboratory

at the Department of Animal Production (CAVS). The vacutainers were transferred to the freezer for storage at -21℃ before DNA isolation (Rahila *et al*., 2017).

3.5 Genomic DNA Extraction from blood samples

The DNA extraction was done using a QIAGEN kit. About 20ul of proteinase K was pipetted and added into the micro centrifuge tube (Hanley, 2018). This was later followed by the addition of 200ul of the blood sample in the tube. The volume was adjusted by adding 220ul of PBS buffer into the tube, followed by the addition of 200ul of AL buffer. The tube was vortexes for 5minutes, transferred to the heating block, and incubated for 15 minutes at the temperature of 56℃ (Tabibnejad *et al.*, 2016; kraemer *et al*., 2017). About 200µL of absolute ethanol was added to the tube, mixed and thoroughly vortexes. The subsequent procedures were followed as per the manufacturer instructions.

3.6 Visualization of genomic DNA

One percent Agarose gel was prepared in a buffer solution at its optimal pH (8.0), 89mM of Tris-borate, and 89mM of EDTA (per litre). It was run in a horizontal gel electrophoresis setup. The sample of DNA was thoroughly mixed with loading buffer and then loaded into the wells on the agarose gel. The electric current was switched on and allowed to pass through at 100 voltages for 30 minutes. Subsequently, the agarose gel was coloured with ethidium bromide (0.5µL/ml) for 29 minutes and finally visualized on U.V light with its photograph captured by a digital camera (Lee *et al*., 2012).

3.7 PCR Amplification for DNA templates

The two pairs of oligo primers were used in the amplification of the beta-lactoglobulin and lactoferrin genes respectively. The amplification reactions had a final volume of 25µL, which

was aggregated as 2µL (10x PCR buffer), 3µL (5x Q solution), 0.2µL of each primer, 0.4µL dNTPs mixture, 0.2µL Taq DNA polymerase, 15µL Nuclease-free water, and 4µL DNA template (100ng/µL). The reactions for (beta-lactoglobulin) were performed in a thermal cycler (MJ RESEARCH, INC.) with the following conditions; initialization at 94℃ for 5minutes, denaturation at 94℃ for 60 seconds, annealing of primers at 61℃ for 60 seconds, extension at 72℃ for 60 seconds and in 30 cycles. The final extension was performed at 72℃ for 7 minutes, and PCR products were stored at 4℃. The primers used for amplification of the lactoferrin gene fragment were (forward) and (reverse) (Sharma *et al*., 2015).

The genomic DNA samples of lactoferrin were amplified in polymerase chain reactions (MJ RESEARCH, INC.) with the following conditions; initialization at 95℃ for 5 minutes, denaturation at 95℃ for 60 seconds, annealing of primers at 57℃ for 60 seconds, extension at 72℃ for 60 seconds and carried out in 35 cycles. The final extension for both genes was performed at 72℃ for 7 minutes and PCR products were stored at 4℃ (Azam *et al.,* 2017). PCR products formed were subjected to gel electrophoresis of two percent agarose at 100 volts for 40 minutes. The bands were later visualized on ultraviolet Transiluminator and photos were taken using a digital camera. The size of bands were measured with DNA ladder 100bp plus marker (Kaplan, 2018).

Table 2: The two pairs of primers which were used in the amplification of Beta-lactoglobulin and Lactoferrin genes respectively

Types of sequence	Primer sequence
Oligo LGB – F	5'- GTCCTTGTGCTGGACACCGACTACA -3'
Oligo LGB - R	5'- CCCAGGACACCGGCTCCCGGTATAT-3'
Oligo LTF - F	5'- GCCTCATGACAACTCCCACAC -3
Oligo LTF -R	5'- CAGGTTGACACATCG GTTGAC -3'

F -Forward Primer, R - Reverse Primer, Lactoglobulin (LGB) and Lactoferrin (LTF)

3.8 Bioinformatics analysis of the DNA sequence data

3.8.1 Quality control of the raw DNA sequence data

The sanger sequenced nucleotides were received results from Microgen Inc., in the multi-fasta sequence files, which were downloaded from the web account of "Macrogen Inc." as fasta text sequence file. The DNA nucleotide sequences were then edited manually in MEGA. The poorly sequenced portions of the nucleotide sequences were trimmed off from the main sequence. The nucleotide sequences were run in BLAST to confirm the genes of interest.

The nucleotide sequences were run in the NCBI blast as query sequences in comparison with nucleotide sequences available in the database. The low quality sequences which did not respond to the respective genes of the study were removed, leaving high quality nucleotide sequences which matched with the genes of interest.

3.8.2 Sequence alignments

The fasta text sequence files of the DNA nucleotide sequences were aligned by Molecular Evolutionary Genetics Analysis (MEGA) using ClustalW with default parameters. The aligned

DNA sequences were rearranged and trimmed for the quality. The phylogenetic tree was constructed by test maximum likelihood tree to access the evolutionary relationship between different cladograms of the genes.

3.8.3 Sequences Submission to Genbank

The BLAST analyses of the nucleotide sequences were carried out in NCBI (Blastn) to confirm whether sequence corresponded to the genes of interest. The Blast homepage was opened, and the nucleotide sequences were entered as the query sequence. The formatting options and alignment view of pairwise with dots identities were selected. This was an attempt to examine the differences between query and subject sequences. Then, the coding region (CDS), exons, and introns of the sequence were annotated and eventually sequences were deposited in the Genbank via the Banklt platform. The submitted sequences were made up of both beta-lactoglobulin (16) and lactoferrin (22) genes with the following accession numbers; MN239407 and MN239408 for Complement MN325091 to MN325104, and MN337970 to MN337991 for beta-lactoglobulin and lactoferrin genes respectively.

CHAPTER FOUR: RESULTS

4.1 The California mastitis test scores for the udder quarters of lactating crossbred dairy cows

A total of 152 quarters were examined for subclinical mastitis by California Mastitis Test kit (CMT) as in the Fig: 2, the result of screened udders' quarters observed were as follows; 55.01 % were negative for subclinical mastitis, 43.67 % Trace and 1.32 score one plus were positive subclinical mastitis. A total of 85 quarters were free from infections while about 67 quarters were infected. There were no case of clinical mastitis registered at the farm (fig 2).

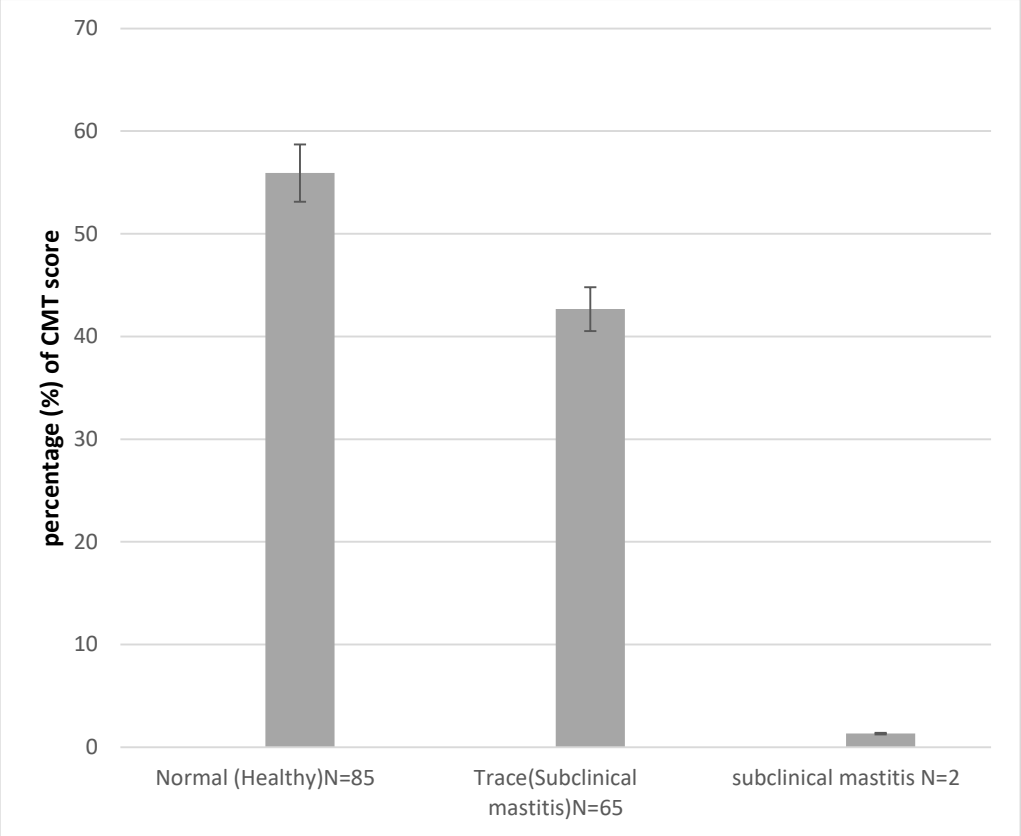


Figure 2: Infection status of the udder quarters for lactating dairy cattle genotypes.

Key; CMT = California Mastitis Test, N = Number of Udder quarters,

4.1.1 Association between the California mastitis test scores and somatic cell scores

Tukey's test was carried out to compared means' associations and determine if there were significant differences between CMT scores and Somatic cell scores (SCS). The result revealed that there were significant associations between California Mastitis Test scores and Somatic Cell Scores at $P \leq 0.05$. The Tukey's analysis for means comparisons CMT and somatic cell scores were greater by 0.23. In general the result have shown that there were significant associations between CMT scores and SCS of crossbred dairy cattle genotypes (Table 3).

Table 3: Multiple Comparison of the effect CMT and SCS across udder's quarters of the crossbred dairy cattle genotypes

CMT SCORE	**Normal**	**T**	1^+ score
Normal (healthy)	—	0.863* ± 0.1	0.46* ± 0.0023
T	—	—	0.403* ± 0.101
1^+ score	—	—	—

Normal =Negative score for subclinical mastitis, T = Trace (subclinical mastitis), & 1^+ score (subclinical mastitis)

* The significant difference when $P \leq 0.05$ level of significance

CMT Score = California Mastitis Test Scores, SCS = Somatic Cell Scores

4.2 Association between somatic cell scores and lactation stage

Table: 4, presents the comparison of means between SCS and lactation stages in order to find out if they were associated. The results found out that there were associated between first and second lactation stages (0.25±0.11); and second and third lactation stages (0.27±0.0118) were significant associations at P≤0.05. Thus, SCS and lactation Stages were significantly associated and this can be used to predict level of SCS in milk as per lactation stage. There was no association in somatic cell scores between second and first; and second and third lactation stages (table 4).

Table 4: Mean difference between Somatic Cell Score and Lactation stages of the crossbred dairy cattle.

Lactation stage	First	Second	Third
	Mean ± SE	Mean ± SE	Mean ± SE
First	—	0.25* ± 0.11	0.021 ± 0.097
Second		—	0.27* ± 0.0118
Third			—

* This indicated the result was significant at P≤0.05

4.3 Somatic cell count

Figure 3; the four main crosses of dairy cows were used to assess the level of SCC in milk, these crosses were; Ayshire, Friesian, Guernsey, and Jersey. The means' distributions of SCCs and standard error means were as; 246.64±15.177 cells/ml, 178.59±10.008 cells/ml,

174.67±13.212 cells/ml, and 243.45±21.997 cells/ml, respectively. The amounts SCC varied from one breed to one another (Ayrshire = 1.06 x 10^5 to 4.0 x 10^5 cells/ml; Friesian = 9.7 x 10^4 to 3.12 x 10^5 cells/ml; Guernsey = 9.2 x 10^4 to 3.1 x 10^5 cells/ml; and Jersey = 9.2 x 10^4 to 3.94 x 10^5 cells/ml). Ayshire genotype of the crossbred dairy cattle had the highest numbers of Somatic Cell Counts (246.64±15.177 cells/ml), and Jersey genotype had the least amount of SCC (174.67±13.212 cells/ml). Ayshire and Jersey genotypes were resistant to mastitis incidence while as Friesian and Guernsey genotypes were moderately resistance.

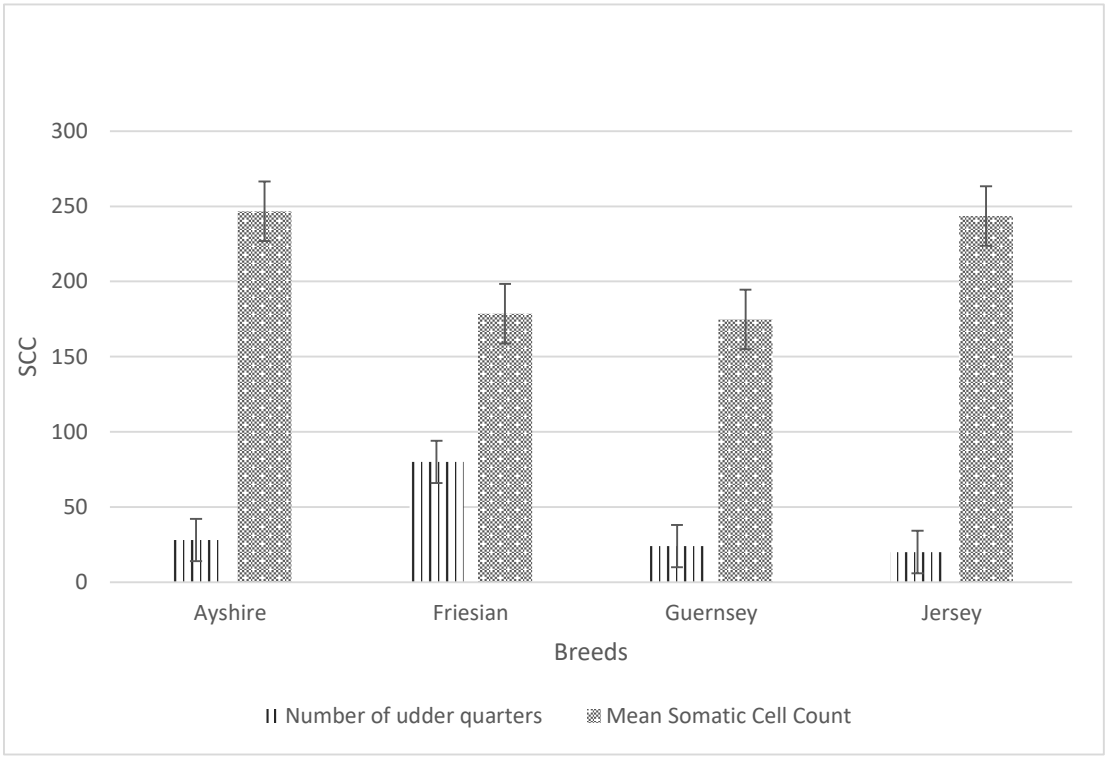


Figure 3: Number of udder quarters and Mean of Somatic Cell Counts for the four crossbred dairy cattle genotypes

4.3.1 The average of somatic cell count in the udder quarter of four genotypes of the crossbred dairy cows

In table 5; the means' separations of SCC between Ayshire and Friesian; Ayshire and Guernsey; Friesian and Jersey and Guernsey and Jersey were significantly different at $P \leq 0.05$. The Tukey's test found out that each significant result was greater by 56.162 cells/ml. The result showed that there were significant differences between Ayshire and Friesian; Ayshire and Guernsey; Friesian and Jersey and Guernsey and Jersey. However, no significant differences were recorded between Ayshire and Jersey; and Friesian and Guernsey Genotypes as in table 5.

Table 5: Means separation of somatic cell counts between four genotypes of crossbred dairy cows

Breed	Friesian	Guernsey	Jersey
	Mean ± SE	Mean ± SE	Mean ± SE
Ayshire	68.055* ± 18.821	71.97*± 23.844	3.193 ± 25.095
Friesian	—	3.921 ± 19.949	64.863* ± 21.429
Guernsey		—	68.78* ± 25.952
Jersey			—

* The mean is significant at $P \leq 0.05$, SEM; standard error mean

The post hoc analysis of pairwise comparisons of somatic cell counts among quarters of crossbred dairy cattle genotypes have showed were not significant at P≤0.05. The standard errors of somatic cell count among were relative larger than means, thus, signifies that there were no significantly different among the udder quarters. These were showed that means of SCC were found to be less than the mean value of 22.66. Therefore, there were no significant differences among the udder quarters of the crossbred dairy cattle genotypes (Table 6).

Table 6: SCC means across the udder quarters of the four genotypes of crossbred dairy cows

Quarter	Left front	Left hind	Right front	Right hind
		Mean ± SE	Mean ± SE	Mean ± SE
Left front	–	19.76 ± 23.681	20.87 ± 23.681	21.76 ± 23.681
Left hind		–	1.105 ± 23.681	2.0 ± 23.681
Right front			–	0.895 ± 23.681
Right hind				–

There were a strong correlations between somatic cell scores and udder quarters for the four crossbred breeds of dairy cattle. The result has showed that SCS and udder quarters were closely associated in term of intra-mammary infections. Therefore, a higher scores were observed across all the quarters, Left front and Right hind quarters while as Left hind and Right front quarters were moderate but they were all significantly correlated at P≤0.01 (Table 7).

Table 7: Correlation between somatic cell score and the udder quarters of crossbred dairy cattle genotypes.

	somatic cell count (x1000 cells/ml)			
Quarter	Left front	Left hind	Right front	Right hind
Left front	0.831**	0.993**	0.742**	0.751**
Left hind	1.00**	0.848**	0.937**	0.904**
Right front	0.900**	0.763**	0.942**	1.00**
Right hind	0.902**	0.761**	0.938**	1.00**

** Correlation is significant when P≤0.01 level of significance (2-tailed)

4.3 Gel Electrophoresis

The genomic DNA for beta-lactoglobulin and lactoferrin genes were run in a polymerase chain reactions using two pairs of oligo specific primers of LGB 252bp and LTF 301bp respectively. These yielded 23 and 27fragments of PCR products for beta-lactoglobulin and lactoferrin genes respectively, they were then loaded into the wells on 2% agarose gel stained ethidium bromide, and placed in gel tank containing 1XTBE buffer. A molecular weight marker of 100bp plus was used to measure the sizes of these genes. The bands on the gel were visualized under UV Transiluminator and photographed using a digital camera (Figure 4 and 5)

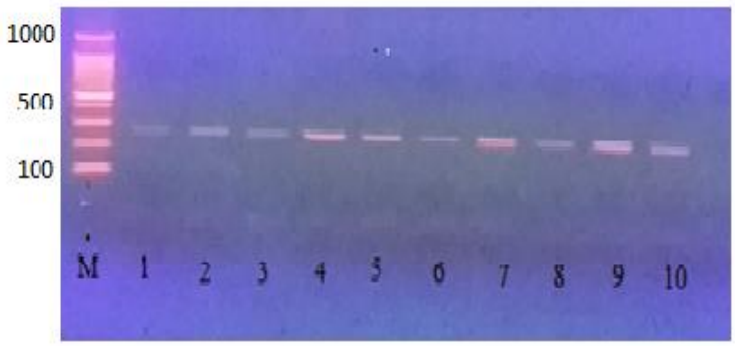
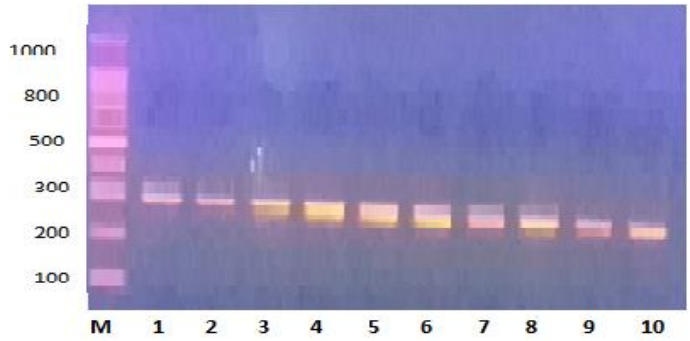


Figure 4: The image of amplified PCR Products of Beta-lactoglobulin gene.



Key; M represents DNA ladder used as a marker, Bands 1 to 10 represents fragments of beta-lactoglobulin gene at 252bp

Each band represents 100 base pairs

The figure below shows the image of 10 fragments of the amplified PCR products of the lactoferrin gene viewed under gel electrophoresis. These fragments were having similar weight of 301bp and DNA ladder of 100bp plus was used and viewed on 2% agarose gel.

Figure 5: the image of amplified PCR Products for Lactoferrin gene

Key; M represents DNA ladder of 100 Base pairs (bp) plus,

Bands 1 to 10 represents fragments of the lactoferrin gene on gel agarose slab

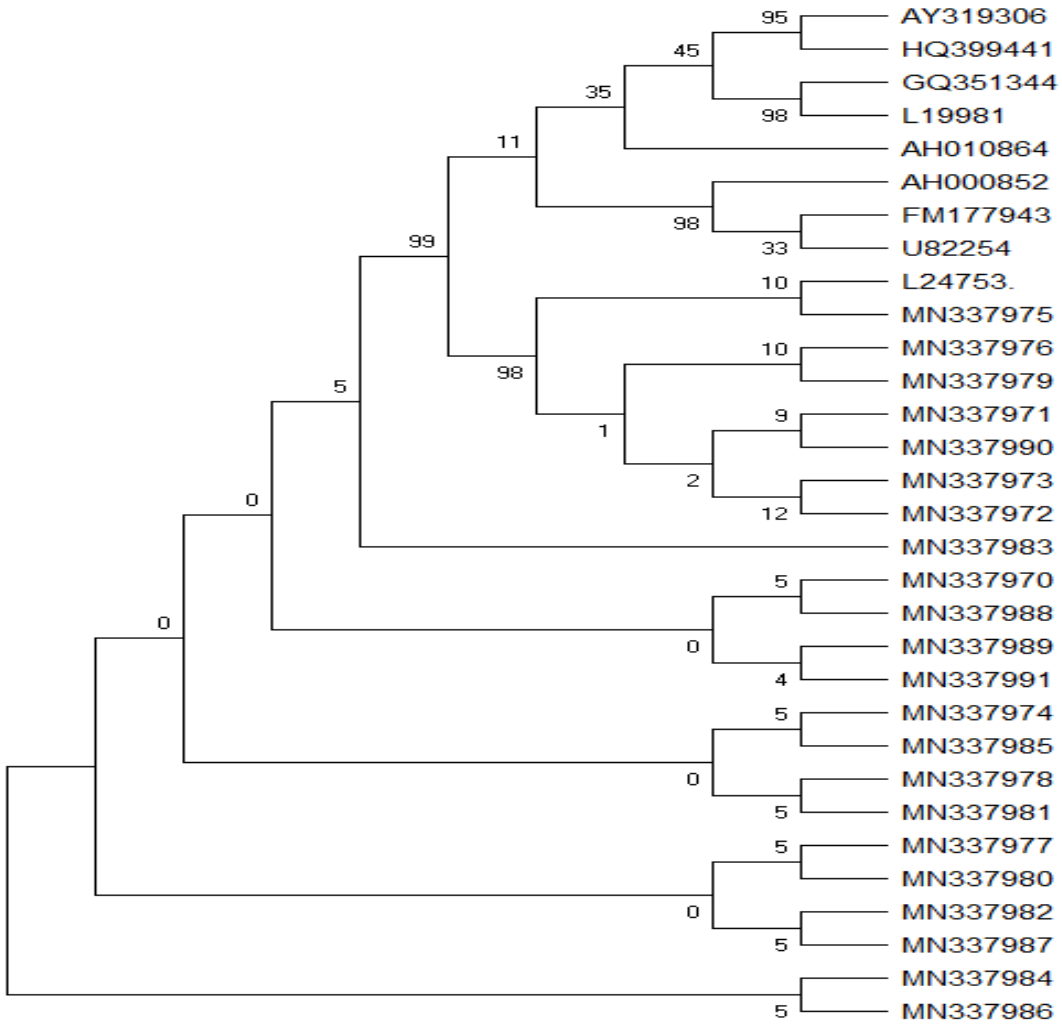
Each band represent 100 base pairs

4.4 Evolutionary relationship between Sequences of lactoferrin gene

The phylogenetic tree was constructed using maximum likelihoods, and ClustalW analysed the gene's taxa by default (Figures 5 and 6). They have clustered into several subunits of taxa joined by a node to each other. The phylogenetic tree bootstraps' consensus replicated 1000 times to account for both evolutionary differences and similarities among gene taxa in the studied population of the admixed breed dairy cows (Figures 5 and 6). The population of 31 sequences for lactoferrin gene taxa of both crossbred dairy cow genotypes was studied in comparison to other related breeds of dairy cattle globally. These were represented by the following accession numbers (L24753, U82254, FM177943, AH00852, AH010864, L19981, CQ351344, HQ399441, and AY319306) Fig 6.

The allelic similarities among the lactoferrin gene's taxa were attributed to the free exchange of genetic materials through gene flows, which were facilitated by artificial insemination services. Artificial insemination involves the movement of genetic materials from one isolated zone to another zone in the form of either frozen semen or germplasm. This lactoferrin gene's taxon (L24753) was similar to those of crossbred dairy cattle genotypes (MN337979, MN337976, and MN337975). Some of these gene's taxa of crossbred dairy were neither related nor similar to other lactoferrin gene's taxa of other breeds of dairy cattle. They were MN337984, MN337986, MN337987, MN337982, MN337980, MN337977, MN337981,

MN337978, MN337985, MN337974, MN337991, MN337987, MN337988, MN337970,



MN337983, MN337972, MN337973, MN337990, and MN33797, (Figure 6). These lactoferrin gene's taxa of other ecological zones were compared with those of crossbred dairy cattle, did not shows any similarities (U82254, FM177943, AH00852, AH010864, L19981, CQ351344, HQ399441, and AY319306). The differences were a result of geographical isolation, which created a barrier to mating.

Figure 6: The maximum likelihood tree for 31 lactoferrin gene sequences

4.5 Evolutionary relationship between Sequences of beta-lactoglobulin gene

The evolutionary distance among the beta-lactoglobulin gene's taxa was used to construct a maximum likelihood tree and was analysed by ClustalW default (fig 7).

The bootstraps' consensus were replicated 1000 times to account for evolutionary events between the taxa of the beta-lactoglobulin gene's taxa. According to their allelic similarities, the beta-lactoglobulin gene's taxa were clustered into several subtaxa of lineages supported by phylogenies of the beta-lactoglobulin gene's taxa.

These functional taxonomic units are composed of one node joining several others (Figure 7). The allelic similarities observed among the gene's taxa indicates that these breeds of crossbred dairy cows under study (MN325102, MN325099, MN325091, MN325101, MN325092, MN325100, MN325096, MN32509, MN325093, MN325098, MN325103 and MN325097, MN325094, and MN325104) had similar ancestral backgrounds to other breeds of dairy cows (HQ589927, AY96418, HQ589925, & AY971526). The closer alleles are in a population, the more similar individuals are. They could result from the free movement of genetic materials from one isolated zone to another through artificial inseminations.

The differences among the beta-lactoglobulin gene's taxa of the crossbred dairy cows studied and for other taxa such as; AY971146, AY97152893, NC_037338, KR329338, KR73293, KP687164, M27732, M19088, and X14712. The allelic differences among these taxa and those of admixed dairy cows resulted from several factors among reproductive isolations, genetic drifts, mutation, and different geographical locations.

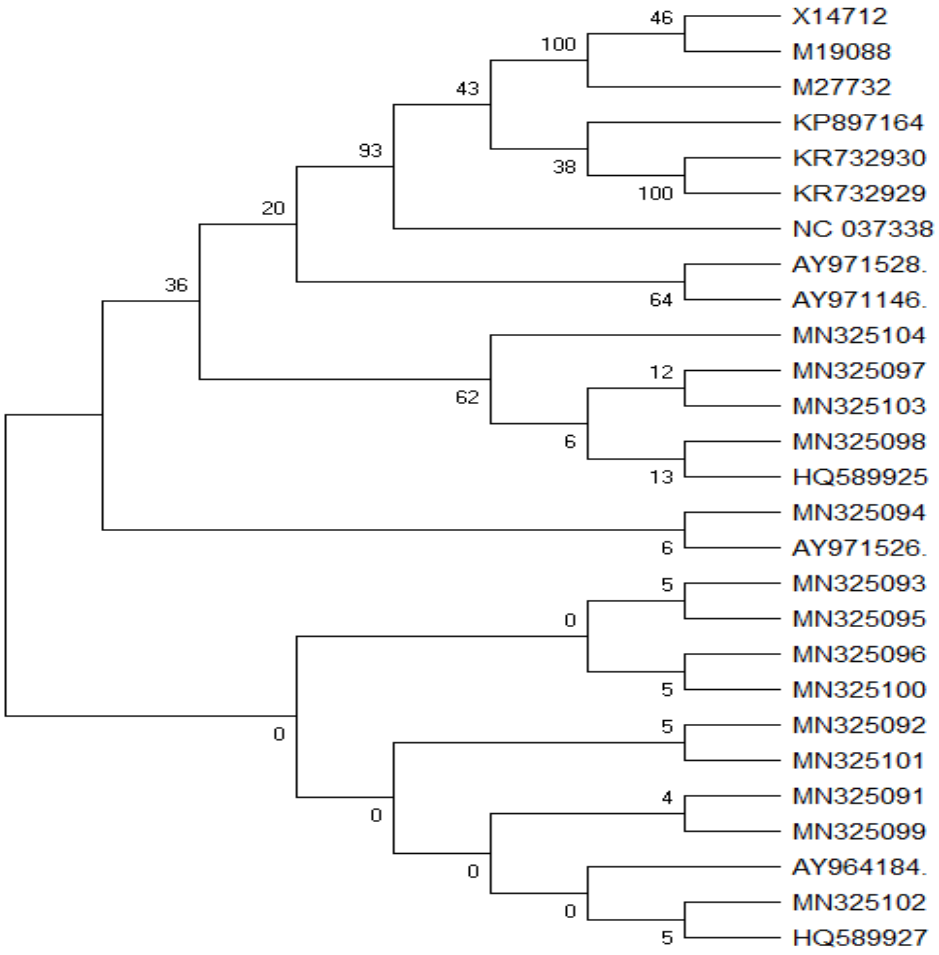


Figure 7: The maximum likelihood tree for 27 beta-lactoglobulin gene sequences

4.6 The association between Somatic cell count and the lactoferrin gene

In table 8; a total of 20 DNA nucleotide sequences of the lactoferrin gene were represented by accession numbers. Accession numbers were obtained from Sanger PCR DNA sequencing

results, which were deposited in the Genbank. They were representatives of four crossbred dairy cows of Ayshire; MN337971*, MN337983**, MN337984**, and MN337985**; (b) Friesian; MN337970*, MN337972**, MN337973*, MN337974**, MN337977**, MN337978*, MN337980*; MN337986*, MN337989*, and MN337990*; (c) Guernsey; MN337975*, MN337976* and MN337988*; and (b) Jersey; MN337979*, MN337982**, and MN337987*.

The sequences' variations were observed between 50 and 100 positions of nucleotide bases within the coding region of the nucleotide sequences and variations were found to be oriented in both transitions and transversions. These orientations of nucleotide bases were caused by mutation due to tautomeric shifts in the nucleotide sequences and the genetic polymorphisms were both in transitions and transversions. The variations were identified as single polymorphism nucleotides include; A>G (50, 54 63, 67, and 98), G>A (50, 54, 63, 67, 96, and 98), C>T (63, 67, 69, 83, and 100), and T>C (69, 80, 83, 94, 96, 98, and 100); and A>C (50, 54, 63, 73, 75, 80, 83, 94, 96, and 100), C>A (63, 80, 83, 94, 96, and 100), G>C (50, 54, 63, 75, 83, 94, 96, and 98), C>G (50, and 63), A>T (54, 63, 67, 69, 80, 83, 94, 98, and 100), T>A (54, 63, 67, 75, 80, 94, and 96), G>T (54, 67, 69, 75, 80, and 98), and T>G (67, 69, 80, 83, and 96) respectively.

Table 8: Accession Numbers for Different Sequences of Crossbred Dairy Cattle and Nucleotide base sequence variations from 50 to 100 base pairs for Lactoferrin gene

	Nucleotide Base Positions Within the Sequences											
ACESSION No.	50	54	63	67	69	75	80	83	94	96	98	100
MN337970*	A	G	G	A	G	T	T	A	C	A	C	A
MN337971*	C	T	T	G	G	A	A	T	A	G	T	C
MN337972**	G	T	A	T	A	T	C	C	C	C	A	T
MN337973*	A	A	A	G	T	C	T	A	T	C	G	C
MN337974**	C	C	A	C	T	G	G	A	T	G	A	C
MN337975*	A	A	T	A	G	T	C	G	T	T	A	A
MN337976*	A	A	G	G	G	A	G	A	A	C	C	C
MN337977**	C	T	C	T	A	C	A	T	T	A	T	A
MN337978*	C	G	A	A	G	G	T	A	A	T	A	G
MN337979*	A	T	A	G	G	C	A	A	A	A	T	C
MN337980*	A	A	G	A	C	T	C	A	C	C	T	G
MN337981*	G	T	G	T	G	C	G	T	A	A	G	A
MN337982**	T	A	T	G	T	A	T	T	A	C	C	A
MN337983**	A	G	G	A	G	T	A	A	C	A	C	A
MN337984**	C	C	T	T	C	C	T	C	A	A	G	C
MN337985**	G	A	A	A	T	T	G	G	C	A	C	A
MN337986*	G	C	G	T	T	A	T	T	C	C	C	T
MN337987*	C	C	T	T	A	T	T	A	G	C	A	A
MN337988*	C	G	A	A	G	G	T	A	T	C	A	G
MN337989*	A	T	G	A	G	T	A	T	G	G	A	C
MN337990*	A	G	C	A	A	T	T	A	A	G	T	C
MN337991*	C	A	A	G	A	A	A	A	C	A	C	G

* Healthy cows

** Mastitic cows- Subclinical Mastitis

4.7 The association between Somatic cell count and beta-lactoglobulin gene

A total of 14 nucleotide sequences were represented by their accession numbers obtained after they were deposited in Genbank (table; 9). These accession numbers represented four crosses of dairy cows, which include; NM325091, NM325092, NM325093, NM325094, NM325095, NM325096, NM325097, NM325098, NM325099, NM325100, NM325101, NM32502, NM325103, and NM325104. The sequence variations were between 9 and 195 positions of the sequence nucleotides. The nucleotide bases were in either transitions or transversions, and point mutations brought these about due to tautomeric shifts in the nucleotide sequences. These polymorphisms among the nucleotide sequences were found in following locations; A>G (136, 159, and 165), G>A (165, 174), C>T (9, 138), T>C (32); and C>A (136, 159, 163), G>C (9, 14), C>G (116, 138, 138, 163), G>T (9, 136, 138), T>G (32), they were presented in transitions and transversions respectively. These polymorphisms were found to be highly associated with mastitis resistance. Thus, these can be applied as markers in associate studies.

Table 9: Accession Numbers for Different Sequences of Crossbred Dairy Cattle and Nucleotide base variations from 09 to 195 base pairs for the beta-lactoglobulin gene

	NUCLEOTIDE POSITION WITHIN THE SEQUENCE											
Accession No.	9	14	32	116	136	138	159	163	165	174	178	195
MN325091*	G	G	G	G	A	C	G	G	G	G	C	G
MN325092*	G	G	G	G	A	C	G	G	G	G	C	G
MN325093*	C	G	T	T	C	G	C	G	A	G	G	G
MN325094*	T	G	C	G	G	G	A	G	A	G	C	A
MN325095**	T	C	C	G	G	G	A	G	A	G	C	A
MN325096**	C	C	G	T	T	T	C	A	A	A	A	T
MN325097*	C	G	G	G	G	G	A	G	G	T	C	G
MN325098*	G	G	G	G	A	C	G	G	G	G	C	G
MN325099*	G	C	G	G	A	C	G	G	G	G	C	G
MN325100*	C	G	T	T	C	G	C	G	A	G	G	G
MN325101**	G	G	G	G	A	G	G	G	G	G	C	G
MN325102**	G	G	G	G	A	C	G	C	G	G	C	G
MN325103*	G	G	G	G	A	C	G	G	G	G	C	G
MN325104*	G	G	G	G	A	C	G	G	G	G	C	G

* Healthy, ** Subclinical Mastitis

CHAPTER FIVE: DISCUSSION

5.1 The California mastitis test scores

A total of 152 quarters were screened for subclinical mastitis, 55.01% were free from subclinical mastitis, 43.67% positive subclinical mastitic (Trace), 1.32 % were positive for subclinical mastitis (score 1+) in Fig. 2. These results were in disagreement with the findings of Bhutto *et al.* (2012) and Hoque *et al*. (2015) stated that CMT results are 25% Negative and 75% positive for subclinical mastitis. The differences in results can be attributed to climatic variations, geographical, and sometimes breed differences. Hoque *et al.* (2015) also reported that CMT results positive for subclinical mastitis are usually less than 25% compared to the findings of this study, which were 44.99%. These are contrary to the findings of this study, as shown in Fig 2. Iraguha *et al*., (2014) also reported that 60% and above of the udder quarters were positive for subclinical mastitis. The aforementioned is contrary to this study's findings, which found out that only 44.08% of the udder quarters were positive subclinical mastitis (Fig 3).

CMT is a simple, affordable, reliable, and economically viable technique that requires minimum expertise to apply (koziel & Leifsson, 2016). These factors make CMT a suitable tool that most smallholders' dairy farmers can use for early detection of mastitis. Early detection of mastitis helps farmers prevent associated economic losses (Chakraborty *et al*., 2019). Thus improving hygiene, quality, and quantity of milk produced agrees with Abebe *et al*. (2016). The associations between the California Mastitis Test (CMT) score and Somatic Cell Score (SCS) across the udder quarters were significantly associated (Table: 3). The findings agree with Das *et al*. (2018) that CMT scores and Somatic cell scores varied significantly across udder quarters. The associations between CMT scores and SCS could be utilized as a marker-assisted selection in selective breeding for mastitis resistance or tolerance (Table: 3). Meredith et al. (2012) and Republic (2017) reported that SCS could be applied as a pseudo phenotypic

trait for selecting mastitis resistance because SCS has higher heritability than both mastitis and SCC. The Least Significant Differences (LSD) of mean differences between the California Mastitis Test (CMT) scores and the lactation stage were significantly different at $P \leq 0.05$. These results are in agreement with Jadhav *et al*. (2018)

5.2 The role of somatic cell scores in the udder quarters

In table 4, Tukey's test for somatic cell scores and lactation stages have shown significant associations between first and second lactation, second and third lactation stages at $P \leq 0.05$. The result agreed with Alam *et al.* (2015) that associations between Lactation stages and SCS were in the range of 0.62 and 0.99. There were significant associations between lactation stages and SCS in milk. It indicated greater resistance against mastitis in crossbred dairy cows. This statement agreed with Alam *et al.* (2015) and Rainerd *et al.* (2018), which stated greater the lactation somatic cell scores, the greater resistance against mastitis by crossbred dairy cows. It helps an animal resists infection in case its immune system is impaired. They further stated that Somatic Cell Scores vary between 0.10 and 0.16, and it is higher in early lactation periods than late lactation (Alam *et al.*, 2015; Rainard *et al.*, 2018).

5.3 Somatic cell count as an indicator of milk quality

Table 5; Tukey's means separations have found out that there were significant SCC associations among the four crosses of dairy breeds of cattle recruited in the study. These associations were found between Ayrshire and Friesian, Ayshire and Guernsey, Friesian and Jersey, and Guernsey and Jersey with means of 68.055±18.821, 71.79±23.844, and 64.863±21.429 and 68.78±25.952 cells/ml, respectively. These associations could be used as a diagnostic tool to address matters related to milk quality and hygiene, this is line with Nyman *et al. (*2014). In Figure 3, have shown that number of somatic cells for healthy cows were between 0 to 200,000

cells/ml, this is not agreement in with Hemati Doust *et al.* (2014) which stated that the number range between 250,000 cells/ml and 300,000 cells/ml on average.

In table; 5, the Tukeys' analysis of the amount of Somatic Cell Count (SCC) among the udders' quarters in dairy cows, Ayshire and Friesian were 68.055±18.821 cells/ml, Ayshire and Guernsey were 71.97±23.844 cells/ml, and Friesian and Jersey were 64.863±21.429 cells/ml were significantly different at $P \leq 0.05$. A breed's ability to produce significant amounts of somatic cells in milk has treated an indicator of robust immune responses to mastitis infections. This can be applied as a first-line response to reduce a disease's further spread across udder quarters. An increase in SCCs can facilitate a rapid and effective response to an intramammary infection (IMI) in dairy herds (Hussein *et al.*, 2018).

Table 7; the correlations between somatic cell count and udder's quarters indicate that quarters' infection can occur randomly regardless of its positions. They bring up the agreement that the disease of quarters can occur randomly, as shown.

The even distributions of somatic cell counts in all quarters have demonstrated that infection rates of intra-mammary are usually faster. If no immediate intervention is done, this is in line with the study by Das *et al.* (2018), who stated that SCC increases rapidly in all quarters.

In small holders' farms, SCC should be checked regularly to minimize subclinical mastitis incidences, which usually affects production economically. It is vital to monitor and evaluate intramammary infections (IMI) in dairy herds (Hussein *et al.*, 2018). The direct microscopic examination is the most suitable and straightforward technique for evaluating the somatic cell count level in milk. The percentages of macrophages in milk can be reckoned for differential somatic cell counts (Etter, 2001; Nyman *et al.*, 2016). The somatic cell count between Ayrshires and Jersey was slightly above the threshold of 246,640 cells/ml and 243,450 cells/ml, respectively. The somatic cell count for Friesian and Guernsey was below the threshold level 178,590 and 174,670 cells/ml, respectively (Fig: 3).

However, the decline in milk productions have been attributed to other factors such as physiological aspects of lactation, lactation stage, feeds, climatic conditions, and geographical locations of an area that influences the production of milk (Maraya, 2019; MOHAMED, 2019; Lees *et al*., 2019). The occurrence of mastitis in mammary glands trigger inflammation of udder tissues, which eventually interrupts the release of oxytocin hormone in the udder during milking hence less milk produced (Lovegrove, 2017). Therefore, somatic cell count in udder quarters is an important trait that may be used in selective breeding for mastitis resistance in dairy herds (Davis and South, 2015). This is because of the higher heritability it possessed as compared to clinical mastitis. Somatic cell scores in e udder quarters are also another strategy that can be explored to select mastitis resistance or tolerance in the dairy herd (Lynn, 2012).

5.4 The significances of the lactoferrin and the beta-lactoglobulin genes

In table: 8, the sequences of the lactoferrin gene were highly polymorphic within its flanking region. The region harbours genetic polymorphisms that significantly affect genome variability, the finding agreed with (Sharma *et al*., 2015; Ateya *et al*., 2016). The region has several Single Nucleotide Polymorphisms (SNPs) associated with disease presence in the regulatory region of the gene. This tends to influence the gene's activities and is called flanking SNPs of a sequence. This statement agrees with the findings of Pawlik *et al.* (2014) and Nanaei *et al.* (2016), who documented that the bovine lactoferrin gene plays diverse roles in milk. Its regulatory regions are also associated with the production of oxytocin hormone in the udder, which influences milk let-down (Lovegrove, 2017).

On the other hand, the gene's flanking site is associated with mastitis and SCC in milk. The sequence variations were found within flanking regions of the nucleotide sequences for cows positive for subclinical mastitis as well as from healthy dairy cows. This was in line with Olsen *et al.* (2016).

In table; 9, the beta-lactoglobulin gene has shown variability between sequences of those cows infected with subclinical mastitis and healthy ones such as; A>G (136, 159, and 165), G>A (165, 174), C>T (9, 138), T>C (32); and C>A (136, 159, 163), G>C (9, 14), C>G (116, 138, 138, 163), G>T (9, 136, 138), T>G (32). The gene has multiple effects in milk, such as ferrous-binding, antimicrobial action, and inhibitory nature against microorganisms. Table 9 these properties play crucial roles in regulating the growth and multiplications of pathogenic agents and maintaining milk quality against bacterial; this is agreed with Ateya et al. (2016). Furthermore, the gene's inhibitory nature reinforces mammary gland immune mechanics when the innate immune system is impaired and provides a faster, more efficient, and timely defence against intra-mammary gland infections (Lukač *et al.*, 2013). The innate immune responses act against mastitis pathogens and reduce bacterial loads present in milk naturally. This gene plays a key role in addressing challenges caused by mastitis, and selecting for it can improve animal milk safety and increase milk production.

5.5 Genetic parameters underlying similarities between nucleotide sequences

Figures 6 and 7 suggested that there were gene flows between these admixed dairy breeds' populations, which were suggested to have been brought about by free access to genetic material globally. This study does not reject the incidences of incongruence among the sequence nucleotides (Accession Numbers). The relationship between sequences varies in the phylogenetic tree, which agrees with Tak *et al.* (2018). The genetic polymorphisms in the DNA remain variable from one sequence to another sequence, which agrees with Boushaba *et al.* (2019). Breed differences were a result of individual genetic variations. They support the argument that genotype and allele frequencies remain variable from one population to another. As a result, other factors like mutations which unevenly within all populations of organisms as in table 8 and 9, respectively. Their relative nucleotide sequences continue to differ from one

breed to another. They might have been by insertion, substitutions deletion of nucleotide bases of the sequence. However, the similarities among the nucleotide base sequences of the lactoferrin and beta-lactoglobulin genes resulted from gene flows, from one isolated population to another through artificial inseminations (Figures 5 and 6). The process of AI lessens the mating barrier between one isolated population cattle breeds to another, thus, allowing easy movement of genetic materials. It is in line with the study done by Smiltina and Grislis, (2018) and Tak *et al.* (2018).

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, the study found out that the presence of subclinical mastitis in a farm dairy is unavoidable unless early diagnoses are made. Out of 152 udder quarters screened by California mastitis kit (CMT) for subclinical mastitis, 44.99 % were found out to be positive, and 55.01% were free from infections. Subclinical mastitis cases were closely associated with a high level of somatic cell count, and somatic cell scores among the four main genotypes of the crossbred dairy cows studied. The results of CMT scores and SCS were highly associated with occurrences of subclinical mastitis in the udder quarters of the crossbred dairy cattle. It could be used as one technique to assess the presence of subclinical mastitis or intra-mammary gland infections of the udder quarters. Somatic cell scores in the milk of these dairy cows' crosses were dependent on lactation stages as recorded. Instead, first and second lactation stages and second and third lactation stages were closely associated with a high level of somatic cell scores present in milk. SCS was strongly correlated with udder quarters. They increase evenly across the udder quarters.

The SCC level varied among udder quarters of the same mixed dairy cows to other breeds of crossbred dairy cattle. These differences resulted from different immune responses to intramammary infections of the udder quarters by micro-organisms, which are causal agents of mastitis in a dairy herd. The crosses of Ayshire breed obtained the highest followed by Jersey, Friesian, and the lowest Guernsey. Those who registered a higher SCC level in their milk were treated to have a robust immune response (resistance) to mastitis. In contrast, those with the SCC level were treated as having a weak immune response, and they were susceptible to mastitis infections.

The gene taxa of beta-globulin and lactoferrin of crossbred dairy cows in Kanyariri, University of Nairobi Veterinary teaching farm were similar to those of other studies conducted in

different parts of the globe. These gene taxa were (HQ589927, AY96418, HQ589925, and AY971526); and (L24753, U82254, FM177943, AH00852, AH010864, L19981, CQ351344, HQ399441, and AY319306) for beta-lactoglobulin and lactoferrin genes, respectively. Similarities between the nucleotide sequences of the lactoferrin and beta-lactoglobulin genes were found out to the result of gene flows between populations. Artificial insemination was considered to be the major element that facilitated gene flows from one population to another. These crossbred dairy cows' populations were related to allelic similarities between dairy cows on the farm and other sequences of related studies represented by Accession numbers.

However, some sequences of similar studies were not related to either sequence of beta-lactoglobulin or lactoferrin of the crossbred dairy cattle. These sequences were (U82254, FM177943, AH00852, AH010864, L19981, CQ351344, HQ399441, and AY319306) and (AY971146, AY97152893, NC_037338, KR329338, KR73293, KP687164, M27732, M19088, and X14712). The sequences' differences were believed to result from mutation, reproductive isolations, genetic drifts, geographical differences, and many others.

The genetic polymorphisms found out between sequences of beta-lactoglobulin and lactoferrin genes for those affected with subclinical mastitis and those free from infections were identified as single nucleotides polymorphisms (SNP). The findings support the fact that there were associations between genes (beta-lactoglobulin and lactoferrin) and subclinical mastitis in the udder quarters of the crossbred dairy cattle.

6.2 Recommendations

The study recommended that the following measures be adopted to address mastitis's significant challenges in dairy industries.

- The study recommends that there should be massive awareness about subclinical mastitis. Subclinical mastitis causes severe losses in the dairy farm. Subclinical mastitis causes severe economic losses such as reduced milk produced, increased SCC, and low-quality dairy due to its asymptomatic nature. The disease does not show any clinical symptoms, therefore impact on dairy negatively.
- The study also recommends that somatic cell count and somatic cell scores can be used to select mastitis-resistant dairy breeds. SCS and SCS have higher heritability as compared to clinical mastitis.
- The study suggested that associations of somatic cell scores and California mastitis test scores could monitor udder quarters' health status. They will help to enhance udder health and milk quality.
- The study strongly suggests that the novel candidate' genes of beta-lactoglobulin and lactoferrin can be used for the selection of mastitis resistant breeds of crossbred dairy cows in dairy farms.

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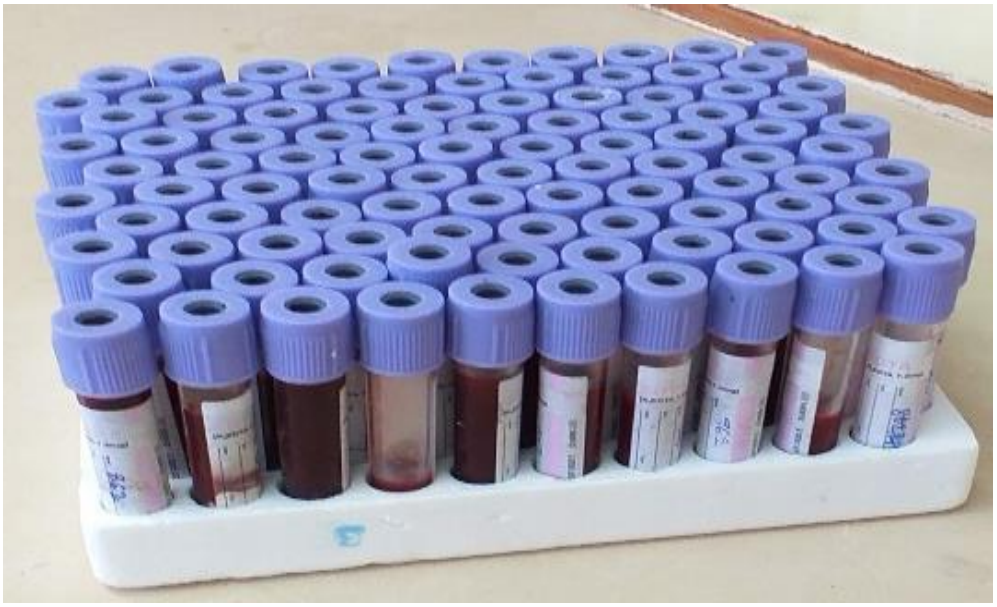
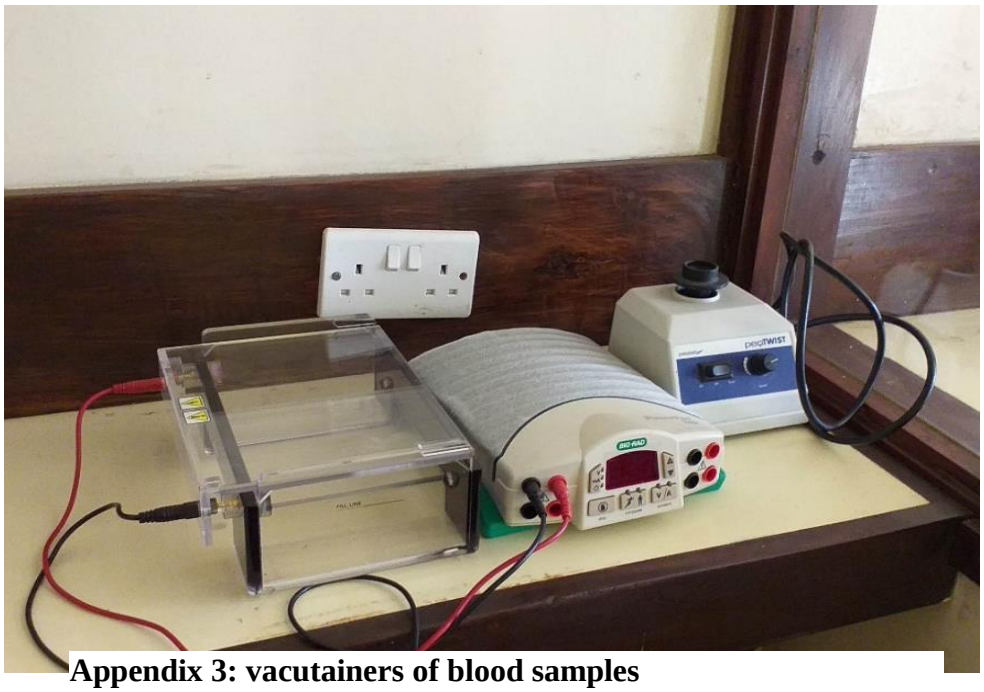
APPENDICES



Appendix 1: Consumables used during DNA extractions

Appendix 2: DNA Mini Extraction kit

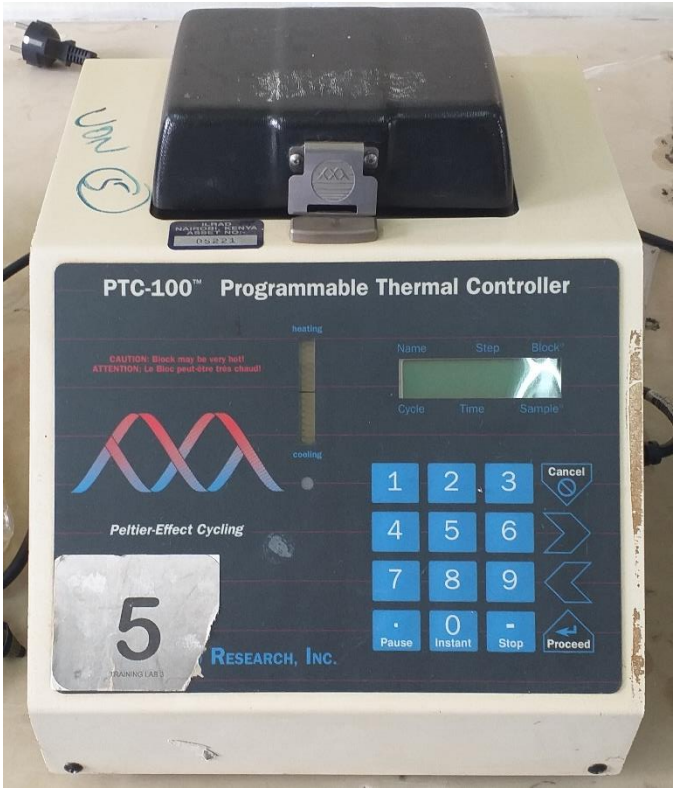


Appendix 3: vacutainers of blood samples

Appendix 4: Milk Samples in sterilized bottles

Appendix 5: Gel electrophoresis tank

Appendix 6: Some of Crossbred dairy cattle recruited in study



Appendix 7: A Polymerases Chain Reactions (PTC-100™ Programmable Thermal Controller), RESEARCH, INC. Controller), RESEARCH, INC.

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Appendix 8: A California Mastitis Test Kit (Paddle)

Appendix 9: Heat block which used for incubation of blood samples before extraction of Genomic DNA (ThermoStat plus)