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Molecular characterization and association of lactoferrin gene to subclinical mastitis in goats (*Capra hircus*)

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ABSTRACT. The study characterized the lactoferrin (Lf) mRNA gene in different goat breeds in the Philippines and determined its association with subclinical mastitis (SCM). The study involved collection of milk at second week of lactation (n=75) and blood samples (n=5) to obtain extracted RNA and using cDNA to amplify Lf gene through polymerase chain reaction. The nucleotide and amino acid sequences were determined and used as reference in the evaluation of phylogenetic relationship. Amplified products were utilized for RFLP analysis before determining the association of the gene with SCM. Results of the study demonstrated that Lf gene in goats registered a molecular weight of 2135. Nucleotide and amino acid sequence of Lf gene revealed high similarity (99%) in Saanen, Anglo-Nubian and Philippine native goats with that of *Capra hircus* (U53857) Lf gene submitted to GenBank. Phylogenetic studies showed that Lf gene of Anglo-Nubian, Saanen and Native goats clade together with Lf gene of *C. hircus* (U53857). Three genotypes in goats were documented using the restriction enzymes *AluI* and *HaeIII*. Based on the Statistical analysis, association (comp 5.65, p = 0.0308) has been established between the Lf genes of goats with genotype BB to SCM using *HaeIII* restriction enzyme.

Keywords: Anglo-Nubian; Lf gene; Philippine native goat; Saanen.

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Introduction

Milk coming from dairy animals meets the basic requirements of the body. The significant increase of milk, milk fat, and protein in dairy animals is a result of advanced researches in the past two decades (Tsuda et al., 2000).

Mastitis is an economically significant infectious disease of dairy animals. Losses are due to decreased quantity and quality of milk, heightened by medicine and labor cost (Hogeveen &Van der Voort, 2017; Waminal, Tubalinal, & Mingala, 2019). Limiting the occurrence of subclinical mastitis (SCM), therefore, can be a vital strategy in reducing economic losses. To understand the mechanism on the susceptibility of animals to mastitis, genetic traits for milk production should be considered. The use of genetic markers in the selection of animals for breeding has also be considered (Rupp & Boichard, 2003; Gholizadeh, Mianji, & Zadeh, 2018).

Lactoferrin (Lf) gene, also known as 'red protein from milk', is an iron-binding protein that is found in most fluids in the body (Marchweka, Roterman, Strus, Spiewak, & Majka, 2012) and is the most important iron-binding protein in milk (Collins, Flores, Wang, & Anderson, 2018). Neutrophils and inflamed tissues release Lf and considered to have direct antimicrobial properties (limits bacterial proliferation and adhesion to microbes) and have a role in innate immunity (Van Der Strate, Belijaars, & Molema, 2001; Valenti & Antonini, 2005; Legrand et al., 2008; Walker, 2010; Siqueiros-Cendon et al., 2014). Furthermore, Lf possesses abundant health benefits such as anti-fungal, anti-parasitic, anti-viral, and anti-tumor properties (Niaz et al., 2019).

The potential of Lf gene (Lonnerdal & Iyer, 1995) as genetic marker for mastitis resistance in dairy animals needs to be elucidated in order to validate its role in mastitis (Pawlik, Sender, & Korwin-Kossakowska, 2009). The selection of superior individuals that show resistance or susceptibility to mastitis based on data generated through molecular studies of the Lf gene will be necessary for the selection of animals for breeding (Sharma, Chakraborty, & Gupta, 2015). This strategy may boost the economic condition of the farmer and nurture the dairy industry as a whole while promoting a mastitis-free dairy herd.

Currently, the information about Lf gene in goats (*Capra hircus*) is limited. Genetic characterization of Lf gene would augment their possible role in occurrence, onset and disease resistance to SCM (Pawlik et al., 2009). The potential of Lf gene as a marker for mastitis resistance in dairy animals is important and will augment existing marker-assisted breeding and selection resulting in improved dairy performance of dairy animals (Shimazaki & Kawai, 2017; Waminal et al., 2019). In cattle production, molecular characterization and genetic diversity of Lf gene have been associated with different traits and disease resistance and have been included in their breeding and selection techniques (Sharma et al., 2015).

The main goal of this study is to characterize and identify Lf gene as a genetic marker for SCM resistance in goats through molecular analysis and association of nucleotide polymorphisms with the presence of SCM.

Material and methods

The collection of samples for analysis was conducted from goats in selected provinces in Central Luzon, the Philippines.

Sample collection

A total of 75 goats (30 Anglo-Nubian, 30 Saanen and 15 mixed breeds) on their second week of lactation onwards and 5 Philippine native goats (not lactating) were selected from previously identified goat farms in Luzon (Farm 1 – 15.6310157, 120.5991522; Farm 2 – 15.4557851, 121.3377025; Farm 3 – 15.7409607, 120.9273197). Housing, feeding, and overall management of the animals per farm were not altered or changed for the conduct of this study. In addition, the farms included in the study housed their animals in concrete housing in group pens with elevations. It was during the summer season (March-May 2018) in the Philippines during the collection of the samples. The test animals were manually milked from the udder and pooled to collect 30 mL of milk sample for ribonucleic acid (RNA) extraction. The milk was collected in a 50 mL conical tube and was placed in a cooler with ice transported to the laboratory for processing. In case the milk was not processed immediately, it was placed in a refrigerator and processed the following day. For the five (5) native goats, RNA was extracted from blood as these animals were not lactating. Five (5) mL of blood was collected from the jugular veins of the animals using EDTA tube. Samples were processed that day after collection.

RNA Extraction

RNA extraction from milk and blood samples from the goats was done following the Promega protocol with some modifications. For milk, 30 mL of raw milk was centrifuged at 4,000 rpm for 20 min. After centrifugation, the fat layer and the supernatant were discarded, and afterwards the pellet was suspended in one (1) mL PBS (phosphate-buffer-saline, a pH of 7.4). This suspension was centrifuged again at 4,000 rpm for 10 min. After this step, the pellet was collected into 2 µL microtube after removing the supernatant fluid. One (1) mL of PBS was mixed again with the pellet and was centrifuged for 14,000 rpm for 1 min. This process was repeated twice and the supernatant was removed to collect the pellet.

For blood, 500 µL of the buffy coat was aspired after centrifugation at 4,000 rpm for 5 min and transferred into the 2 µL microtube, and then added with 2-3 volumes of 0.14 NH_4Cl. The mixture was mixed by sonication and centrifuged at 14,000 rpm for 1 min. Supernatant was removed and the process was repeated twice to collect the pellet.

The pellet from the milk and the blood was added with 1 mL cell lysis solution and was subjected to sonication to mix the pellet and cell lysis. After which, it was centrifuged to 14,000 rpm in 1 min. The supernatant was discarded and 500 µL of nuclei lysis and 300 µL protein precipitation solution was added next and centrifuged for 14,000 rpm for 1 min. After discarding the supernatant fluid, isolated cells were dissolved with 1 mL Trizol reagent and incubated for 10 min at room temperature. Then 200 µL of chloroform was added. The mixture was centrifuged at 14,000 rpm for 15 min. at 4°C. The solution was decanted and pellets were washed by 1 mL 75% ethanol and centrifuged for 5 min at 8,000 rpm at 4°C. Pellets were dried and reconstituted with 30 µL diethyl pyrocarbonate (DEPC) treated water.

Reverse-transcription - polymerase chain reaction (RT- PCR)

RT-PCR kit (TaKara™) was used to synthesize the cDNAs from the total RNA samples extracted. One µL of dT random primers were mixed with 1 µL dNTP, 5 µL of RNAse free water and 3 µL of RNA template. The solution was incubated for 5 min at 65°C before running for PCR. The prepared RNA primer mix was mixed

with 4 µL 5x buffer, 0.5 µL RNAse inhibitor, 1 µL reverse transcriptase, and 4.3 µL RNAse free water. This was subjected to PCR run for segment 1, 10 min. at 30°C; segment 2, 45 min. at 50°C; and segment 3, 5 min. at 95°C. The β-actin was used to determine if the DNA has been produced through PCR.

PCR assay

Primers (Table 1) were designed using Primer3 server and Primer-BLAST using the Caprine Lf gene (Accession number: U53857) stored from National Center for Biotechnology Information (NCBI).

Table 1. Primers used for the amplification of cDNA.

Gene	Accession Number	Primer Sequence (5'-3')	Fragment Length (bp)
	Primer 1	Forward- AGACATGAAGCTCTTCGT Reverse- GAGTACGGACAACACCGGGC	834
	Primer 2	Forward- ATCCTTCGCCCGTTCTTGAG Reverse- CGCCGAATCTACTTTTGAGG	561
	Primer 3	Forward- GCCCAGGTCCCTTCTCATG Reverse- GGGCACAGCTCTGACTAAAG	736
	Primer 4	Forward- AGAAAGCAAATGAGGGGCTC Reverse- TTACCTCGTCAGGAAGGCCG	749

All PCR assays were performed in 20 µL reaction volume containing 2 µL of genomic DNA template, 10 pmol of each primer and PCR master mix. The amplification of the Lf gene was carried out in a thermocycler (SimpliAmp, Thermofisher) under the optimized conditions. Briefly, initial denaturation at 94˚C for 5 min., followed by 40 cycles consisting of denaturation at 94˚C for 1 min., annealing at 58˚C for 1 min. and extension at 72˚C for 1 min., with final extension at 72˚C for 5 min.

After amplification, 1 µL of the PCR product was electrophoresed in 1.5 % agarose gel containing 1X TAE buffer at 70 volts for 30 min. and visualized under ultraviolet light using UV transillumination advance imaging system. To ensure that amplification products are of the expected size, a 1 kb plus DNA ladder was run simultaneously as a marker.

Gene sequencing and phylogenetic tree construction

Lf products were submitted for sequencing. Sequences were assembled using Mega 7 software. The forward and reverse sequences for each primer were assembled to form contigs of the respective regions. The gene sequences were compared with the Lf mRNA sequences to annotate different exonic regions putatively to identify SNPs in respective regions. The contiguous Lf gene nucleotide sequence was subjected to Basic Local Alignment Search Tool (BLAST) at NCBI database to determine the sequence similarity with the corresponding regions of other species. DNA nucleotide sequence was also conceptually translated using MEGA 7 software and compared with that of NCBI Genbank for Caprine and Bubaline Lf gene to detect amino acid changes. Phylogenetic trees were derived using MEGA 7 software using Maximum Likelihod method with 1000 bootsrap replications (Tamura et al., 2011).

Restriction fragment length polymorphism (RFLP) analysis

The Lf PCR products using primer 2 (23 µL) were subjected to endonuclease digestion in goat to amplify exon 2, 3 and 4 of Lf gene with an amplicon size of 561 bp.

Restriction enzymes (Table 2) that could cut the fragments were analyzed using Sequence Manipulation Suite: Restriction map (Stothard, 2000). RFLP was conducted by preparing the reaction mixture composed of 7 µL of PCR product, 5.15 µL of sd2H_20, 0.35 RFLP buffer and 0.075 µL for each enzyme. Samples were incubated at 37°C for 4h. Restriction fragments were resolved on 2% agarose gel in horizontal electrophoresis. The restriction-digested gene fragments were visualized and documented using a UV transilluminator (FlourChemE by ProteinSimple ™). The differences in fragment yielded by various restriction enzymes that would indicate polymorphism in a particular gene were analyzed and compared.

Table 2. Restriction enzymes used for the RFLP analysis.

Restriction Enzymes	Lf Gene Loci
AluI (AG/CT)	21, 53, 78, 295, 418, 465, 472
HaeIII (GG/CC)	353, 380, 492

Association of Lf gene with SCM

Samples of amplified Lf gene from the milk of goats where polymorphisms shown by different genotypes of the target gene previously exhibited after RFLP were taken as reference in tracing back the goats suspected for SCM and for California mastitis test (CMT) evaluation utilizing their milk as samples. A total of 69 goat samples were used in the study that associated expression of Lf gene and in the occurrence of SCM. Physical assessment for SCM covered the evaluation of the mammary gland and milk that involved visual observations and palpation.

CMT was used as an indirect method of measuring SCC as the indicator of intramammary infection as it has the advantage of being a quick, cheap, and simple 'animal side' test (Persson & Olofsson, 2011). CMT scores for all animal subjects were classified as non-mastitic if CMT score result is 1 or lower and mastitic if CMT score result is 2 or higher.

Statistical analysis

Univariate analysis on the possible association between the genotypic frequency and the occurrence of SCM was examined using Chi-square ($\chi 2$) by a certified statistician (Petrie & Watson, 2006).

Results and discussion

Of the 80 samples collected, only 4 (5%) samples produced complete Lf mRNA sequences in goats. It has been shown that the mRNA sequence of Lf gene was detected in the milk samples of Anglo-Nubian and Saanen and in the blood samples of the Native goats. Target primers 1, 2, 3, and 4 were amplified and generated an amplicon size of 834 bp, 561 bp, 736 bp, and 749 bp, respectively.

Native goat Lf mRNA sequence resulted in an average of 2133 bp while there were 2134 bp in Anglo-Nubian and 2135 bp in Saanen. Goat Lf nucleotide sequences were aligned with other ruminants and other species. Statistical nucleotide pair frequency of the 14 aligned nucleotide sequences showed an average of 1334 identical pairs, 751 transitional pairs, and 60 transversional pairs. Among the different goat breeds' nucleotide pair frequencies, the average identical pairs were 2118 with 2 transitional pairs and 6 transversional pairs. There was a high similarity of nucleotide sequences between the breeds of goats being studied (Table 3). It shows that there is a high similarity in the nucleotide and amino acid sequence of different goats. This may be due to the highly conserved region that can be found in the Lf gene. This is essential to maintain their distinctiveness from other species.

Table 3. Nucleotide and amino acid sequence percentage similarity of Native, Anglo-Nubian and Saanen breeds in reference to other ruminant species.

Species (Accession No.)	Nucleotide Sequence			Amino Acid Sequence		
	Native	Anglo Nubian	Saanen	Native	Anglo Nubian	Saanen
C. hircus (U53857)	99.24%	99.44%	99.58%	99%	99%	99%
O. aries (NM_001024862.1)	97.74 %	97.97 %	98.03 %	97 %	98 %	98 %
B. grunniens (DQ387455.1)	93.94 %	94.03 %	94.26%	93%	94 %	94 %
B. bubalis (JF825526.1)	94 .09%	94.28%	94.38%	93%	94 %	94 %
B. indicus (GU059864.1)	93.91%	94.14%	94.14%	93%	93%	94 %
B. Taurus (FJ589071.1)	94.04%	94.05%	94.29%	93%	93%	94 %
E. caballus (NM_001163974.1)	77.12%	77.09%	77.19%	77 %	77 %	77 %
S. scrofa (M81327.1)	72.80%	72.97%	73.02%	72%	72%	72%
H. sapiens (U076343)	73.28%	73.11%	73 %	72%	72%	72%

Maximum Likelihood algorithm with 1000 NJ bootstrap resampling revealed the clustering of all breeds of goats' Lf nucleotide sequence with that of *C. hircus* (U53857.1). Anglo-Nubian and Saanen claded together while Native goats claded with other breeds (Anglo-Nubian and Saanen). All breeds of goats' Lf sequences claded together with *C. hircus* (U53857) sequence. *O. aries* (NM_001024862.1) Lf sequence, on the other hand, separated from the clade of goats. While *B. taurus* (FJ589071.1), *B. indicus, B. grunniens,* and *B. bubalis* (JF825526.1) Lf sequences have much greater separation from the clade of small ruminants. *S. scrofa* (M81327.1), *E. caballus* (NM_001163974), and *H. sapiens* (U07643.1) Lf nucleotide sequence have been used to show an outlier from other ruminants (Figure 1).

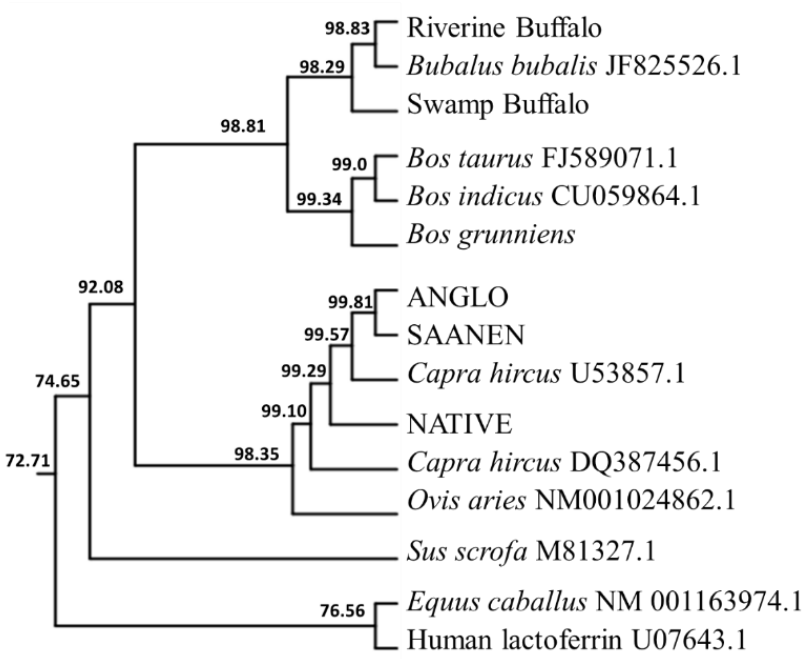

Figure 1. Phylogenetic tree showing the relationship of Lf gene between breed of goats, water buffaloes, other ruminants' and human.

The coding sequence of Lf gene in goats was analyzed for differences in nucleotides and functional amino acid substitution. Furthermore, restriction enzyme capable of digesting the sequence to identify the nucleotide polymorphism and distinguish the genotype has been identified, and the results are herein presented. Sequence analysis revealed seven (7) nucleotide polymorphism in native in the coding sequence of Lf in native goat at locus 551, 552, 553, 701, 910, 939 and 1069 with 5 amino acid substitutions, three (3) in Saanen at locus 1069, 1526 and 1527 with 2 amino acid substitutions and four (4) in Anglo-Nubian breed at locus 910, 1069, 1526 and 1527 with 3 amino acid substitutions.

The nucleotide polymorphism at locus 1069 of Native, Saanen, and Anglo Nubian goat Lf gene sequences resulted in a functional amino acid substitution from leucine to valine. Kaminski et al. (2008) reported that the higher milk protein yield was related to polymorphism at position +216 when it occurs along with another polymorphism. The substitution of valine which is a growth hormone receptor gene can increase the milk yield as well as elevate the milk protein and fat yield.

This is in consonance with the previous studies (Lee et al., 1997; Martin-Burriel, Osta, Baredse, & Zaragosa, 1997; Li & Chen, 1999; Kaminski, Oleński, Brym, Malewski, & Sazanov, 2006; Daly, Ross, Giblin, & Buckley, 2006) that polymorphisms in Lf gene reportedly occur in the coding and regulatory regions and polymorphisms are encoded in exons and introns. To develop rapid recognition of polymorphic site of Lf gene, restriction enzymes *AluI* and *HaeIII* were used to digest 561 bp that was amplified by primer 2. This 561 bp segment covered exons 2 and 3 of the coding region based on the SMS Restriction Digest application of 4 samples with complete Lf gene nucleotide sequence.

The availability of these restriction patterns on extracted Lf gene was examined by using PCR product that was subjected to *AluI* (Figure 2) and *HaeIII* (Figure 3) digestion and electrophoresed. Results showed restriction patterns that were almost the same based on the conceptualized expected pattern (Chang-hong, Gaoming, Yanliang, & Zhaoxia, 2008).

The presence of three restriction patterns using *AluI* seen in gel conferred that there were more polymorphic nucleotides for Lf gene and had to be discovered. High polymorphism in Lf was quite expected because of its function to bind peptides derived from exogenous antigens and in mediating immune response. *AluI* produced cut at AGT/ACT site. Results showed no cut in genotype AA producing 561 bp fragment size of the gene, while genotype AB had one cut producing two bands with 300 bp and 217 bp amplicon size products. Genotype BB produced 3 bands with 561 bp, 300 bp, and 217 bp fragment sizes.

Another restriction enzyme used to test the restriction pattern of Lf gene in goats was *HaeIII* which produced cuts at AGT/ACT site. Results showed no cut in genotype AA producing 561 bp fragment size of the gene, while genotype AB had 5 cuts producing four bands with 561 bp, 312 bp, 122 bp, and 70 bp amplicon size products. Genotype BB produced 3 bands with 312 bp, 122 bp, and 70 bp fragment sizes (Figure 3)

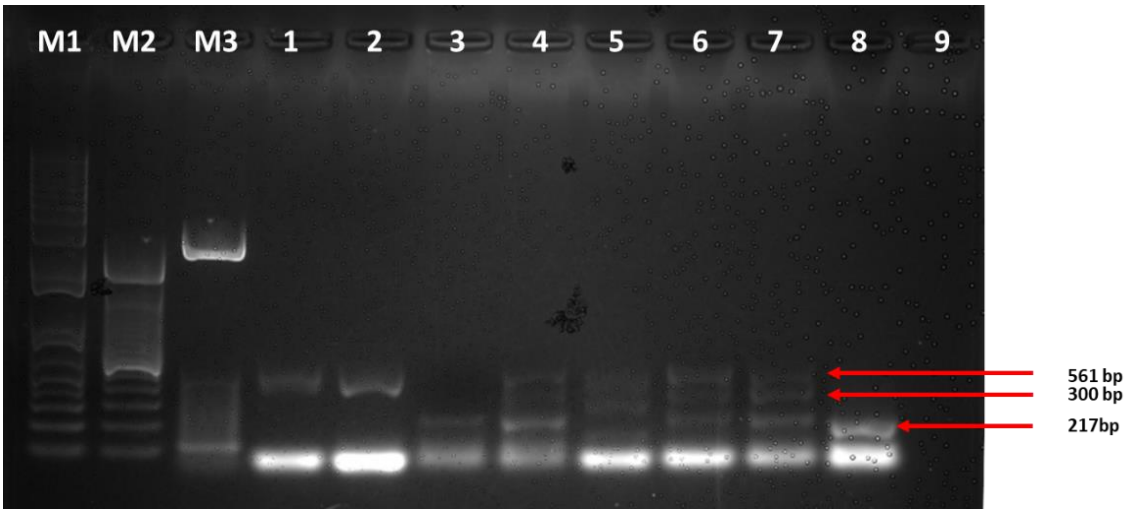

Figure 2. Restriction patterns of caprine Lf gene PCR products using *AluI* in 2% agarose gel. M1. 1000 bp + ladder, M2 100 bp ladder M3 25 bp ladder, Lane 1 and 7-genotype AA, Lanes 2 to 5 genotype AB, Lanes 6 and 8 –genotype BB.

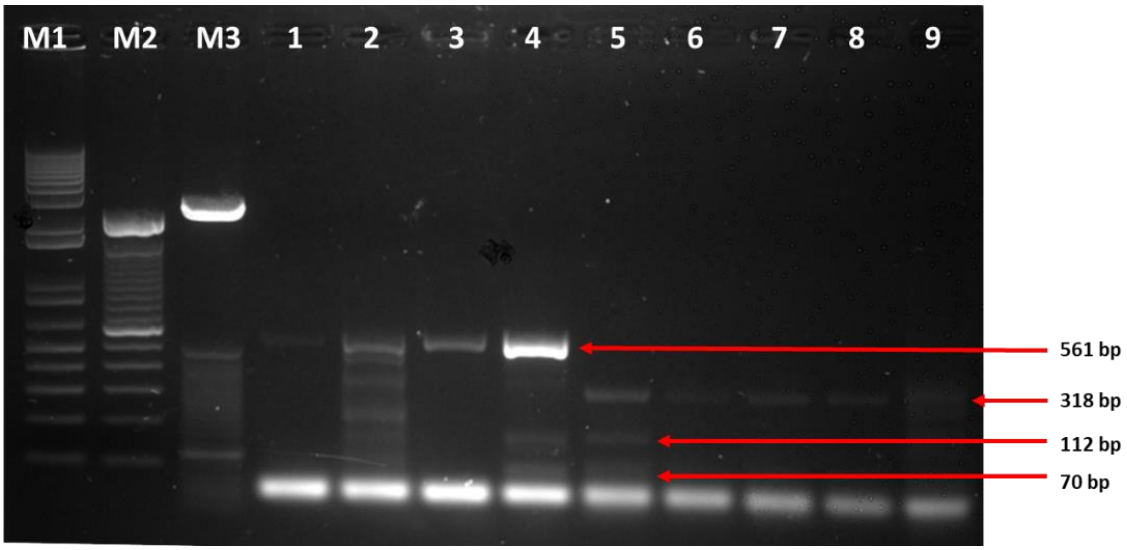

Figure 3. Restriction patterns of caprine Lf gene PCR products using *HaeIII* in 2% agarose gel. M1. 1000 bp + ladder, M2 100 bp ladder. M3 25 bp ladder, Lanes 1 and 3-genotype AA, Lanes 2 and 4-genotype AB, Lanes 5 to 9–genotype BB.

Restriction enzyme *Alul* that can cut AG/CT nucleotide site was selected to examine restriction fragment pattern in the nucleotide sequence of Lf gene based on the SMS Restriction Digest application of the Lf complete nucleotide sequence.

The different genotypes found in the Lf gene are not significantly associated with the occurrence of mastitis using other restriction enzymes. No prior studies have been conducted in support of the three different genotypes found in goats using *HaeIII* and *AluI* restriction enzyme, hence this study will be the baseline for other researchers in identifying mastitic resistance genotypes.

The 561 bp was used for the association study. It showed the frequency of Lf genotypes in non-mastitic and SCM goats. Although chi-square test revealed no significant effect to make an association between genotypes and mastitic incidence (Table 4 and 5), it was clearly presented that genotype BB had a high frequency in SCM compared to non-mastitic animals. Animals having a Genotype BB may or may not increase the tendency on the occurrence of SCM as compared to genotype AA and AB.

Table 4. Chi-Square Analysis on the Association of Genotypes and Sub-clinical mastitis in Goats using Restriction Enzyme *AluI*.

Genotype	Expected Frequencies			Chi-square			Chi-square computed	Chi-square tabulated
	AA	AB	BB					
Non-mastitic	11.88	17.23	11.88	0.10	0.18	0.69	2.43	5.99
Sub-clinically Mastitic	8.11	11.76	8.11	0.15	0.26	1.02	2.43	5.99

Table 5. Chi-Square Analysis on the Association of Genotypes and Sub-clinical mastitis in Goats using Restriction Enzyme *HaeIII*.

Genotype	Expected Frequencies			Chi-square			Chi-square computed	Chi-square tabulated
	AA	AB	BB					
Non-mastitic	10.84	16.57	16.57	0.43	0.35	1.26	5.658	5.99
Sub-clinically Mastitic	6.15	9.42	9.42	0.75	0.62	2.22	5.65	5.99

Percentage frequencies of Lf *AluI* and *HaeIII*-based genotype in non-mastitic and SCM goats are presented in Table 6 and 7.

Table 6. Percentage frequency of Lf *Alu1*-based genotype in non- mastitic and sub-clinically mastitic goats.

Genotype (base pair size)	Category of Animal				Total	
	Non- mastitic		Sub-clinically mastitic			
	%	(n)	%	(n)	%	(n)
AA (561bp)	65.00	13	35.00	7	28.99	20
AB (300 bp, 217 bp)	65.58	19	34.48	10	42.02	29
BB (561 bp, 300 bp, 217 bp)	45.00	9	55.00	11	28.99	20
Total	59.42	(41)	40.58	(28)	100	69

Table 7. Percentage frequency of Lf *HaeIII*-based genotype in non-mastitic and sub- clinically mastitic goats.

Genotype (base pair size)	Category Of Animal				Total	
	Non- mastitic		Sub-clinically mastitic			
	%	(n)	%	(n)	%	(n)
AA (561bp)	76.47	13	23.53	4	24.64	17
AB (561bp, 318bp, 112bp, 70bp)	73.08	19	26.92	7	37.68	26
BB (318bp, 112 bp, 70 bp)	46.15	12	53.85	14	37.68	26
Total	63.77	44	36.23	25	100	69

Association analysis based on the frequency of genotypes revealed that there is an association between genotypes and mastitic incidence. The frequency of the occurrence of digestion patterns were produced by *HaeIII* enzyme with SCM and non- mastitis regardless of breed. The Lf gene in goats has a bactericidal and bacteriostatic activity that could lessen the bacterial population in the milk through phagocytic killing (Tsuda et al., 2000). However, this may not be enough to sequester the microbial flora in the mammary gland of the animals. Underlying factors such as climate, housing system, type of bedding, and rainfall and wetness in the vicinity of dairy premises interact to influence the degree of exposure of teat and tend to increase mastitis pathogens that cause SCM (McEwen & Cooper, 1947).

Conclusion

The whole mRNA coding sequence of goats had an average molecular weight of 2134 bp. Both nucleotide and translated amino acid sequences of Lf were highly similar (98-99%), and phylogenetic analysis found an evolutionary relationship with goats from NCBI GenBank.

The Lf gene of goat established three different genotypes (*AA, AB, and BB*) using restriction enzymes *Alu1* and *HaeIII;* however, Chi-square analysis revealed no association between Lf gene and the occurrence of SCM using *HaeIII* restriction enzymes.

These initial findings are applicable in the field of immunity and disease resistance. The incidence of association between restriction sites and clinical parameters converts PCR-RFLP into a powerful tool in relating specific amino acid substitution at a critical position to possible disease resistance. Moreover, the selection of disease resistance genes will provide potential avenues for improving the health status of the animal and increasing productivity.

The polymorphic nature of the genes merits further investigation for other nucleotide loci and their significance to disease resistance or susceptibility. Identification of the blood composition of the upgraded goats would help establish the bloodline of animals. Expression analysis can also assist in the understanding of how the genes progress in disease protection.

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