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

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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 PstI and TaqI in two separate reactions. The results showed that TLR4|AluI was monomorphic and fixed with allele T in all three breeds while DRB|PstI and DRB|TaqI loci were found polymorphic for all breeds. Heterozygosity expected (He) and PIC were found low at both DRB|TaqI and DRB|PstI loci in PE and Saanen. X2 results showed that DRB|PstI in PE and DRB|TaqI in PESA were not in H-W equilibrium and did not display homoygous recessive genotype. The results declared that TLR4|AluI was not a good marker for diseases resistance whereas DRB|TaqI and DRB|PstI memberikan harapan untuk dapat digunakan sebagai marker resistensi berdasarkan nilai PIC yang didapatkan.

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

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unable 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 interleukin-1 (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.

**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 MgCl₂ 1.5 µl 10x buffer, 10.85 µl dH₂O 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 MgCl₂ 1.5 µl 10x buffer, 10 15 µl dH₂O for PE DNA samples.

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

**RFLP and Agarose Gel Electrophoresis**

TLR4 PCR product was digested with AluI for 16 h at 37 °C. PCR product (4 µl) was digested with AluI (2 µl) restriction endonuclease (RE) cocktail that was

| Locus   | Annealing temperature (°C) | PCR product | Primer sequence       |
|---------|----------------------------|-------------|-----------------------|
| TLR4    | 57                        | 382 bp      | F:5’-AGACAGCATTTCACTCCCTC  |
|         |                            |             | R:3’-ACCACCGACACACTGATGAT |
| CaLA-DRB| 60 and 58                  | 284 bp      | DRB1F TACCCCTCTGCGACAGCATTTCT |
|         |                            |             | DRB1R TCGCCGCTGCACACTGAAACTCTC |

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**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            |

**Table 2. Amplification conditions and primers to be used**
composed of dH2O (1 µl), buffer (0.7 µl) and AluI RE (0.3 µl).

DRB PCR product was digested with TaqI and PstI in two separate reactions. DRB amplicon (4 µl) was digested with PstI RE (2 µl) mix that consisted of dH2O (1µl), buffer (0.7 µl) and PstI restriction endonuclease (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 TaqI 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 (PopGene52) version 1.0380.20651 was used to determine fitness to Hardy-Weinberg (H-W) equilibrium. The formulae for allele frequency (Nei & Kumar, 2000):

\[ x_i = x_{ii} + \frac{1}{2} \sum_{j=1}^{m} x_{ij} \]

where: X1 is the allele frequency for the ith allele; \( \sum \) summation of xij over all j’s except for j= i, j≠i.

Expected heterozygosity

\[ h = 1 - \sum_{i=1}^{m} x_i^2 \]

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

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Figure 1. The 382 bp TLR4 PCR amplification product

Figure 2. The 285 bp DRB PCR amplification product

Sequence 1. TLR4 amplicon sequence; Genbank HM627213.2; AluI 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 |
|-----------|------------|------------------|------------------|--------------------|
|           |            |                  | A₀ | A₁ | A₀A₀ | A₀A₁ | A₁A₁ |
| TLR4/AluI | 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/PstI  | 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/TaqI  | 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.

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

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

guous recessive pattern in PESA while Egyptian goat displayed three genotypes. DRB/PstI 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/TaqI in PE and PESA was low (PIC<0.25), while the same loci in PESA, PE and DRB/PstI loci was moderately informative (0.25< PIC<0.5) in all goat breeds. PIC for DRB/PstI in Saanen and DRB/PstI in PESA have almost the same values as in Sanhe cattle Chinese Holstein and Simmental (Wang et al., 2007) while DRB/PstI in PE and Saanen were slightly lower. Chi square analysis showed that DRB/PstI in PE and DRB/TaqI 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/TaqI and DRB/PstI loci (Table 3) displayed three genotypes in all three breeds while Egyptian goat did not display a homozygous dominant genotype for DRB/PstI 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/AluI 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...
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|PstI and DRB|TaqI loci rendering it exploitable for future selection purposes. PIC showed that (except for DRB|TaqI in PESA and PE) moderately high implying that they are more informative markers.

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