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The Genetic Diversity of TLR4 MHC-DRB Genes in Dairy Goats Using PCR-RFLP Technique

M. Petlane[a],*, R. R. Noor[b], & R. R. A. Maheswari[b]
[a]Major Program of Animal Production and Technology, Graduate School, Bogor Agricultural University
[b]Department of Animal Production and Technology, Faculty of Animal Science, Bogor Agricultural University
Jln. Agatis, Kampus IPB Darmaga, Bogor 16680, Indonesia
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ABSTRAK

Penelitian ini bertujuan untuk mempelajari keragaman genetik gen TLR4 dan MHC-DRB pada kambing perah [(saanen, persilangan peranakan etawah-saanen (PESA), and peranakan etawah (PE)] dengan menggunakan metode PCR-RFLP. Kedua gen tersebut berperan dalam sistem imunitas yang penting dalam pengenalan patogen serta membawa patogen tersebut ke sel-T dan sel CD4. PCR digunakan untuk mengamplifikasi fragmen DNA genom dari gen TLR4 (382 bp) dan CaLA-DRB (285 bp). Keragaman genetik pada TLR4 dideteksi dengan memotong amplimer dengan enzim restriksi Alu1 sedangkan pada DRB, amplimer dipotong menggunakan enzim restriksi *Pst*I dan *Taq*I pada reaksi yang berbeda. Hasil penelitian ini menunjukkan bahwa TLR|*Alu*I tidak menunjukkan keragaman genetik (monomorfik) dan hanya ditemukan satu alel pada ketiga tipe peranakan. Nilai-nilai heterozigositas (He) dan PIC ditemukan rendah pada kedua lokus DRB|*Taq*I and DRB|*Pst*I di PE dan saneen. Nilai X^2 menunjukkan bahwa DRB|*Pst*I di PE and DRB|*Taq*I di PESA tidak berada dalam kesetimbangan H-W dan tidak ditemukan adanya genotype homozigot resesif. Hasil penelitian menunjukkan bahwa TLR4|*Alu*I bukan merupakan marker yang tepat untuk menggambarkan resistensi penyakit sedangkan DRB|*Taq*I dan DRB|*Pst*I memberikan harapan untuk dapat digunakan sebagai marker resistensi berdasarkan nilai PIC yang didapatkan.

Kata kunci: keragaman genetika, TLR4|AluI, DRB|PstI, DRB|TaqI, kambing perah

ABSTRACT

This research was aimed at evaluating the genetic polymorphism of TLR4 and MHC-DRB genes in dairy goats [(Saanen, Etawah Grade-Saanen Crossbred (PESA), and Etawah Grade (PE)] using PCR -RFLP. The two genes are involved in immunity where they play a crucial role in pathogens recognition and presentation to T-cells and CD4 cells. PCR was used to amplify genomic DNA for TLR4 (382 bp) and CaLA-DRB (285 bp) genes fragments. Genetic polymorphism was detected by digesting TLR4 amplimer with AluI while DRB amplimers were digested with *Pst*I and *Taq*I in two separate reactions. The results showed that TLR4|*Alu*I was monomorphic and fixed with allele T in all three breeds while DRB|*Taq*I and DRB|*Pst*I loci were found polymorphic for all breeds. Heterozygosity expected (He) and PIC were found low at both DRB|*Taq*I and DRB|*Pst*I loci in PE and Saanen. X^2 results showed that DRB|*Pst*I in PE and DRB|*Taq*I in PESA were not in H-W equilibrium and did not display homozygous recessive genotype. The results declared that TLR4|*Alu*I was not a good for marker for diseases resistance whereas DRB|*Taq*I and DRB|*Pst*I gave hope for resistance based on their PIC.

Key words: genetic diversity, TLR4|AluI, DRB|PstI, DRB|TaqI, dairy goats

INTRODUCTION

Chronic mastitis becomes the common and main problem in dairy goats because it goes unnoticeable hence left unattended unless it advances to clinical stage. Bacteria that causes mastitis enters mammae through teats canal and causes inflammation that is observable from outside in extreme cases (clinical mastitis). Because of udder abscesses and pain, mastitic goats may often refuse milking and or refuse nursing their offspring. Chemical composition and physical characteristics of milk from such animals are altered; rendering their milk

*Corresponding author:
Lesotho Agricultural College, Maseru 100 Lesotho
E-mail: ppetlane23@yahoo.com

unusable for processing because of low shelf life and off flavors. This eventually leads to unplanned and early culling of young animals.

Although mastitis cannot be completely eliminated from the herd due to re-infections, infection levels can be kept minimal by keeping the environment clean and observing hygienic milking procedures. The host immune system also plays a very crucial role in protecting the animal by differentiating pathogenic foreign materials from its own tissues.

As opposed to dairy cows, research on genetics of mastitis resistance in dairy goats is still superficial. So this research will explore the genetic diversity of toll like receptor4 (TLR4) gene and the DRB region of goat leucocyte antigen (GoLA) in three breeds Saanen, Etawah Grade goat (referred to as PE in the text) and PESA, as genetic diversity is always associated with fitness.

Toll like receptor4 (TLR4) gene is conserved over evolution resulting in high degree of homology between species (Jungi *et al.,* 2010). It is a type I transmembrane proteins characterized by an extracellular leucine-rich domain and a cytoplasmic domain referred to as Toll/IL-1R domain (or TIR domain) because of its homology with cytoplasmic domain of the mammalian interleukin1 (Jungi *et al.,* 2010). TLR4 plays a key-role in innate immune system by recognizing conserved molecules on the surface of various microbes. Its extracellular ligand recognition domain then binds to specific pathogen-associated molecular patterns (PAMPs) (Tirumurugaan *et al.,* 2010) and the recognition signals cascade through intracellular domain to initiate signaling events including translocation of transcription factors, cytokine modulation, and interferon-stimulated gene regulation leading to inflammatory responses and or release of antimicrobial agents (Takeda & Akira, 2003).

The major histocompatibility complex (MHC) is a complex of genes that play a vital role in immune systems. MHC is well known for containing a large number of genes and genetic diversity in alleles found in most vertebrates. Its genes are divided into three classes; classes I and II genes exhibit most genetic variation (Baghizadeh *et al.*, 2009, Zhao *et al.*, 2011). Heterozygosity of MHC genes plays a key role in immune recognition of pathogens and parasites hence improved resistance because of diverse antigens that will be presented to T-cells and by generating a diverse collection of T cells Genetic polymorphisms of class II genes occur predominantly in the first domain (exon 2) that encodes the peptide-binding sites (PBS). This research was aimed at evaluating the genetic polymorphism of TLR4 and MHC-DRB genes in dairy goats [(Saanen, Etawah Grade-Saanen Crossbred (PESA), and Etawah Grade (PE)] using PCR -RFLP.

Table 1. Total DNA samples

Goats sub population	Total samples
Saanen	22
Etawah Grade (EG) (PE)	40
EG-Saanen Crossbred (PESA)	20
Total DNA	82

MATERIALS AND METHODS

The study used a collection of DNA sample (Table 1) from Animal Molecular Genetics Laboratory, Faculty of Animal Science, Bogor Agricultural University. The blood samples were taken from dairy goats reared Bogor Regency, West Java, Indonesia.

The primers used (Table 2) for TLR4 and DRB amplification were designed by Wang *et al*. (2007) and Ahmed & Othman (2006), respectively. Polymerase chain reaction (PCR) thermal conditions and reaction formulae were optimized based on conditions used by the researchers mentioned above. PCR reaction volume for all amplification process was 15 µl; and for most of the amplification the cocktail was made of 0.05 µl Taq polymerase, 0.3 µl of forward and reverse primer, 0.3 µl dNTPs, 1 µl $MgCl_2$, 1.5 µl 10x buffer, 10.85 µl dH_2O and 1 µl DNA sample. Most of DRB fragment from PE could not be amplified using the same formula hence it was modified as follows; 0.4 µl of forward and reverse primer, 0.4 µl dNTPs, 1.5 µl $MgCl_2$, 1.5 µl 10x buffer, 10 15 µl dH_2O for PE DNA samples.

TLR4 was annealed at 57 ^oC while DRB was annealed at 60 ^oC and 58 ^oC for some PE samples. Other PCR conditions were constant and similar for all samples, viz cycling for 5 min at 95 ^oC during denaturation and 1 min at 72 ^oC during extension. GeneAmp PCR system 9700 Applied Biosystem was used for amplification process.

RFLP and Agarose Gel Electrophoresis

TLR4 PCR product was digested with *Alu*I for 16 h at 37 ^oC. PCR product (4 µl) was digested with *Alu*I (2 µl) restriction endonuclease (RE) cocktail that was

Table 2. Amplification conditions and primers to be used

Locus	Annealing temperature (^oC)	PCR product	Primer sequence
TLR4	57	382 bp	F:5′- AGACAGCATTTCACTCCCTC
			R:3′-ACCACCGACACACTGATGAT
CaLA-DRB	60 and 58	284 bp	DRB1F TATCCCGTCTCTGCAGCACATTTC
			DRB1R TCGCCGCTGCACACTGAAACTCTC

composed of dH_2O (1 µl), buffer (0.7 µl) and *Alu*I RE (0.3 µl).

DRB PCR product was digested with *Taq*I and *Pst*I in two separate reactions. DRB amplicon (4 µl) was digested with *Pst*I RE (2 µl) mix that consisted of dH_2O (1µl), buffer (0.7 µl) and *Pst*I restriction endonulease (0.3 µl). The mix was then incubated for 16 h at 37 °C, while in another reaction the same amount of amplicon was digested with *Taq*I at 65 °C for 16 h.

The RFLP digestion products were then separated on 2% agarose / 0.5X TBE stained with 2.5 µl of ethidium bromide (EtBr) and calibrated with 100 bp ladder marker for both genes. Electrophoresis chamber was run on 100 V power supply for forty minutes. The gel was finally visualized under UV transilluminator.

Data Analysis

Genotype frequencies and the expected heterozygosity values were determined following Nei & Kumar (2000). Cervus (3.0) software was used to determine allele frequencies and PIC while population genetics simulation software (PopGeneS²) version 1.0.3380.20651 was used to determine fitness to Hardy-Weinberg (H-W) equilibrium. The formulae for allele frequency (Nei & Kumar, 2000):

$$x_i = x_{ii} + \frac{1}{2}\sum_{j \neq i} x_{ij}$$

where: X1 is the allele frequency for the ith allele; Σ summation of xij over all j's except for j= i. j≠i.
Expected heterozygosity

$$h = 1 - \sum_{i=1}^{q} x_i^2$$

Where:
h = heterozygosity
q = number of alleles observed
x_i = population frequency of i-th allele

RESULTS AND DISCUSSION

The 382 bp partial fragment of third exon of TLR4 gene and 285 bp of second exon of DRB gene in sample population showed in Figure 1, Sequence 1 and Figure 2, Sequence 2, respectively. All three breeds are monomorphic for TLR4|*Alu*I (Figure 3) restriction sites with only one allele (A) available thus resulting in only "AA" genotype (Table 3). In case there was polymorphism, the restriction patterns were expected to be 260 bp, 109 bp and 13 bp for allele A and 142 bp, 118 bp, 109 bp and 13 bp for allele B (Wang *et al.*, 2007).

DRB|*Pst*I (Figure 4) restriction analysis showed two alleles (P and p alleles) in all three breeds. The restriction patterns were 226 bp and 44 bp and 15 bp (for P restriction site) or 270 bp and 15 (p restriction sites). DRB|*Taq*I restriction analysis also showed polymorphism with T and t alleles in all three breeds (Figure 5) displayed on as 163 bp and 122 bp fragments (T restriction site) or the undigested fragment at 285 bp for 't' restriction site.

There is limited research done in goats about TLR4 as compared to cattle hence there is limited information. Genetic polymorphism of TLR4|*Alu*I was reported in Chinese Holstein, Chinese Simental, and Sanhe cattle (Wang *et al.*, 2007, Jungi *et al.*, 2010). Contrary to that, this study found TLR4|*Alu*I locus monomorphic and fixed with allele "A" in all three breeds. This monormophism renders less usable for future prospects of genetic diversity exploitation.

Heterozygosity values (Table 4) showed that DRB|*Taq*I in PE had the least heterozygosity (0.1576) while DRB|*Taq*I Saanen had the highest heterozygosity (0.4985). DRB|*Pst*I had the lowest heterozygosity value (0.4032) in Saanen and high in (0.4753). On average, DRB|*Pst*I had higher heterozygosity in sample population as compared to DRB|*Taq*I.

With exception to DRB|*Taq*I in Saanen, all the sample population have heterozygosity values lower than that of Sanhe cattle, Chinese Holstein, and Simmental (Wang *et al*., 2007). DRB|*Taq*I did not display homozy-

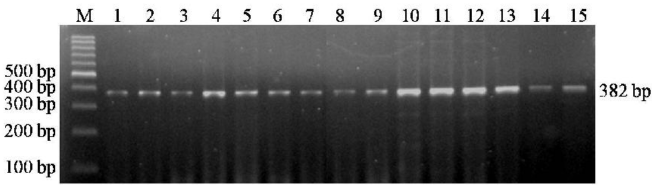

Figure 1. The 382 bp TLR4 PCR amplification product

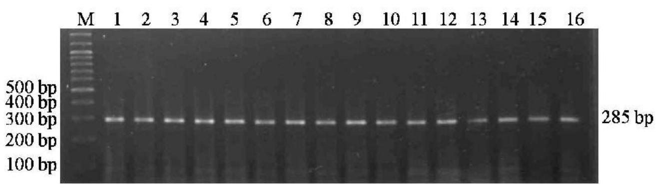

Figure 2. The 285 bp DRB PCR amplification product

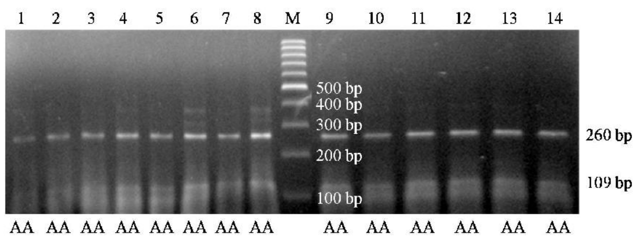

Figure 3. The RFLP product for TLR4|*Alu*I

```
2461 gtgtcaactg gaacaggtgt cctggacggc atttcactcc ctccctagcc ttcaggtgct
2521 gaatatgagt cacaacaaac tcttgtcatt ggatacattt ctttatgaac cactccactc
2581 gctccggatc ctagactgca gtttcaaccg tatcacggcc tctaaggagc aagaactacg
2641 gagtttgcca aggaacctca cttggctaaa tcttactcag aatgaatttg cttgtgtttg
2701 tgaacatcag agtttcctgc agtgggtcaa ggaccagagg cag|ctcttgg tgggag|ctga
2761 gcaaatgatg tgtgcagagc ctttagatgt gaaggacatg ccagtgcttg gtttcaggaa
2821 tgccacttgt cagatgagca agacgatcat cagcgtgtcg gtggtcactg tactcctggt
```

Sequence 1. TLR4 amplicon sequence; Genbank HM627213.2; *Alu*I restriction site AG|CT. The primer sites are underlined and the restriction sites are bolded.

Table 3. Allelic and genotypic frequencies values of dairy goat breeds

Locus	Breed	Number of sample	Allele frequency		Genotype frequency		
			A_1	A_2	A_1A_1	A_1A_2	A_2A_2
TLR4|*Alu*I	Saanen	37	1.000	0.000	1.000	0.000	0.000
	PE	41	1.000	0.000	1.000	0.000	0.000
	PESA	33	1.000	0.000	1.000	0.000	0.000
DRB|*Pst*I	Saanen	25	0.720	0.280	0.520	0.400	0.080
	PE	37	0.650	0.350	0.380	0.540	0.081
	PESA	9	0.611	0.389	0.220	0.780	0.000
DRB|*Taq*I	Saanen	18	0.472	0.528	0.280	0.390	0.330
	PE	26	0.912	0.086	0.828	0.172	0.000
	PESA	16	0.846	0.154	0.846	0.154	0.000

Note: PE= Etawah Grade; PESA= Etawah Grade-Saanen Crossbred.

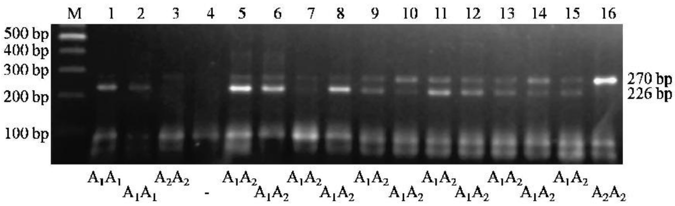

Figure 4. The RFLP product for DRB|*Pst*I

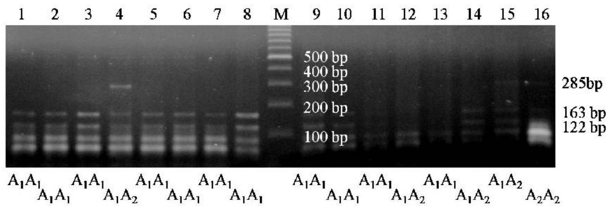

Figure 5. RFLP product for DRB|*Taq*I

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 1.  TATCCCGTCTCTGCA|GCACATTTCCTGGAGTATCATAAGAGCGAGTGTCATTTCTTCAAC
61   GGGACCGAGCGGGTGTGGTACCTGGACAGATACTTCTATAATGGAGAAGAGTACGTGCGC
121  TTCGACAACGACTGGGGCGAGTACCGGGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAG
181  TACTGGAACAGCCAGAAGGAGATCCTGGAGCGGAAGCGGGCCAATGTGGACACGTACTGC
241  A|GACACAACTACGGGGTCGGTGAGAGTTTCAGTGTGCAGCGGCGAT
```

Sequence 2. DRB amplicon and *Pst*I restriction sites, 286 bp; ACCESSION: X83367; *Pst*I restriction sites (CTGCA|G). The primer sites are underlined and the restriction sites are bolded.

```
 2.  ATCCCGTCTCTGCAGCACATTTCCTGGAGTATCATAAGAGCGAGTGTCATTTCTTCAAC
62   GGGACCGAGCGGGTGTGGTACCTGGACAGATACTTCTATAATGGAGAAGAGTACGTGCGC
122  TT|CGACAACGACTGGGGCGAGTACCGGGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAG
182  TACTGGAACAGCCAGAAGGAGATCCTGGAGCGGAAGCGGGCCAATGTGGACACGTACTGC
242  AGACACAACTACGGGGTCGGTGAGAGTTTCAGTGTGCAGCGGCGAT
```

Sequence 3. DRB amplicon and *Taq*I restriction sites, 285 bp; ACCESSION: X83367; *Taq*I restriction sequence (T|CGA). The primer sites are underlined and the restriction sites are bolded.

gous recessive pattern in PESA while Egyptian goat displayed three genotypes. DRB|*Pst*I in PESA and PE did not show homozygous recessive genotypes as well, while Egyptian goat did not display homozygous dominant genotypes in the same loci (Ahmed & Othman, 2006).

Polymorphism information content measures the degree of informativeness of a mutation as a candidate marker for linkage studies Botstein *et al.* (1980). It is the index of probability that a certain marker allele of an offspring will enable one to deduce which of the two marker alleles it received from the affected parent. PIC results (Table 4) showed that DRB|*Taq*I in PE and PESA was low (PIC<0.25), while the same loci in PESA, PE and DRB|*Pst*I loci was moderately informative (0.25< PIC<0.5) in all goat breeds. PIC for DRB|*Pst*I in Saanen and DRB|*Pst*I in PESA have almost the same values as in Sanhe cattle Chinese Holstein and Simmental (Wang *et al*., 2007) while DRB|*Pst*I in PE and Saanen were slightly lower. Chi square analysis showed that DRB|*Pst*I in PE and DRB|*Taq*I in PESA were out of Hardy-Weinberg equilibrium since the critical value (X^2, 0.05, 1) was 3.84 while all other restriction sites were still within H-W equilibrium. The results for DRB|*Taq*I and DRB|*Pst*I loci (Table 3) displayed three genotypes in all three breeds while Egyptian goat did not display a homozygous dominant genotype for DRB|*Pst*I locus (Ahmed & Othman, 2006).

Since these genes are involved in immune systems, this calls for a broader research that will associate with milk quality parameters with these mutations and hence find how animals with less mastitis susceptibility can be bred. This research found TLR4|*Alu*I locus less informative to impede infection against mastitis or bacterial infections, hence other restriction techniques should be tried because TLR4 is being associated with reproduction and production parameters (Kannaki *et al.,* 2011), and recognition of PAMP (Jilling *et al.*, 2011; Hoshino *et al.*, 2010).

Alleles in DRB region are being associated with either susceptibility or resistance to mastitis (Swiderek *et al.*, 2005; Yoshida *et al.*, 2009). This then justifies why

Table 4. Heterozygosity, H-W and PIC values of dairy goat breeds

Locus	Breed	Hetero-zygosity (He)	H-W* X2 0.05, 1	PIC
TLR4 ǀ AluI	Saanen	0.0000	-	0.0000
	PE	0.0000	-	0.0000
	PESA	0.0000	-	0.0000
DRB ǀ PstI	Saanen	0.4032	0.0015	0.3220
	PE	0.4558	1.2787	0.3520
	PESA	0.4753	7.2892	0.3620
DRB ǀ TaqI	Saanen	0.4985	0.8697	0.3740
	PE	0.1576	0.2581	0.1450
	PESA	0.2604	12.9997	0.2260

Note: *Df= 1; critical value 3.84; PE= Etawah Grade; PESA= Etawah Grade-Saanen Crossbred.

a research that will associate DRB alleles with resistance or susceptibility is a necessity.

Additional research explored TLR4 using other restriction techniques is recommended, therefore the reported association with PAMPs recognition is exploited. Another study associated DRB alleles with mastitis and SCC is also needed.

CONCLUSION

TLR4 is monomorphic in all three breeds while DRB is found to be polymorphic in all the three breeds for both DRB ǀ *Taq*I and DRB ǀ *Pst*I loci rendering it exploitable for future selection purposes. PIC showed that (except for DRB ǀ *Taq*I in PESA and PE) moderately high implying that they are more informative markers.

REFERENCES

Ahmad, S. & Othman. 2006. A PCR-RFLPmethod for the analysis of Egyptian goat MHC class IIDRB gene. J. Biotechnol 5: 58-61.

Baghizadeh, A., M. Bahaaddini, M. R. Mohamadabadi, & N. Askari. 2009. Allelic variations in exon 2 of Caprine MHC Class II DRB3 Gene in Raeini Cashmere goat. Am-Eurasian J Agric. Environ. Sci. 6: 454-459.

Botstein, D., R. L. White, M. Skolnick, & R. W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics 32: 314-331.

Jilling, T., D. Simon, J. Lu, F. J. Meng, D. Li, R. Schy, R. B. Thompson, A. Soliman, M. Aditi, & M. Caplan. 2006. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. J. Immunol. 177: 3273- 3282.

Jungi, T. W., K. Farhat, I. A. Burgener, & D. Werling. 2010. Toll-like receptors in domestic animals. J Cell Tissue Res. DOI 10.1007/s00441-010-1047-8.

Hoshino, K., T. Osamu, K. Taro, S. Hideki, O. Tomohiko, T. Yoshifumi, T. Kiyoshi, & A. Shizuo. 2010. Cutting edge: toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. J. Immunol. 99:0022-1767.

Kannaki, T. R., M. Shanmuam, & P. C. Verma. 2011. Toll-like receptors and their role in animal reproduction . J. Animal. Reprod. 125: 1-12.

Nei, M. & S. Kumar. 2000. Molecular Evolutionary Genetics. Columbia University Press, New York.

Swiderek, W. P., K. M. Charon, A. Winnicka, & J. Gruszczynska. 2005. Relationship between blood lymphocyte phenotype, drb1 (MHC CLASS II) gene polymorphism and somatic cell count in ewe milk. J. Vet. 50: 73-77.

Takeda, K. & S. Akira. 2003. TLR signaling pathways. J Smim.16:3-9. http://dx.doi.org/10.1016/j.smim.2003.10.003

Tirumurugaan, K. G., G. D. Raj, A. Raja, K. Kumanan, & V. Ramaswamy. 2010. Differential expression of toll-like receptor mRNA in selected tissues of goat (*Capra hircus*). J. Vet. Immunol. Immunopathol. 133: 296-301.

Wang, X., X. Shangzhong, G. Xue, R. Hongyan, & C. Jinbao. 2007. Genetic polymorphism of *TLR4* Gene and correlation with mastitis in cattle. J. Genet. Gen. 34: 406-412.

Yoshida, T., H. Mukoyama, H. Furuta, Y. Kondo, S. N. Takeshima, Y. Aida, M. Kosugiyama, & H. Tomogane. 2009. Association of BoLA-DRB3 alleles identified by a sequence-based.typing method with mastitis pathogens in Japanese Holstein cows. J Anim. Sci. 80:498-509. http://dx.doi.org/10.1111/j.1740-0929.2009.00663.x

Zhao, Y., H. Xu, S. Zhang, & J. Zhang. 2011. Polymorphisms in exon 2 of MHC class II DRB3 gene of 10 domestic goats in Southwest China**.** Asian-Aust. J. Anim. Sci 24: 752-756.