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EVALUATION OF GENES ASSOCIATED WITH MASTITIS IN CROSSBRED DAIRYCATTLE

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ABSTRACT

Mastitis affects dairy production economically. It also associated with increased somatic cells in milk, these cells include; neutrophils, macrophages, lymphocytes, and mammary epithelial cells. They are used as an indicator to monitor udder health status of dairy cattle. Somatic cell count has higher heritability as compared to mastitis, thus it can be used as a trait for selection of mastitis resistance. The objective of this study was to evaluate genes associated with mastitis in crossbred dairy cattle. Blood samples were collected from 96 crossbred cattle for DNA analysis, and a total of 152 milk samples were collected from full udder quarters of 38 lactating crossbred dairy cattle for SCC analysis. Genomic DNA of beta-lactoglobulin and lactoferrin genes were amplified using two pairs of oligo primers 252 bp and 301 bp respectively. Their amplified products yielded 27 fragments at the 301-bp and 23 fragments at 252-bp. Results of PCR-DNA sequencing found out that there were several genetic variations in sequences, which were identified as Single Nucleotide Polymorphism (SNPs) associated with mastitis susceptibility. This study strongly suggests that beta-lactoglobulin and lactoferrin are novel candidate genes for selection of mastitis resistance in crossbred dairy cattle.

Keywords: Crossbred, Gene, Mastitis, Somatic Cell Count, Susceptibility

1. INTRODUCTION

Mastitis affects dairy production economically through its associated costs and effects on production (Peter *et al.,* 2015; Martin *et al.,* 2018). These costs may include: cost of drugs, reduced milk production and household income through discarding of milk in times of treatment, sudden death and premature culling of dairy cattle (Jingar *et al.*, 2017). Mastitis also brings about changes in milk constituents due to the increase in somatic cells. These cells include;

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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 sign of mastitic conditions in the udders of dairy cattle. They are also used internationally to determine milk quality (Li *et al.*, 2014; Jadhav *et al*., 2018). Dairy cows 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 a large number of somatic cell count due to inadequate milking and management. Mastitis is usually associated with a decline in the quantity produced and bacterial load in milk (Sharma *et al*., 2011; Azmi *et al.*, 2017). The frequent use of antibiotic drugs in mastitis treatment and prevention results in their residues accumulation in animal bodies. These residues find their way into milk making it unfit for human consumption, and raising public health concerns (Jadhav *et al*., 2016).

Mastitis is a polygenic trait related to milk production, proteins, and milk quality traits. This it very complicated for molecular markers to be used in selective breeding for mastitis resistance breeds in dairy cattle (Tiezzil *et al.*, 2015). Mastitis has low heritability as compared to SCC and SCS. This makes not suitable for selection of mastitis resistance. Selective breeding can help to lower the incidences of mastitis, when suitable candidate genes associated with mastitis resistance are identified (Cai *et al.*, 2018).

Beta-lactoglobulin and lactoferrin genes have a bactericidal and bacteriostatic actions on pathogenic agents of mastitis. This study offers important insight into evaluation of genes associated with mastitis in crossbred cattle, and identify potential genetic markers that could be used for selection of mastitis resistance in order to improve milk quality.

2. MATERIALS AND METHODS

2.1 DNA Samples

About 5ml of blood samples were obtained aseptically from the external jugular vein of each of the 96 crossbred cattle dairy at Kanyariri Veterinary Farm for DNA analysis. Blood samples were placed into EDTA vacutainer tubes and transported under ice to a Molecular Genetics Laboratory at the Department of Animal Production (CAVS). The vacutainers were stored at -21℃ prior to analysis. DNA was extracted from whole blood sample using QIAGEN DNA extraction kit (DNeasy® Blood & Tissue kit), the manufacturer instructions were strictly followed.

2.2. PCR Amplification for DNA templates

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Optimization of PCR equipment were carried out prior to identify optimum temperature to be used (Baker *et al.*,2017). Two pairs of oligo primers were used for amplification of beta-lactoglobulin and lactoferrin genes respectively (Table 1). The amplification reaction was carried out in 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).

Table 1: Primer pairs 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)

The reaction was performed in a thermal cycler (MJ RESEARCH, INC.) by Taq PCR Core kit 250 units (QIAGEN). The steps were; initialization at 94℃ for 5minutes, denaturation at 94℃ for 60 seconds for 30 cycles, annealed at 61℃ for 1 minute, extended at 72℃ for 60 seconds. The final extension performed at 72℃ for 7 minutes, and PCR products were stored at 4℃ for beta-lactoglobulin (Sharma *et al*., 2015).

For lactoferrin reactions were carried out in thermal cycler (MJ RESEARCH, INC.) with the following conditions; initialization at 95℃ for 5 minutes, denaturation at 95℃ for 60 seconds followed by 35 cycles, annealing of primers at 57℃ for 60 seconds, and extended at 72℃ for 60 seconds. The final extension for both genes were was performed at 72℃ for 7 minutes and PCR products were stored at 4℃ (Azam *et al.*, 2017). Its products were then subjected to 2% agarose gel electrophoresis at 100 volts for 40 minutes. The PCR products formed were viewed under ultraviolet transiluminator to its integrity, and photographed using a digital camera. The similar bands were for each gene and measured with DNA ladder 100bp plus (Kaplan, 2018).

2.3. Sequences Analysis

The BLAST was carried in NCBI results showed 85 to 100 % resemblance to Bos Taurus, Bos Indicus and other species of cattle. The following accession numbers; MN239407 and MN239408 for Complement MN325091 to MN325104, and MN337970 to MN337991 were deposited in Genbank for beta-lactoglobulin and lactoferrin genes respectively.

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3. RESULTS

These phylogenetic tree was constructed by a maximum likelihoods and composed of functional taxonomic units of one node jointed to several others. The larger percentage of sequences of both beta-lactoglobulin and lactoferrin were found to be similar. The bootstrap consensus tree was replicated 1000 times to account for beta-lactoglobulin and lactoferrin genes. These sequences were compared with available sequences available in Genbank (Figure 2, & 3).

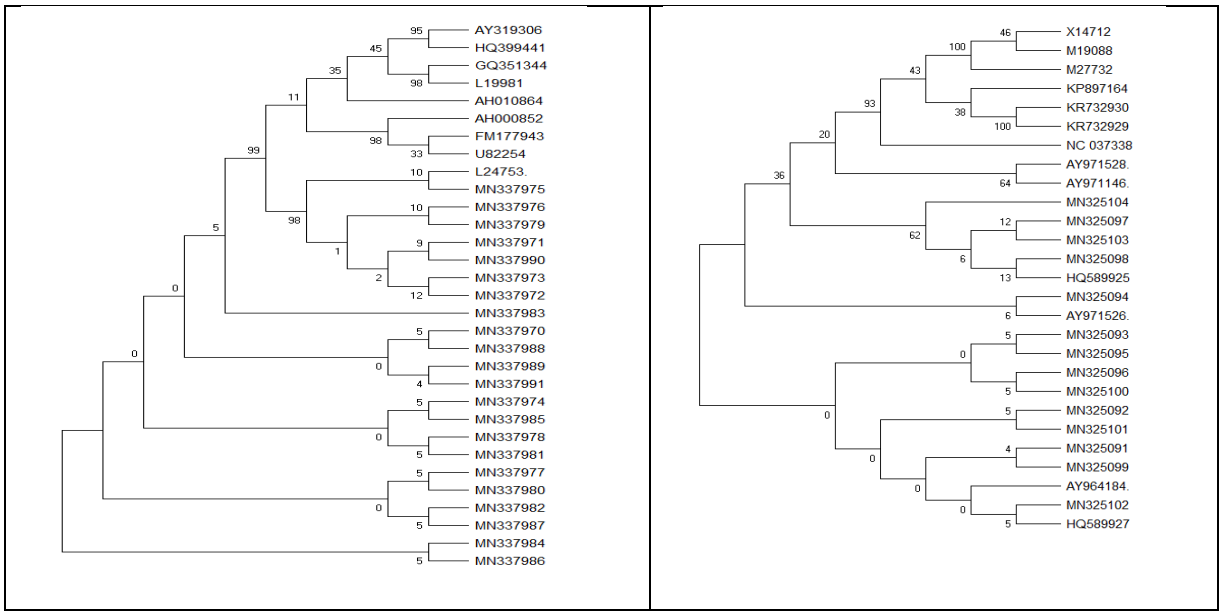

Figure 2 & 3: phylogenetic tree constructed 5' flanking sequence of lactoferrin and beta-lactoglobulin respectively. Figures at nodes were represented by bootstrap values of 1000 replicates in data set.

The lactoferrin gene sequences were both homogenous and heterogeneous in nature for a population of 31 Nucleotide sequences. Beta-lactoglobulin gene composed of 27 sequences, which were aggregated into two larger lineages namely: (Clade 1 and 2) and (clade 3 and 4). These nucleotide sequences were clustered.

A total of 22 sequences of the lactoferrin gene were represented by their accession numbers in table 2, sequences were varied between 50 to 100 positions within its flanking region. These sequences were derived from the sequenced PCR products of four main breeds of dairy cattle include;

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In table 2 evaluation of genetic variability was carried out in between 50 to100 base position. These genetic variants were identified as SNPs, which can be used as marker for selection of mastitis resistance dairy cattle. The polymorphisms found were both transitions and transversions in position 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 1: 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

A total of 14 sequences represented by their accession numbers deposited in Genbank (table 3). These sequence were found to varied between 9 to 195 positions. These polymorphisms were 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) in transitions and transversions position respectively. These polymorphisms were found to be highly associated to mastitis susceptibility. Thus, these can be applied as markers in associate studies.

Table 2: 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 cows

** Subclinical Mastitis

4. DISCUSSION

The 5'flanking region of lactoferrin gene harbours genetic polymorphisms, which significantly attributes to its sequence genetic variabilities, this statement was agreement with (Sharma *et al.*, 2015; Ateya *et al.*, 2016). This property largely contributes to diverse actions of the gene on micro-organisms, through iron binding nature of the gene which deprives mastitis causal agents

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iron needed for their growth. On other hands, bactericidal and bacteriostatic nature of lactoferrin gene on micro-organisms, has made it appropriate candidate gene for selection for mastitis resistance. Thus, its flanking region are sometimes referred as flanking SNPs and also associated with the presence of mastitis and SCC in milk (Yuan *et al.*,2013; Pawlik *et al.*, 2014; Ateya et al.,2016).

The production of lactoferrin gene in udder quarter is triggered by presence of infections, which prompts into an increase in number of cells which include; neutrophil which at some time produces lactoferrin gene in milk. The diverse nature of the regulatory region of lactoferrin gene with presence of the promoter sequences, TATA box, and multiple transcription binding sites, made it suitable gene to be used in genetic selection of mastitis resistance (Zabolewicz *et al.*,2012; Zabolewicz *et al.*,2014).

Beta-lactoglobulin gene is a vital whey protein in milk. It has iron-binding, antibacterial and its inhibitory nature, which affects the growth of mastitis causing agents in milk such as; Staphylococcus ssp, Staphylococcus ssp and many others. This reduces their populations in the udder, thus improves milk quality to be consumed by human (Chaneton *et al.*, 2010; Ateya *et al.*,2016; Martin *et al.*,2018). The presence of mastitis causal agents in udder quarter triggers an increase of SCC in milk, which eventually are used to monitor udder health in dairy cows (Singh *et al.*,2014). The application of SCC as monitor for udder's health status is simple, affordable and it can be handled by smallholder dairy farmers without expertise supports. Early detection of subclinical mastitis is essential to maximize their profits through reduction of the cost of disease control and prevention.

This gene was found out to be highly polymorphic at the following positions in its sequences; 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) in transitions and transversions position respectively. Table 3 these polymorphisms play tremendous roles in regulating the growth and multiplications of pathogenic agents and maintaining the quality of milk against bacterial. Furthermore, the inhibitory nature of these genes reinforce mammary gland immune mechanics, when the innate immune system is impaired. This provides a faster, more efficient, and timely defence against intra-mammary gland infections (Lukač *et al.*, 2013; Ateya et al.,2016). Thus, reduces bacterial loads present in milk. This action triggers an increase in SCC in milk, as a result of immune response mechanism. Therefore, it plays a key role in addressing challenges imposed by mastitis, and it can be to used improve animal milk safety and increase milk production.

Figures 1 and 2 suggested that there were gene flows between these admixed breeds of dairy cows. This study does not reject the incidences of incongruence among the sequences (Tak *et*

al., 2018). The genetic polymorphisms in the DNA remains variable from one sequence to another sequence. Breed differences were due to an individual genetic made up, and this supported the argument that genotype and allele frequencies remain variable from one population to another (Boushaba *et al.,* 2019). However, the similarity between the sequences were attributed by Artificial Insemination (AI), which allows easy gene flow and lessens the mating barrier between several breeds of dairy cattle. This was in line with Smiltina & Grislis, 2018; Tak *et al.,* 2018).

5. CONCLUSIONS

The study found out significant associations among beta-lactoglobulin, lactoferrin genes and somatic cell counts, these manifested mainly in udder quarters of dairy cows positive for subclinical mastitis. The sequences of two genes were also found to be related with other sequences of Bos Taurus, Bos Indicus and other breeds of cattle dairy in globally. This study also mitigates problems related to milk quality as far as mastitis is concerned, which in turn aids hygienic milk fit for human consumption.

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