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# Molecular characterization and gene expression of MHC II DRB3 gene associated with subclinical mastitis in goats

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**Abstract:** The major histocompatibility complex (MHC) genes play a vital role in the immune response of vertebrates by encoding molecules involved in self and non-self-discrimination (spelling) and antigen presentation. This study focuses on the MHC II DRB3 gene in goats, specifically comparing its nucleotide and amino acid sequences with those of other ruminant species (*Capra hircus*, *Ovis aries*, *Bos taurus*, *B. grunniens*, and *Bubalus bubalis*). Four different goat breeds (Philippine Native, Upgraded (Philippine Native x Exotic), Anglo-Nubian, and Saanen) were analyzed, and their MHC II DRB3 sequences were aligned with sequences from the NCBI GenBank. The nucleotide sequences exhibited high similarity between the different goat breeds and other ruminant species, indicating a conserved structure across mammalian species. Results from sequence comparisons revealed varying degrees of similarity between goat MHC II DRB3 sequences from *C. hircus*, *O. aries*, *B. taurus*, *B. grunniens*, and *Bu. bubalis*. Phylogenetic analysis further supported the close (95-99%) relationship between goats and other ruminants. Different functional domain regions in the MHC II DRB3 gene across different breeds of goats and other ruminants were characterized using SMART online tool. Moreover, polymorphism analysis using the *Hae*III restriction enzyme highlighted genetic variability within the MHC II DRB3 gene in goats. This study enhances our understanding of the goat MHC II DRB3 gene, its similarities to other ruminant species, and its potential role in immune response.
**Keywords:** Genotypes; Goats; Mastitis; MHC II DRB3; PCR-RFLP; +2.

## 1. Introduction

Goat (*Capra hircus*) production serves as a significant source of income for smallholder farmers in the Philippines (Orden et al., 2023), and Indonesia (Petlane et al., 2012). Goats exhibit excellent adaptability to extreme weather conditions and can subsist on crop residues, agro-industrial by-products, and locally available fodder (Koluma, 2023). In dairy goats, subclinical mastitis (SCM) stands out as one of the most challenging diseases, leading to economic production losses attributed to treatment costs and animal loss through culling (Jabbar et al., 2020). SCM can result in reduced milk production, compromised milk quality, and poor milk hygiene, with physical, chemical, microbiological, and pathological changes occurring in the udder and milk (Mishra et al., 2018), rendering the milk unsuitable for processing due to its short shelf life and off-flavors (Owusu-Kwarteng et al., 2020).

Somatic cell count (SCC) is a valuable predictor of intramammary infection (IMI) in dairy cattle, as evidenced in research by Sharma et al. (2011). The California Mastitis Test (CMT) offers a prevalent and practical indirect method for measuring SCC, facilitating the on-site detection of inflammatory infections in cows (Persson and Olofsson, 2011; Robertson and Muller, 2005). Mastitis in dairy cattle is commonly caused by bacterial pathogens such as Staphylococcus aureus, Escherichia coli, and Streptococcus uberis. Environmental factors, poor milking practices, and inadequate barn hygiene can also significantly contribute to the incidence of the disease.

Recent studies expand on these findings, highlighting the variability in susceptibility to mastitis across different ruminant species. For instance, buffaloes, which are pivotal to dairy production in regions like South Asia, exhibit a unique profile of mastitis prevalence and pathogen susceptibility. Pegolo et al. (2023) suggests that buffaloes have lower SCC thresholds for diagnosing mastitis compared to cattle, indicating species-specific immune response mechanisms. This points to the necessity of developing tailored diagnostic and management strategies for different ruminant species to effectively control mastitis.

Genetic research has furthered our understanding of immune responses in ruminants. Studies on the bovine leukocyte antigen DRB3 (BoLA-DRB3), part of the major histocompatibility complex (MHC) genes, have been instrumental in ruminants. MHC molecules play a critical role in immunological defenses against pathogens, influencing disease resistance and variability in immune responsiveness (Ahmad et al., 2022). In goats, similar studies on caprine lymphocyte antigen (CLA) or goat lymphocyte antigen (GoLA) demonstrate that MHC genes also govern susceptibility to diseases like mastitis in non-bovine ruminants (Sbalamurugan et al., 2021).

Genes associated with immune response have been investigated for the presence of single nucleotide polymorphisms (SNPs) and their associations with mastitis-related traits. The bovine leukocyte antigen DRB3 (BoLA-DRB3), which belongs to the major histocompatibility complex (MHC) genes, has been studied in ruminants. MHC molecules play a significant role in immunological defense against pathogens (Vandre et al., 2014), disease resistance, and immune responsiveness variability (Paracha et al., 2015). The MHC of the goat, also known as caprine lymphocyte antigen (CLA) or goat lymphocyte antigen (GoLA), is similar to that of

sheep and cattle (Zhao et al., 2011). Limited literature is available on the molecular characterization of the MHC II DRB3 gene in goats. Molecular characterization offers enhanced detection of diversity in genotypes that can be utilized in selecting dairy animals with genotypes for SCM resistance, thus mitigating production losses associated with SCM. This study aimed to characterize and detect polymorphisms in the MHC II DRB3 gene and determine the genotypes associated with SCM in goats.

## 2. Materials and Methods

### 2.1. Characterization of MHC II DRB3 Gene in Goats

A total of 99 goats (17 Philippine Native, 33 Upgraded (Philippine Native x Exotic), 29 Anglo-Nubian, and 20 Saanen) from goat farms in Luzon, Philippines were included in the study. Ribonucleic acid (RNA) extraction from milk and blood samples was performed following the TRIzol™ protocol (California, USA) with some modifications. Reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted using the TaKaRa TM kit (California, USA) to synthesize complementary DNA (cDNA) from the extracted RNA samples. Primer sets were designed based on the caprine MHC II DRB3 (NM_001314217.1) messenger RNA (mRNA) from NCBI GenBank using the Primer3 server (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primer1 (forward: 5'-ATGGTGTGCCTGTATTTCTCC-3' and reverse: 5'-CAGACCGTGCCCTCCATT-3') yielded a product of 664 base pairs (bp), while Primer2 (forward: 5'-AGAATGGAGACTGGACCTT-3' and reverse: 5'-CCCTGTTGGCTGAAGTGTAG-3') produced a product of 257 bp.

### 2.2. Gene amplification

Polymerase chain reaction (PCR) was performed using a thermocycler (SimpliAmp, ThermoFisher) (Massachusetts, USA) in a 20 µl reaction volume containing, 2 µl of genomic DNA template, 10 pmol of each primer, and PCR master mix. Optimized amplification cycles were conducted for this study with initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 sec, annealing at 58°C for primer1 and 56°C for primer2, 38 cycles for 30 sec, extension at 72°C for 35 sec, and a final extension at 72°C for 5 min. Visualization of the 1 µl PCR product was performed after electrophoresis in a 2% agarose gel containing 1X tris-acetate-EDTA (TAE) buffer at 100 volts for 30 min (MYGEL mini, Accuris Instruments) (New Jersey, USA) using ultraviolet (UV) transillumination (FlourChem E by ProteinSimple) (Minneapolis, USA) with 1kb plus DNA ladder as a marker.

### 2.3. Gene sequencing and analysis

PCR products were submitted for sequencing and assembled using MEGA 7.1 software (Kumar et al., 2016). Gene sequences were compared with the MHCII DRB3 coding DNA sequences (CDS) of *Capra hircus* (NM_001314217.1), *Ovis aries* (NM_001123402.1), *Bos taurus* (NM_001012680.2), *Bos grunniens* (KY682173.1), and *Bubalus bubalis* (LC210724.1) from the NCBI GenBank database using BLASTn.

### 2.4. Restriction Fragment Length Polymorphism (RFLP) Analysis

Amplified PCR products (15 µl) underwent endonuclease digestion using *Hae*III (GG|CC) restriction enzymes to cleave the gene fragments, which were then visualized on a UV transilluminator (FlourChem E, ProteinSimple) (Minneapolis, USA) alongside 100kb, 50kb, and 25kb ladders as markers. The differences in fragment length yielded by the restriction enzymes, indicating polymorphism in the gene, were analyzed and compared. The genotype frequency of fragments was determined through direct counting.

### 2.5. Association of Polymorphism of MHC II DRB3 Gene in Goats and the Occurrence of Subclinical Mastitis

A total of 65 milk samples were used to assess the association of subclinical mastitis and the *Hae*III enzyme. All animal subjects were classified as non-subclinical mastitic if the California Mastitis Test (CMT) score was 1 or lower or subclinical mastitic if the CMT score was 2 or higher (Scruton et al., 2009; Escobar, 1999). Additionally, the Porta SCC goat milk test was employed to determine the somatic cell count and categorize animals as non-subclinical mastitic if SCC<1,500,000 cells/ml or subclinical mastitic if SCC≥1,500,000 cells/ml.

### 2.6. Statistical Analysis

Univariate analysis of the potential association between genotypic frequency and the occurrence of subclinical mastitis was examined using the Chi-square ($X^2$) test for goodness-of-fit (Petrie and Watson, 2006). Additionally, odds ratios were computed in Microsoft Excel 2013 (Redmond, USA) to determine the strength of the association (Deeks and Higgins, 2010).

### 2.7. Determination of Genetic Expression of MHC II DRB3 Gene in Goats with and without subclinical mastitis

#### 2.7.1. Quantitative Gene Expression Analysis

Real-time PCR for MHC II DRB3 was performed using an ABI 7500 sequence detection system (Massachusetts, USA) with SYBR green PCR master mix (Applied Biosystems, CA) (Massachusetts, USA). The 10 µl reaction mixture for RT-PCR consisted of 1 µl of cDNA, 0.15 µl each of the forward (upstream) primer: 5'-CTAAGAGCGAGTGTCATTTC-3' and reverse (downstream) primer: 5'-CCGACCCCGTAGTTGTGT-3' (10 nmol/L), 5 µl of SYBR Green Real-time PCR master mix (2x), and 3.7 µl of ddH2O, using glyceraldehyde phosphate dehydrogenase (GAPDH) as an endogenous control. The cDNA template (10µl) was used for each gene quantification after sequential 10-fold dilution. RT-PCR was run using the diluted samples as a gradient and template.

The RT-PCR amplification was performed with an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing for 45 sec at 63°C for MHC II DRB3, with a reaction efficiency of 90-100%, a quantification cycle (Cq) value of 31.07, and a correlation coefficient ($R^2$) value of 0.92. GAPDH had an annealing temperature of 65°C with a reaction efficiency of 90-100%, a Cq value of 29.36, and an $R^2$ value of 0.996. The melt curve stage involved heating to 95°C for 15 sec, followed by cooling to 65°C for 30 sec, and an extension to 95°C for 15 sec.

### 2.7.2. Statistical analysis

Significant differences in MHC II DRB3 gene expression between animals with or without subclinical mastitis were compared using a two-sided Student's t-test. Comparative CT values methods (2 ddCt) were computed based on their mean Ct values concerning those in non-mastitic counterparts.Statistical Package for the Social Sciences, version 20, was used in this study by IBM (New York City, USA)

## 3. Results

### 3.1. Sequence analysis of MHC II DRB3

Fourteen samples produced good sequences: four contigs were completed in Native goats, four in Upgraded goats, four in Anglo-Nubian goats, and two in Saanen goats. The average length of the Native goat MHC II DRB3 nucleotide sequence was 790 bp, while the averages were 792 bp in Upgraded goats, 791 bp in Anglo-Nubian goats, and 784 bp in Saanen goats.

Goat MHC II DRB3 nucleotide sequences were aligned with those of other ruminants' MHC II DRB3 CDS using the database from NCBI GenBank. Statistical analysis of nucleotide pair frequency in the 19 aligned sequences revealed an average of 748 identical pairs, 20 transitional pairs, and 20 transversional pairs, with a ratio of 1.0. A comparison of nucleotide pair frequencies between different goat breeds showed an average of 755 identical pairs, 15 transitional pairs, and 15 transversional pairs, with a ratio of 1.0. This indicates a high (96%) similarity of nucleotide sequences among the studied goat breeds' MHC II DRB3 genes.

BLASTn results for the MHC II DRB3 nucleotide sequence of Native goats showed 99% similarity to the *C. hircus* (NM_001314217.1) sequence in GenBank, while Upgraded, Anglo-Nubian, and Saanen goats exhibited lower similarity, ranging from 97 to 98% (Table 1). Furthermore, lower similarity was observed when comparing Native goat nucleotide sequences with those of *O. aries* (NM_001123402.1), *B. taurus* (NM_001012680.2), *B. grunniens* (KY682173.1), and *B. bubalis* (LC_210724.1), with 95% similarity each. Similar trends were observed in Upgraded, Anglo-Nubian, and Saanen MHC II DRB3 sequences compared to those of other species, ranging from 94 to 95% similarity, except for Upgraded goats compared to *O. aries* (NM_001123402.1) at 96%. Accession number LC_210724.1 in NCBI GenBank refers to the Philippine carabao, originating from the Philippines.

The higher percentage similarity of MHC II DRB3 nucleotide sequence of *B. bubalis* (LC_210724.1) to cattle compared to goats may be attributed to environmental or locational similarities of the animals studied, as both goats and carabaos are from the Philippines. MHC genes encode essential molecules for self/altered self/non-self-discrimination in the interaction of the organism with its environment, necessary for the effective presentation of various antigens to immunocompetent cells. This interaction creates positive selection pressure on gene variability in the population (Hameed et al., 2006).

| | Nucleotide sequence (%) | | | |
|---|---|---|---|---|
| Species | NATIVE | UPGRADED | ANGLO-NUBIAN | Saanen |
| *Capra hircus* (NM_001314217.1) | 99 | 97 | 98 | 97 |
| *Ovis aries* (NM_001123402.1) | 95 | 96 | 95 | 94 |
| *Bos taurus* (NM_001012680.2) | 95 | 94 | 95 | 94 |
| *Bos grunniens* (KY682173.1) | 95 | 94 | 95 | 94 |
| *Bubalus bubalis* (LC_210724.1) | 95 | 94 | 95 | 95 |

**Table 1 –** Nucleotide percentage (%) similarity of MHC II DRB3 gene in ruminant species from NCBI GenBank concerning Native, Upgraded, Anglo-Nubian, and Saanen goats.

Consequently, lower similarity was observed in the translated amino acid sequence of goat MHC II DRB3 compared to the amino acid sequences of other ruminants from the NCBI GenBank. The Native goat sequence exhibited 96% similarity with the *C. hircus* (NP_001301146.1) sequence from GenBank. Lower similarity of amino acid sequences was also observed in *O. aries* (NP_00116874.1), *B. taurus* (NP_001012698.2), *B. grunniens* (ASG92659.1), and *B. bubalis* (AAZ76544.1).

Similarly, lower similarity of amino acid sequences was observed in Upgraded goats compared to *C. hircus* (NP_001301146.1), *O. aries* (NP_00116874.1), *B. taurus* (NP_001012698.2), *B. grunniens* (ASG92659.1), and *B. bubalis* (AAZ76544.1). Anglo-Nubian goats exhibited a uniform higher similarity with 97% similarity of amino acid sequences, except for *B. grunniens* (ASG92659.1) with 95% similarity. In Saanen goats, a uniform 95% similarity of amino acid sequences was also observed when compared with *C. hircus* (NP_001301146.1), *O. aries* (NP_001116874.1), *B. taurus* (NP_001012698.2), *B. grunniens* (ASG92659.1), and *B. bubalis* (AAZ76544.1), respectively.

### 3.2. Phylogenetic Analysis of MHC II DRB3 in Goats

The Maximum Likelihood algorithm with 1000 bootstrap resampling revealed that the *C. hircus* (NM_001314217.1) MHC II DRB3 nucleotide sequence clades together with Upgraded (NxA)_97 with a 23-bootstrap value, and clades with Native_1, Native_5, and Native_4 nucleotide sequences with a 30-bootstrap value (Figure 1). The Anglo-Nubian_40, Anglo-Nubian, and Anglo-Nubian_19 clade together with Saanen_48 with a 36-bootstrap value. The Upgraded (NxS)_102 nucleotide sequence clades with other goats' nucleotide sequences with a 31-bootstrap value.

On the other hand, the *O. aries* (NM_001123402.1) nucleotide sequence clades together with the goats' (Upgraded (NxA)_98, Saanen_49, Anglo-Nubian_17, Native_3, Upgrade (NxS)_101) MHC II DRB3 sequence with a 25-bootstrap value. While the nucleotide sequences of *B. bubalis* (LC_210724.1), *B. grunniens* (KY682173.1), and *B. taurus* (NM_001012680.2) separate from the small ruminants' cluster with a 28-bootstrap value.

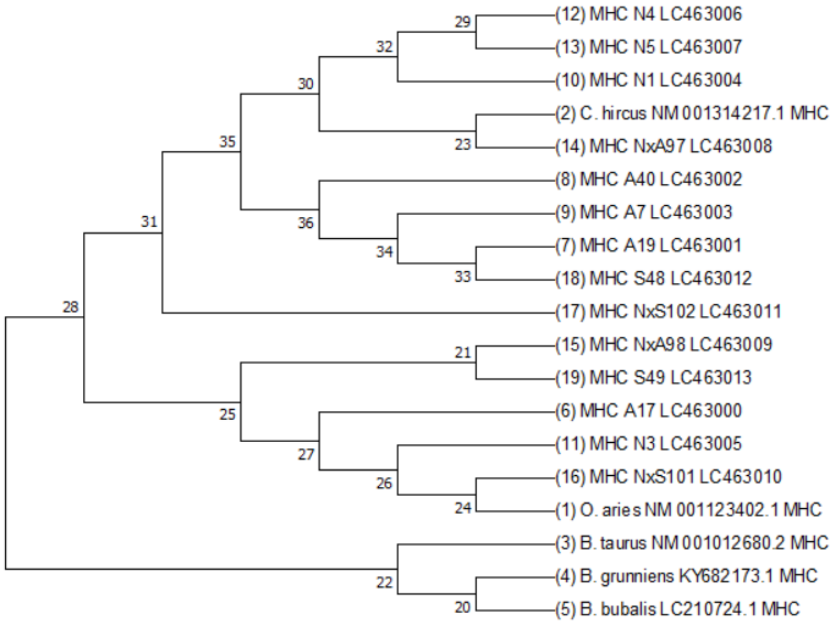


**Figure 1 —** Phylogenetic tree showing the relationship between goats and other ruminants'MHC II DRB3 nucleotide sequence. N = Philippine native; A = Anglo Nubian; S = Saanen; x denotes crossing over.

### 3.3. Predicted functional domains of goat MHC II DRB3 gene

Using the Native goat MHC II DRB3 sequence as the representative animal of this study, a 789 bp nucleotide sequence has a corresponding 263 amino acid translation provided by MEGA 7.1 software. The cleavage site was found to be between positions 29 and 30 amino acid locations (http://www.cbs.dtu.dk/services/SignalP). Functional domains were identified through the SMART application. The first transmembrane domain was identified at amino acid position 12 and ended at amino acid location 29; the MHC_beta domain started at position 42 and ended at position 116; IGc1 started at position 141 and ended at position 212; and another transmembrane region started at position 228 and ended at position 250 (http://smart.embl-heidelberg.de). Domains showed similarities between breeds of goats as well as sheep and cattle (*B. taurus* and *B. grunniens*), and water buffalo.

### 3.4. Polymorphism Analysis for the MHC II DRB3 Gene in Goats

To develop a rapid recognition of the polymorphic site of the MHC II DRB3 gene, the restriction enzyme *Hae*III was used to digest a 664 bp segment amplified by primer 1 of MHC II DRB3 in Study 1. This 664 bp segment covered exons 1, 2, and 3 of the coding regions. The SMS Restriction Digest program (http://www.bioinformatics.org) showed that the *Hae*III enzyme produced eight restriction patterns by cutting nucleotides with the GG/CC sequence. Polymorphisms of the 664 bp amplicon were observed at loci 238, 252, 461, 491, 524, and 654.

The fragment sizes predicted by the SMS Restriction Digest (http://www.bioinformatics.org) produced by possible cuts of *Hae*III on the MHC II DRB3 nucleotide sequence are as follows: restriction pattern a, six bands with fragment sizes 209, 167, 140, 79, 63, 6; pattern b, seven bands with fragment sizes 209, 153, 14, 140, 79, 63, 6; pattern c, 8 bands with fragment sizes 209, 153, 14, 140, 79, 30, 33, 6; pattern d, 8 bands with fragment sizes 209, 153, 14, 130, 10, 79, 63, 6; pattern e, 7 bands with fragment sizes 209, 167, 130, 10, 79, 63, 6; pattern f, 7 bands with fragment sizes 223, 153, 140, 79, 30, 33, 6; pattern g, 8 bands with fragment sizes 157, 52, 153, 14, 140, 79, 63, 6; and pattern h, 6 bands with fragment sizes 304, 157, 140, 30, 33, 6. However, not all patterns were observed in the gel electrophoresis of the samples, as shown in Figure 2.

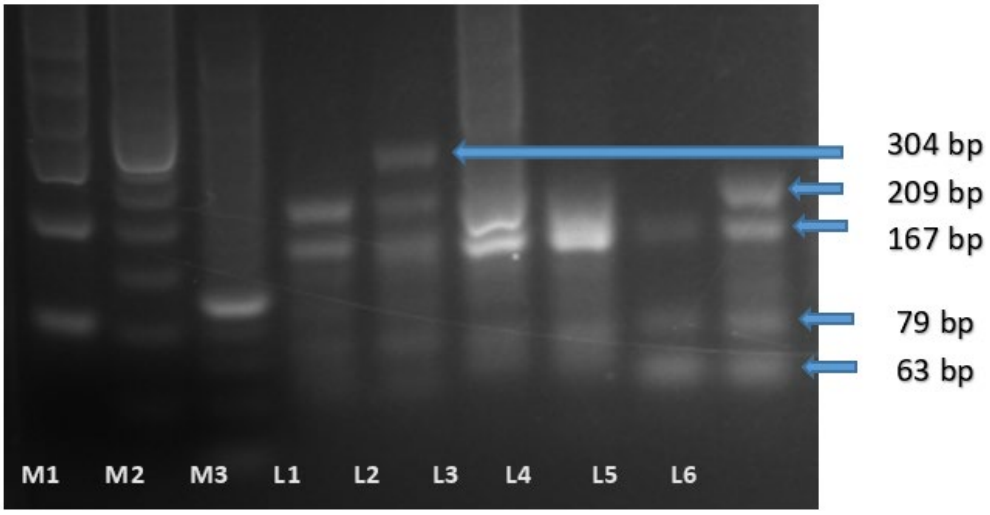


**Figure 2 —** Restriction patterns obtained by digestion of MHC II DRB3 gene PCR products using *Hae*III in 2% agarose gel. M1-100bp, M2-50bp, M3-25bp ladders; Lanes 1, 3, 4, 5, 6 –pattern a (fragment size: 209, 167, 79, 63), Lane 2– pattern h and a (fragment size: 304, 209, 167, 79, 63).

Out of the 65 milk samples tested, 26 were categorized in the subclinical mastitis group, while 39 belonged to the non-subclinical mastitis group. Table 2 shows the frequency of occurrence of digestion patterns (genotypes) produced by the *Hae*III enzyme with subclinical mastitis and non-subclinical mastitis groups. The most frequent genotype was AA with 35 (53.85%), followed by genotype AB with 11 (16.92%), DD with 7 (10.77%), BB with 6 (9.23%), AD with 3 (4.62%), BD with 2 (3.08%), and CC with 1 (1.54%). The Odds Ratio (OR) is a statistical measure used to evaluate whether a particular genotype of a goat increases or decreases the risk of developing mastitis, an inflammatory condition of the mammary glands. Specifically, the OR represents the odds that an outcome (e.g., mastitis) will occur given a particular exposure (e.g., a specific genotype), compared to the odds of the outcome occurring without that exposure. For instance, an OR greater than 1 indicates a higher risk of mastitis associated with the genotype, while an OR less than 1 suggests a protective effect.

The Confidence Interval (CI) for the OR provides a range of estimated values that are likely to include the true OR to a certain level of confidence, typically 95%. A CI that does not include 1 indicates that the OR is statistically significant, suggesting a meaningful association between the genotype and the risk of mastitis. If the CI includes 1, the association is not considered statistically significant, meaning that any observed effect might be due to chance rather than the influence of the genotype.

### 3.5. RFLP analysis for MHC II DRB3

Results showed that seven genotypes/restriction patterns were observed for *Hae*III (Table 2).

### 3.6. Association of MHC II DRB3 gene with the occurrence of subclinical mastitis

Table 2 above shows the frequency of occurrence of digestion patterns (genotypes) produced by the *Hae*III enzyme with subclinical mastitis and non-subclinical mastitis. The frequency of occurrence for subclinical mastitis in genotype AA is 9 compared to 26 for the non-occurrence of subclinical mastitis. Statistical chi-square analysis revealed a significant association of genotype AA with the non-occurrence of subclinical mastitis ($p < 0.05$) in all breeds of goats under study. This indicates that with genotype AA, fewer goats had subclinical mastitis. OR showed that animals with genotype AA had an OR of 0.26 with a 95% confidence interval (CI) of 0.09 (lower limit) to 0.75 (upper limit), with a significant association showing this. On the other hand, the frequency of subclinical mastitic animals with genotype DD was 6, compared to 1 in non-subclinical mastitic animals, with an OR analysis yielding 11.4, indicating a significant association.The percentage frequency of MHC II DRB3 *Hae*III-based genotypes of Anglo-Nubian, Saanen, and Upgraded goats is presented in Tables 3, 4, and 5, respectively, with corresponding odds ratio results and 95% CI, confidence intervals, with no established significant association. Conversely, genotypic frequency in Native goats was not tabulated since only genotype AA appeared in all 7 Native goats in the *Hae*III-based RFLP results. Genotype AA in Native goats had an OR of 0.07, with no significant association established.

| Genotype/ Restriction Patterns (Fragment Sizes) | Category Of Animal | | Total | Odds Ratio (OR) | 95% CI |
|---|---|---|---|---|---|
| | Non-SCM | SCM | | | |
| AA (209, 167, 140, 79, 63, 6) | 26 | 9 | 35[*] | 0.26[*] | 0.09 to 0.75 |
| BB (209, 153, 14, 140, 79, 63, 6) | 4 | 2 | 6 | 0.73 | 0.12 to 4.30 |
| CC (209, 153, 14, 140, 79, 30, 33, 6) | 0 (0.5)[i] | 1 (1.5) | 1 | 4.65 | 0.19 to 118.55 |

| | | | | | |
|---|---|---|---|---|---|
| DD (272, 153, 14, 140, 79, 6) | 1 | 6 | 7 | 11.40* | 1.28 to 101.37 |
| AB (209, 167, 153, 14,140, 79, 63, 6) | 7 | 4 | 11 | 0.83 | 0.22 to 3.18 |
| AD (272, 209, 167, 140, 79, 63, 6) | 0 (0.5)[i] | 3 (3.5) | 3 | 11.77 | 0.58 to 237.97 |
| BD (272, 209, 153, 14, 140, 79, 6) | 1 | 1 | 2 | 1.52 | 0.09 to 25.44 |

[i] 0.5 is added to each cell in the category with zero (0) entries to compute for corrected odds ratio (OR) and standard error (Deeks and Higgins, 2010). [‡]Significant association of the genotype was found in $X^2$ ($p<0.05$). *Significant association was found in the OR ($p<0.05$).

**Table 2** — Frequency of MHC II DRB3 *Hae*III-based genotypes in goats with or without subclinical mastitis (SCM).

| Genotype Restriction Pattern (fragment sizes) | Category of Animal | | Total | Odds Ratio | 95% CI |
|---|---|---|---|---|---|
| | Non-SCM | SCM | | | |
| AA (209, 167, 140, 79, 63, 6) | 9 | 5 | 14 | 0.18 | 0.03 to 1.01 |
| BB (209, 153, 14, 140, 79, 63, 6) | 1 (1.5)[i] | 0 (0.5) | 1 | 0.26 | 0.01 to 7.12 |
| CC (209, 153, 14, 140, 79, 30, 33, 6) | 0 (0.5)[i] | 1 (1.5) | 1 | 2.78 | 0.10 to 74.70 |
| DD (272, 153, 14, 140, 79, 6) | 0 (0.5)[i] | 2 (2.5) | 2 | 5.00 | 0.22 to 115.06 |
| AB (209, 167, 153, 14,140, 79, 63, 6) | 2 | 2 | 4 | 0.83 | 0.10 to 0.867 |
| AD (272, 209, 167, 140, 79, 63, 6) | 0 (0.5)[i] | 3 (3.5) | 3 | 7.61 | 0.35 to 163.83 |
| BD (272, 209, 153, 14, 140, 79, 6) | 0 (0.5)[i] | 1 (1.5) | 1 | 2.78 | 0.10 to 74.70 |

[i] 0.5 is added to each cell in the category with zero (0) entries to compute for the corrected odds ratio and standard error (Deeks and Higgins, 2010)

**Table 3** — Frequency of MHC II DRB3 *Hae*III-based genotypes in Anglo-Nubian goats with or without SCM.

| GENOTYPE RESTRICTION PATTERN (fragment sizes) | CATEGORY OF ANIMAL | | TOTAL | ODDS RATIO | 95% CI |
|---|---|---|---|---|---|
| | Non-SCM | SCM | | | |
| AA (209, 167, 140, 79, 63, 6) | 3 | 3 | 6 | 1.00 | 0.14 to 7.10 |
| BB (209, 153, 14, 140, 79, 63, 6) | 1 (1.5)[i] | 0 (0.5) | 1 | 0.30 | 0.01 to 8.35 |
| DD (272, 153, 14, 140, 79, 6) | 1 | 4 | 5 | 6.40 | 0.55 to 74.89 |
| AB (209, 167, 153, 14,140, 79, 63, 6) | 3 | 1 | 4 | 0.25 | 0.02 to 3.04 |
| BD (272, 209, 153, 14, 140, 79, 6) | 0 (0.5)[i] | 1 (1.5) | 1 | 1.00 | 0.05 to 18.92 |

[i] 0.5 is added to each cell in the category with zero (0) entries to compute for corrected odds ratio and standard error (Deeks and Higgins, 2010).

**Table 4** — Frequency of MHC II DRB3 *Hae*III-based genotypes in Saanen goats with or without SCM.

| GENOTYPE RESTRICTION PATTERN (fragment sizes) | CATEGORY OF ANIMAL | | TOTAL | ODDS RATIO | 95% CI |
|---|---|---|---|---|---|
| | Non-SCM | SCM | | | |
| AA (209, 167, 140, 79, 63, 6) | 7 | 1 | 8 | 0.19 | 0.01 to 2.50 |
| BB (209, 153, 14, 140, 79, 63, 6) | 2 | 2 | 4 | 4.50 | 0.37 to 54.16 |
| AB (209, 167, 153, 14,140, 79, 63, 6) | 2 | 1 | 3 | 1.50 | 0.10 to 23.07 |

**Table 5** — Frequency of MHC II DRB3 *Hae*III-based genotypes in Upgraded goats with or without SCM.

### 3.7. Genetic Expression of MHC DRB3 in Goats

The recurring nature of subclinical mastitis has sparked interest in genetic resistance to subclinical mastitis in dairy animals. Although the cause of the condition is multifactorial, resistance still relies on the ability of the immune system to combat the recurring infection (Alfonseca-Silva et al., 2021). One of the vital immune effectors responsible for the early detection and capture of the infectious agent is MHC II DRB3 (Medina et al., 2019). This is the introduction or part of a discussion.

The expression analysis of MHC II DRB3 genes in lactating dairy goats was performed to provide insight into how these receptors are modulated by the body to confer resistance to infections such as subclinical mastitis. The optimized RT-qPCR assay was used to quantify the expression of MHC II DRB3 in goats. Figure 3 displays the optimized MHC relative expression analysis. Quantification cycles (Qc) of different samples were validated by their characteristic melting curves showing only one peak. The relative quantification of mRNA transcripts of MHC II DRB3 in goats was evaluated. The fold change represents the ratio of the normalized mean expression between the positive and negative groups with subclinical mastitis. In this study, the expression of the

MHC II DRB3 gene was 2.09 ± 2.07 or a maximum of 4.16-fold in animals with SCM. A higher expression of the gene was observed in non-SCM goats with 2.54 ± 3.30 or a maximum of 5.84-fold. However, this difference was not statistically significant.

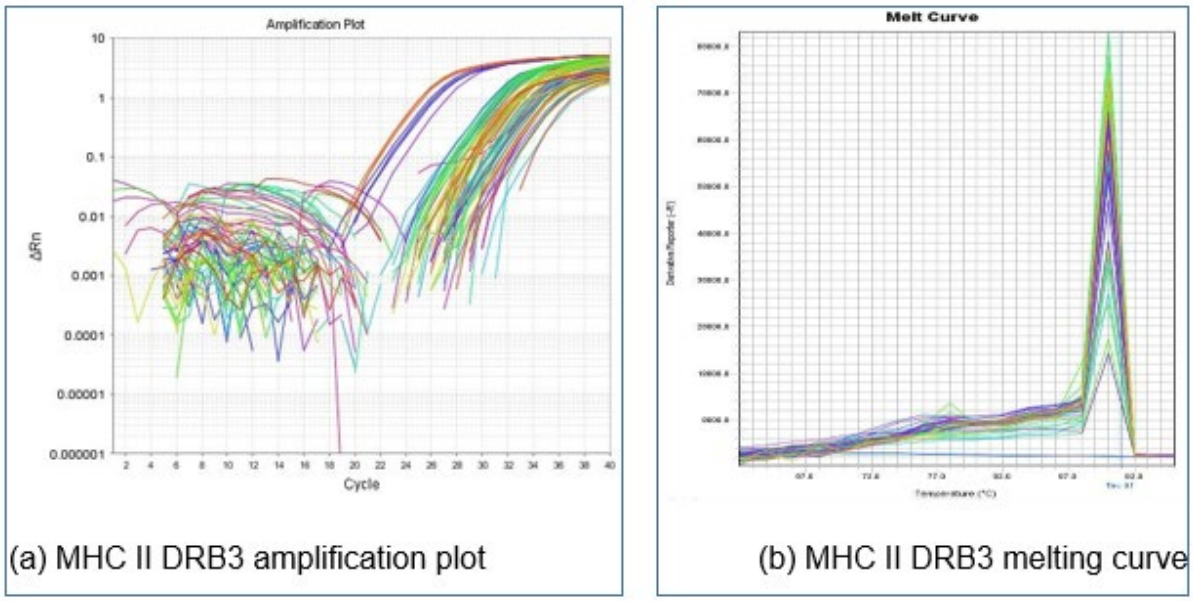


**Figure 3 —** Amplification plot (a) and melting curve (b) of MHC II DRB3 isolated from goats as amplified in qPCR.

## 4. Discussion

This study revealed considerable conservation across the nucleotide and amino acid sequences of the MHC II DRB3 gene among different ruminant species. Notably, the sequences from the goat breeds studied displayed high similarity (89-99%) to those of other ruminants, supporting findings by Medina et al. (2019), which suggest that despite the variability in some regions, the general structure of the MHC is largely conserved across mammalian species. This conservation is particularly evident in the functional domains crucial for immune responses, such as the MHC_II beta and IGc1 domains, as also reported in recent studies (Ivy-Israel et al., 2020; Jurewicz and Stern, 2019).

The identification of significant genetic diversity in the MHC II DRB3 gene among different goat breeds correlates with findings in other ruminants. For instance, the average sequence similarity of 96% within breeds and 89-99% across ruminants aligns closely with the results reported by Smith et al. (2018), who found about 95% sequence similarity in MHC genes among European and Asian breeds of cattle, underscoring a conserved evolutionary pathway across domesticated ruminants.

Our phylogenetic analysis supports these findings, with distinct clades corresponding to different breeds indicating the genetic divergence among populations. This divergence likely influences the observed variability in susceptibility to diseases like mastitis, which is also influenced by environmental factors, as evidenced by the similarity in sequences between goats and carabaos in the Philippines (Medina et al., 2019).

The study also demonstrated a significant association between specific MHC II DRB3 genotypes and the occurrence of subclinical mastitis. For instance, genotype AA was significantly associated with a lower occurrence of subclinical mastitis, showing an odds ratio of 0.26, suggesting a protective effect. Conversely, genotype DD exhibited an odds ratio of 11.4, indicating a higher risk of developing mastitis. These findings reflect trends observed in similar studies, such as Hernandez et al. (2019), who linked the same genotype in dairy cattle with a reduced incidence of mastitis.

Moreover, the restriction pattern analysis using the HaeIII enzyme revealed a wide range of genotypic variability, emphasizing the high degree of polymorphism within the MHC II DRB3 gene. This genetic diversity is essential for the immune system's ability to adapt to and respond to various pathogens. The identification of alleles related to susceptibility or resistance to mastitis can significantly inform selective breeding programs to enhance herd health.

## 5. Conclusions

The nucleotide and translated amino acid sequences of the MHC II DRB3 gene of goats were highly similar to *C. hircus* nucleotide sequences found in the GenBank but were of lower similarity to other ruminants such as sheep, cattle, yak, and water buffalo. Phylogenetic analysis showed an evolutionary relationship between the ruminant species and that some important domains were conserved. RFLP analysis of the gene readily identified polymorphic loci for the gene, especially the peptide-binding region that contains the MHC-beta domain of the MHC DRB3 gene. The genotypes of MHC II DRB3 were based on the digestion patterns produced by the *Hae*III restriction enzyme. The Chi-square test revealed genotype AA in *Hae*III to be associated with the non-occurrence of subclinical mastitis. The OR also showed a significant association in genotypes AA and DD in the occurrence of

subclinical mastitis in goats. Expression of the MHC II DRB3 gene was found to be upregulated in goats with non-SCM, but with no significant difference. This could be attributed to the delayed response of the adaptive immune system.

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