Molecular Characterization and Gene Expression of TLR4 Gene Associated with Mastitis in Goats

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ABSTRAK

Soquila SS, de Guia ACM, Medina NP, Mingala CN. 2022. Karakterisasi molekuler dan ekspresi gen TLR4 yang dihubungkan dengan penyakit mastitis pada kambing. JITV 27(4):159-169. DOI:http://dx.doi.org/1014334/jitv.v27i4364.

Pada penelitian ini dilakukan karakterisasi gen *Toll-like receptor* 4 (TLR4) pada kambing, pendeteksian polimorpis dalam nukelotida, penentuan hubungan identifikasi genotip dengan kemunculan penyakit mastitis subklinis menggunakan Chi-square dan rasio odds, serta penganalisaan ekspresi gen menggunakan Student's T-test dua sisi. Hasil kajian pertama menunjukan kemiripan yang tinggi (99%) pada sekuen nukleotida TLR4 semua rumpun kambing dengan sekuen *C. hircus* (NM_001285574.1) dan domba (*Ovis aries)*, tetapi memiliki kemiripan yang sedikit lebih rendah dengan sapi *(Bos taurus* dan *Bos indicus*) (96%) serta kerbau air (*Bubalus bubalis*) (95%). Pada kajian kedua, analisis polimorfisme panjang berkas restriksi (RFLP) menunjukan tiga genotip dengan sembilan pola restriksi menggunakan enzim AluI. Gen AA memiliki rasio odds secara berurutan 0,28 dan 0,08 pada semua rumpun dan Anglo-Nubian dengan hubungan yang signifikan (P<0,05). Hal itu mengartikan bahwa sebanyak 0,28 dan 0,08 kemungkinan lebih terjadinya mastitis subkilinis pada semua rumpun dan Anglo-Nubian dibandingkan dengan genotip lainnya. Genotip AB menunjukkan rasio odds secara berurutan 3,83; 13,00 dan 2,40 pada seluruh rumpun, Anglo-Nubian dan yang dimutakhirkan dengan hubungan yang signifikan. Hal ini mengindikasikan peluang paparan mastitis subklinis pada seluruh rumpun, Anglo-Nubian dan yang dimutakhirkan lebih tinggi secara berurutan sebesar 3,83; 13,00 dan 2,40 dibandingkan genotip lainnya. Pada kajian ketiga, ekspresi genetik menunjukkan upregulasi maksimum gen TLR4 adalah 3,63 kali lipat pada kambing tanpa mastitis subkilinis dibandingkan dengan hewan dengan mastitis subkilinis yang hanya 0,65 kali lipat. Hal ini menunjukkan peningkatan fungsi gen TKR4 dalam melindungi hewan dari kemungkinan infeksi.

Kata Kunci: Ekspersi Gen, Genotip, Kambing, Mastitis, PCR-RFLP, Gen TLR4

ABSTRACT

Soquila SS, de Guia ACM, Medina NP, Mingala CN. 2022. Molecular characterization and gene expression of TLR4 gene associated with mastitis in goats. JITV 27(4): 159-169. DOI:http://dx.doi.org/1014334/jitv.v27i4364.

In this study characterization of Toll-like receptor 4 (TLR4) gene of goats; detection of polymorphisms in the nucleotides, and determination of the association of identified genotypes with the occurrence of subclinical mastitis was done using chi-square and odds ratio. Analyzing gene expression using two-sided Student's T-test was also done. Results of Study 1 revealed high similarity (99%) of TLR4 nucleotide sequence of all breeds of goats with that of *C. hircus* (NM_001285574.1) and sheep (*Ovis aries*) sequences and slightly lower similarity with cattle *(Bos taurus,* and *Bos indicus*) (96%), and water buffalo (*Bubalus bubalis*) (95%). In Study 2, restriction fragment length polymorphism (RFLP) analysis revealed three genotypes with nine restriction patterns using AluI enzyme. Genotype AA has odds ratio of 0.28 and 0.08 in all breeds, and in Anglo-Nubian, respectively, with significant association (P<0.05) that inferred 0.28 and 0.08 times greater probability in all breeds, and in Anglo-Nubian, respectively, for subclinical mastitis to occur than those of other genotypes. Genotype AB showed odds ratio of 3.83, 13.00 and 2.40 in all breeds, in Anglo-Nubian, and in Upgraded, respectively, with significant association (P<0.05) that indicated 3.83, 13.00 and 2.40 times more likely in all breeds, in Anglo-Nubian, and in Upgraded, respectively, to suffer subclinical mastitis than those of other genotypes. In Study 3, genetic expression analysis showed a significant upregulation of TLR4 gene up to maximum of 3.63-fold in goats without subclinical mastitis compared to subclinically mastitic animals with only 0.65-fold which suggest a prompt role of TLR4 gene in the protection of animal against possible infection.

Key Words: Gene Expression, Genotype, Goats, Mastitis, PCR-RFLP, TLR4 Gene

INTRODUCTION

Breeding and production of goats (*Capra hircus*) in the Philippines suit most biophysical and socio-economic farming conditions of the agricultural sector. Goats have short gestation, short kidding intervals and lower capital requirements compared to cattle and carabaos. Farmers benefit from the incomes of and/or

consumption of goat's meat and milk (Monteiro et al. 2018). Among infectious diseases in dairy ruminants, mastitis is of major importance because of its high frequency and related costs (Rupp et al. 2014). Subclinical mastitis often goes unnoticed; hence it is left unattended unless it advances to clinical stage. Mastitic goats may often refuse to nourish their offspring because of udder pain. Such animals' milk has changed in chemical composition and physical features, making it unsuitable for processing due to its short shelf life and offflavors (Petlane et al. 2012). Intramammary infection (IMI) can be accurately predicted by somatic cell count (SCC) (Petzer et al. 2017). California Mastitis Test (CMT) is common indirect method of measuring SCC (Duarte et al. 2015) that provides practical means to identify inflammatory infections (Seligsohn et al. 2021) in the field.

Genes associated with immune response have been investigated for presence of single nucleotide polymorphism (SNP) and associations with mastitis related traits. Genes studied in bovine is Toll-like receptor 4 (TLR4). TLR4 gene plays key-role in innate immune system by recognizing components on the surface of various microbes that bind to specific pathogen-associated molecular patterns (PAMPs) that initiates signaling events leading to inflammatory responses and release of antimicrobial agents (Petlane et al. 2012).

Scant literature is available on molecular characterization and gene expression of TLR4 in goats. This study aimed to characterize and detect polymorphisms in TLR4 gene, determine the genotypes associated with subclinical mastitis and assess the expression of TLR4 gene in goats.

MATERIALS AND METHODS

Characterization of TLR4 gene in goats

A total of 99 available goats (17 Native, 33 Upgraded, 29 Anglo-Nubian, and 20 Saanen) were included in the study. All goats were derived from goat farms in Luzon. Goat milk and blood samples were collected. For lactating goats, ribonucleic acid **(**RNA) samples were extracted from milk of animals starting on their 2nd week of lactation and above. This prevented collecting high somatic cell count that normally occurred on the first week of lactation. For non-lactating Native and Upgraded crossbred goats, RNA was extracted from blood. Results showed that genotypes found using deoxyribonucleic acid (DNA) from blood matched those obtained from milk.

RNA extraction from milk and blood samples was done by following the Promega® protocol with some modifications; while RT-PCR kit (TaKaRa ™) was used to synthesize complementary DNA (cDNA) from the extracted RNA samples. Primers 1. Forward: 5'-AGACAGAGGGTCATGCTTT-3' Reverse: 5'-CTGTAAACTTGATAGCCCAGA-3' (440bp), 2. Forward: 5'-ATGCGAAATTAAGATTATTGAAG-3' Reverse: 5'-TACTGAAGGCTTGGTAGCTC-3' (870bp), 3. Forward: 5'-CTCACGGAACGATACAGACT-3' Reverse: 5'-ATGTTCACAAACACAAGCAA-3' (874bp), 4. Forward: 5'-GCAGTTTCAACCGTATCAC-3' Reverse: 5'-GGATTCTCCTCCTCAGGT-3' (887bp) were designed using Primer3 server (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) based on the caprine sequence of TLR4 (NM_001285574.1) mRNA from National Center for Biotechnology Information (NCBI) GenBank. Designed primers were analyzed for self-annealing and loop properties using Oligo analyzer software (https://sg.idtdna.com/calc/analyzer). Primer-BLAST server (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was also used to ensure that each set of primer amplifies specific gene segment that are targeted.

Gene amplification

PCR was performed using a thermocycler (SimpliAmp, Thermofisher). The 20µL reaction volume used for all PCR tests contained 2µL of genomic DNA template, 10 pmol of each primer, and PCR master mix. Amplification cycles were carried out in thermocycler optimized for this study: initial denaturation 94ºC for 10 min, denaturation 94ºC for 1 min, annealing for 1 min: Primer 1, 55ºC (35 cycles); Primer 2, 56ºC (37 cycles); Primers 3 and 4, 57ºC (38 cycles), extension 72ºC for 1 min and final extension 72ºC for 10 min.

After amplification, 1µL of PCR product was electrophoresed in 2 % agarose gel containing 1X TAE buffer at 100 volts for 30 minutes (MYGEL™ mini, Accuris Instruments) and visualized under ultraviolet light using UV transillumination advance imaging system (FlourChem E by ProteinSimple ™). To ensure that amplification products are of the expected size, a 1kb plus DNA ladder was run simultaneously as marker.

Gene sequencing and analysis

TLR4 PCR products were submitted for sequencing. Sequences were assembled using MEGA 7.1 software (Kumar et al. 2016). Each primer's forward and reverse sequences were put together to create contigs. Gene sequences were compared with the TLR4 coding DNA sequence (CDS) of *Capra hircus* (NM_001285574.1), *Ovis aries* (NM_001135930.1), *Bos taurus* (NM_174198.6), *Bos indicus* (KX138607.1), and *Bubalus bubalis* (NM_001290903.1) from the database found in NCBI GenBank.

The DNA nucleotides were conceptually translated using Molecular Evolutionary Genetic Analysis (MEGA) 7.1 software and again compared with database found in NCBI Genbank for caprine TLR4 to detect amino acid changes. Contiguous TLR4 nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) to determine sequence similarity with corresponding regions of other ruminant species. Phylogenetic tree was constructed using the Maximum Likelihood method. Confidence in the groups was estimated by bootstrap of data using 1000 replications. Genetic distances and phylogenetic trees were derived using MEGA 7.1 software. Functional domains of the gene were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services /SignalP) (Petersen et al. 2011) and Simple Modular ArDZchitecture Research Tool (SMART) server (http://smart.embl-heidelberg.de/) (Letunic & Bork, 2018).

Polymorphism in TLR4 gene in goats

Polymorphism analysis

Amplified TLR4 PCR products (15µL) from Study 1 were subjected to endonuclease digestion. New set of primers were designed to amplify ligand-binding region in exon 3 of TLR4; forward primer: 5'-GTATTCAAGGTCTGGCTGGTT-3' and reverse primer: 5'-GTCATTGAAGCTCAGATCTAAAT-3'.

Restriction enzymes that can cut the fragments were identified using Sequence Manipulation Suite (SMS): Restriction Map, and Restriction Digest (http://www.bioinformatics.org) (Stothard 2000). AluI enzyme (AG/CT) was selected to cut segments of 494 bp ligand-binding region of TLR4 at loci 312, 378, 431and 485. Restriction-digested gene fragments were visualized on UV transilluminator (FlourChem E by ProteinSimple ™) and photographed. Differences in fragment length yielded by the various restriction enzymes that would indicate polymorphism in a particular gene were analyzed and compared.

Association of polymorphism of* TLR4 *gene in goats and the occurrence of subclinical mastitis

A total of seventy-six (76) available lactating does were identified from farms mentioned in Study 1. There were 67 samples used for the association of occurrence of subclinical mastitis. Milk was collected manually for CMT scoring and for SCC evaluation using PortaSCC®. CMT scores for all animal subjects were classified as Non-subclinically mastitic if CMT score result was 1 or lower and subclinically mastitic if CMT score result was 2 or higher. Raw milk samples from the animal with CMT scores of 1 or higher were subjected for somatic cell count determination using the Porta SCC® goat milk test. Results were categorized as non-subclinically mastitic if SCC<1,500,000 cells/mL and subclinically mastitic if SCC ≥1,500,000 cells/mL.

PCR and RFLP

RNA was extracted from milk samples and converted to cDNA and determination of polymorphism of TLR4 gene using RFLP was done. Since TLR4 coding region has 2664 bp, certain regions with high polymorphism were identified for RFLP analysis. Similar to Study 2, sequence covering 494 bp by the new TLR4 Primer was amplified and subjected for RFLP. Genotype frequency of fragments/alleles was identified through direct counting.

Statistical analysis

Univariate analysis on possible association between genotypic frequency and occurrence of subclinical mastitis was examined using Chi-square (X2) test for goodness-of-fit (Petrie & Watson, 2006). Odds ratio were also computed in Microsoft Excel 2013 to determine the strength of association.

Determination of Genetic Expression of TLR4 Gene in Goats with and without subclinical mastitis

Quantitative gene expression analysis

Real time PCR for TLR4 gene was performed in an ABI 7500 sequence detection system with SYBR green PCR master mix (Applied Biosystems, CA). The 10µL reaction mixture for RT-PCR consisted of 1µL cDNA, 0.15µL each of the forward (upstream) primer: 5'-AATGCCCCTACTCAACCT-3' and reverse (downstream) primer: 5'-CTTCGCAGAGTCAATCCA-3' (10nmol/L), 5µL SYBR Green Realtime PCR master mix (2x) and 3.7µL of ddH2O using glyceraldehyde phosphate dehydrogenase (GAPDH) as an endogenous control. The cDNA template (10µL) was used for each gene quantification after sequential dilution in 10 folds. Real-time PCR was run using the diluted samples as gradient and template.

The RT-PCR amplification was performed at 95ºC for 5 mins initial denaturation and 40 cycles denaturation at 95ºC for 15 secs, followed by annealing for 45 secs with 57ºC for TLR4 with reaction efficiency of 90-100, quantification cycle (Cq) value of 30.86 and correlation coefficient (R2) value of 0.998 while GAPDH has annealing temperature of 65ºC with reaction efficiency of 90-100, Cq value of 29.36 and R2

value of 0.996. Melt curve stage of 95ºC for 15 secs followed by 65ºC for 30 secs and extension at 95ºC for 15 secs.

Statistical analysis

Significant differences in TLR4 gene expression between animals with and without subclinical mastitis were compared using a two-sided Student's T-test, Comparative C_T values methods (2 ddCt) were computed based on their mean Ct values with reference to those in non-mastitic counterparts.

RESULTS AND DISCUSSION

Characterization of TLR4 gene in goats

Sequence analysis of TLR4

There were 18 samples that produced good sequences for goat TLR4 gene; five contigs completed in Native, nine in Upgraded crossbreds, two in Anglo-Nubian, and two in Saanen. Native goat TLR4 nucleotide sequence resulted to average of 2603 bp, while there were 2597 bp in Upgraded, 2589 bp in Anglo-Nubian and 2645 bp in Saanen.

Goat TLR4 nucleotide sequences were aligned with other ruminant's TLR4 CDS using database from the NCBI GenBank. Statistical nucleotide pair frequency of the 23 aligned nucleotide sequences showed average of 2452 identical pairs, 61 transitional pairs and 61 transversional pairs, with ratio of 1.0. Between different goat breeds' nucleotide pair frequencies, average identical pairs were 2468 with 48 transitional pairs and 61 transversional pairs, with ratio of 0.8. This finding showed that there was high similarity of nucleotide sequences between breeds of goats studied.

Nucleotide BLAST (BLASTn) of TLR4 CDS of the representative goat sample breeds revealed high degree of similarity between the query (sample) sequence and other ruminants' TLR4 nucleotide sequences available in the NCBI GenBank database (Table 1). Native, Upgraded, Anglo-Nubian and Saanen TLR4 nucleotide sequences had 99% similarity with that of *C. hircus* (NM_001285574.1) and *O. aries* (NM_001135930.1). Lower similarity was seen on *B. taurus* (NM_174198.6), *B. indicus* (KX138607.1) and *B. bubalis* (NM_001290903.1) with only 96%, 96%, and 95%, respectively.

Comparatively, protein BLAST (BLASTp) results showed high percentage similarity (99%) of translated amino acid sequence of Native and Upgraded goats to *C. hircus* (NP_001272503.1) sequence from the NCBI GenBank. Lower similarity was observed (98%) on Anglo-Nubian, while Saanen had the lowest similarity with *C. hircus* (NP_001272503.1) (93%). Lower similarity of Native and Upgraded amino acid sequences were also observed on other ruminant species: *O. aries* (NP_001129402.1), *B. taurus* (NP_776623.5), *B. indicus* (ADL28421.1), and *B. bubalis* (NP_001277832.1), with 98%, 94%, 94% and 93% similarity, respectively. Anglo-Nubian had 98%, 96%, 96% and 96% similarity with other ruminant species *O. aries* (NP_001129402.1), *B. taurus* (NP_776623.5), *B. indicus* (ADL28421.1), and *B. bubalis* (NP_001277832.1) respectively. Again, Saanen breed had the lowest similarity of amino acid sequence compared to *O. aries* (NP_001129402.1), *B. taurus* (NP_776623.5), *B. indicus* (ADL28421.1), and *B. bubalis* (NP_001277832.1) with 92%, 89%, 89% and 87% similarity, respectively.

Result showed that even if nucleotide and amino acid sequences were from various species of ruminants and from different locations, there was still high similarity (99%) of the nucleotide and amino acid sequences of TLR4 gene especially in goats. This is in consonance with the study of Goyal et al. (2012), wherein goat TLRs have 61 to 99% similarity with other mammals. TLR sequences are highly conserved and have common evolutionary ancestor. Furthermore, functional domains are conserved due to constraints imposed by necessity to recognize PAMPs that are present in parasitic, bacterial or viral germs. This recognition process is translated rapidly into a meaningful defense reaction (Karaś et al. 2019).

Phylogenetic analysis of TLR4 nucleotide sequence

Maximum Likelihood algorithm with 1000 bootstrap resampling revealed clustering of *C. hircus* (NM_001285574.1) with TLR4 nucleotide sequence of Upgraded (NxA)_98, Native_4 and Native_2 and with 36 bootstrap value (Figure 1). Native_1, Upgraded (NxU)_11, Upgraded (NxA)_97, Native_3, Upgraded (NxU)_15 and Native_5 clade together with 42 bootstrap value. TLR4 nucleotide sequence of Upgraded(NxS)_102 clade with Upgraded (NxS)_101 with 33 bootstrap value and then clade with Upgraded(NxU)_12, Saanen_49, Upgraded(NxA)_99, Saanen_39, Anglo-Nubian_40, Upgraded(NxA)_100 and Anglo-Nubian_41 clade together with 32 bootstrap value. *O. aries* (NM_001135930.1), *B. bubalis* (NM_001290903.1), *B. indicus* (KX138607.1), *B. taurus* (NM_174198.6) TLR4 sequence on the other hand, separated from the clade of goats with 39 bootstrap value.

Predicted functional domains of TLR4 nucleotide sequence

Native_1 goat TLR4 sequence was used as representative animal of this study; the 2603 bp

Table 1. Nucleotide percentage similarity of TLR4 gene in ruminant species with reference to native, upgraded, Anglo-Nubian and Saanen goats

Species	Nucleotide Sequence			
	Native	Upgraded	Anglo-Nubian	Saanen
Capra hircus (NM_001285574.1)	99%	99%	99%	99%
Ovis aries (NM_001135930.1)	99%	99%	99%	99%
Bos Taurus (NM_174198.6)	96%	96%	96%	96%
Bos indicus (KX138607.1)	96%	96%	96%	96%
Bubalus bubalis (NM_001290903.1)	95%	95%	95%	95%

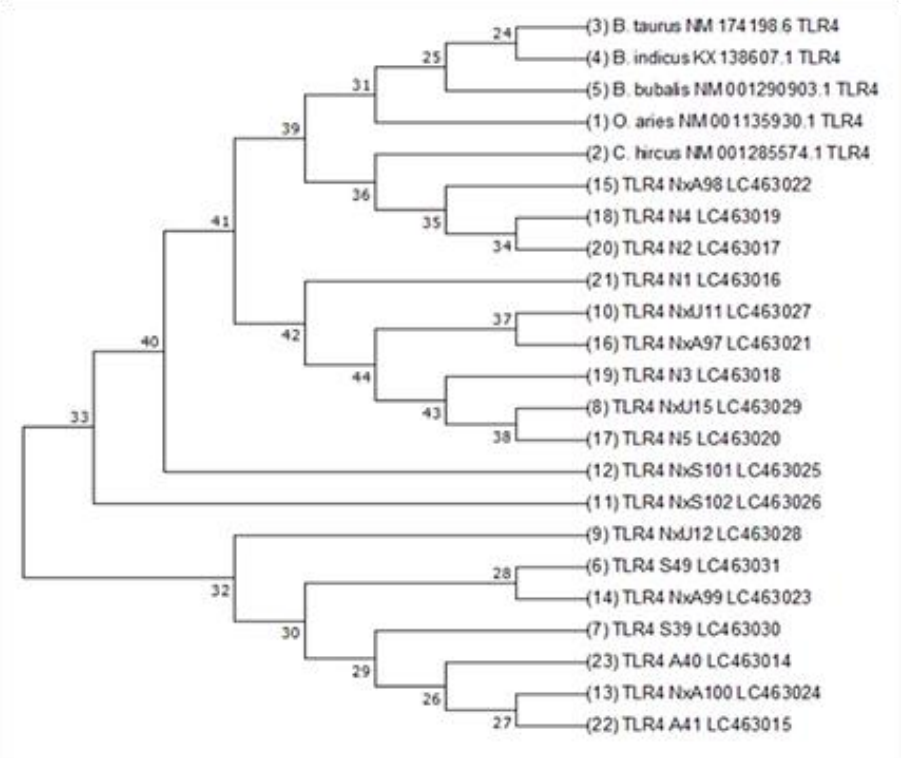

Figure 1. Phylogenetic tree showing relationship between goats and other ruminants' TLR4 nucleotide sequence

nucleotide sequence has corresponding 867 amino acid translation provided by MEGA 7.1 software. Using SignalP 4.1 server, cleavage site was found to be between positions 25 and 26 amino acid location starting from amino acid methionine (http://www.cbs.dtu.dk/services/SignalP). Then, using SMART server, the functional domains were located. Extracellular domains of leucine-rich repeats (LRR) were located starting from amino acid location 95 to 584, followed by single LRRCT (LRR at C-terminal) at positions 597 to 647. Transmembrane protein started at position 653 and ended at 675, followed by globular cytoplasmic domain called the Toll/interleukin 1 receptor (TIR domain) at positions 692 to 837 (http://smart.embl-heidelberg.de). There were typically 13 LRRs present, single globular LRRCT, followed by transmembrane protein and TIR domain. These domains were homologous between breeds of goats, as well as sheep, cattle and water buffalo.

Characterization of TLR4 sequence had elucidated critical regions that were involved in function of the gene. TLR4's function was to bind with specific molecular patterns from pathogens, after which triggered a cascade of events that led to production of

cytokines that initiate further immune response (Mukherjee et al. 2016). This study showed structural domains predicted from translated amino acid. One of these domains was LRR, which contained 20-30 amino acid residues. LRRs are important structural framework for the formation of protein-protein interactions. Proteins containing LRRs included tyrosine-protein kinase receptors, cell adhesion molecules, and extracellular matrix-binding glycoproteins that were involved in variety of biological processes, including transduction, cell adhesion, disease resistance, apoptosis and immune response (Poddar & Banerjee 2020). Result showed 13 LRRs in goat which were similar to that of *O. aries* (NP_001129402.1), while in *B. taurus* (NP_776623.5), *B. indicus* (ADL28421.1), and *B. bubalis* (NP_001277832.1) there is the presence of LRR_TYP (LRR-typical) motif. Dubey et al. (2012) reported that possible variation of LRR motif distribution can occur in various species associated to different diseases with around 21-22 LRRs in buffalo, cattle, sheep and goat.

Polymorphism of TLR4 gene in goats (Native, Upgraded, Anglo-Nubian, and Saanen)

Polymorphism analysis for* TLR4 *gene

From the 2664 bp nucleotide sequence, targeted ligand-binding domain has PCR product of 494 bp that was subjected to restriction enzyme digestion. One heterozygous allele was seen at locus 377 that is part of the ligand-binding region in 494 bp segment. Allele in locus 377 is same as locus 1221, if the start of counting of the nucleotide letters began in exon 1 of the whole CDS of TLR4 mRNA. This polymorphism can either be G or C nucleotide, which can result to either glutamic acid or aspartic acid substitution.

Restriction enzyme AluI that can cut AG/CT nucleotide sequence was selected to examine restriction fragment pattern in the nucleotide sequence of TLR4 gene. Based on SMS: Restriction Digest (http://www.bioinformatics.org) application of the 18 TLR4 complete nucleotide sequence in Study 1, AluI can produce three patterns at nucleotide sequence of 494 bp amplicon. Restriction fragment pattern a, three cuts produced products of 311 bp (1-311), 66 bp (312-377), 107 bp (378-484), and 10 bp (485-494) amplicon sizes; restriction pattern b, four cuts produced products of 311 bp, 66 bp, 53 bp (378-430), 54 bp (431-484) and 10 bp amplicon sizes; and restriction pattern c, three cuts produced products of 311, 119 (312-430), 54 and 10 amplicon sizes. This digestion pattern recognized polymorphism at loci 311, 377, 430, 484 and differentiated animals with allele A or B which can detect amino acid change in locus 377 in the 494 bp amplicon. Thus, restriction digest result fragments with 107 bp will indicate allele B, while absence of cut fragment will indicate allele A.

Figure 2 shows result of digestion of TLR4 gene PCR products using AluI enzyme. From the conceptualized three restriction patterns from nucleotide sequence, nine patterns were observed. Uncut 494 bp, 377 bp (merged of 311 and 66 bp) and 173 bp (merged of 107 and 66 bp) were obtained in addition to conceptualized fragments.

Polymorphism results of this study in ligand-binding region is in consonance with the study of (Singh et al. 2015) using AluI enzyme in PCR-RFLP procedure where polymorphisms in TLR4 gene had been found in Beetal breed but monomorphism in Jamunapari and Black Bengal goats. Furthermore, polymorphism in same domain was also observed by Zhou et al. (2008), who identified five allelic variations in the ligand binding region using PCR-SSCP.

Association of TLR4 Gene in the Occurrence of Subclinical Mastitis in Goats

Detection of subclinical mastitis

A total of 108 milk samples were categorized as non-subclinically mastitic or subclinically mastitic based on CMT scores and PortaSCC® kit results. Non-subclinical mastitis was considered, if CMT score result was 1 or lower, while subclinical mastitis was accounted, if CMT score result was 2 or higher. While in PortaSCC®, results were categorized as non-subclinically mastitic, if SCC<1,500,000 cells/mL and subclinically mastitic, if SCC≥1,500,000 cells/mL.

Association of* TLR4 *gene in occurrence of subclinical mastitis

The 494 bp putative ligand-binding domain described in Study 2 was targeted in this segment of the study. Table 2 shows frequency of TLR4 genotypes in non-subclinically mastitic and subclinically mastitic goats. From the total of 67 milk samples tested using AluI enzyme, 30 was considered subclinically mastitic, while 37 was non-subclinically mastitic. Overall frequency of genotype AA was 17, while genotype AB and BB were 35 and 15, respectively. Frequency of genotype AA in mastitic cases was 4 and genotypes AB and BB were 21 and 5, respectively; while frequency of genotype AA in non-mastitic cases was 13 and genotypes AB and BB were 14, and 10, respectively.

Genotype AA was found to be more frequent in non-subclinically mastitic compared to subclinically mastitic animals. Frequency for occurrence of subclinical mastitis in genotype AA was 4 compared to 13 for non-occurrence of subclinical mastitis. Odds

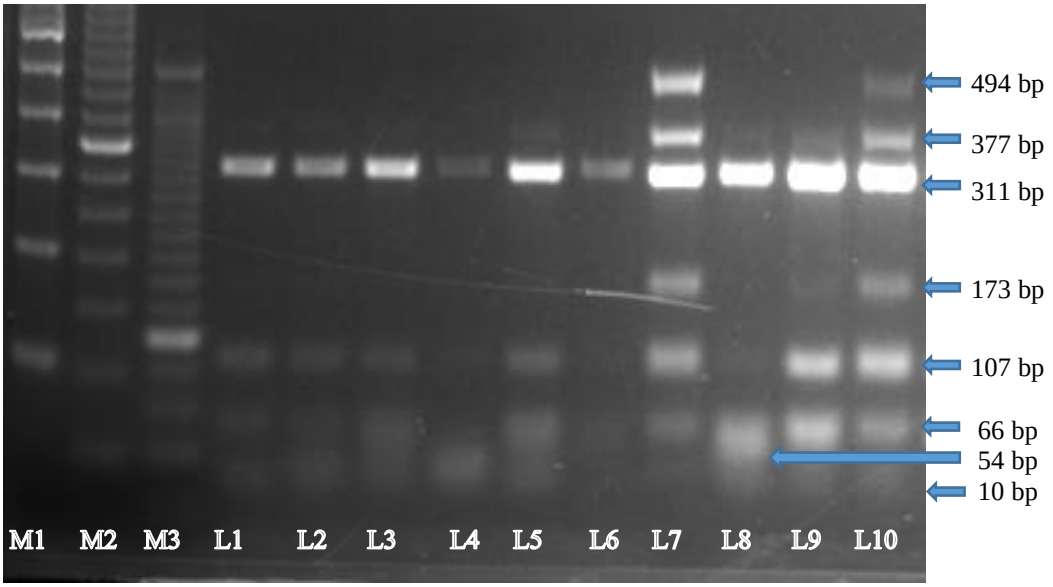

Figure 2. Restriction patterns obtained by digestion of TLR4 gene PCR products using AluI in 2% agarose gel. M1-100bp, M2-50bp, M3-25bp ladders; Lanes 1, 2, 3, 5, 9 –pattern a (fragment size: 311, 107, 66), Lanes 4, 6, 8–pattern b (fragment size: 311, 10 not distinguishable), Lanes 7, 10 – uncut and merged cutting pattern of 494 bp amplicon (fragment size 494, 377 (311+66), 311, 173 (107+66), 107, 66, 10)

Table 2. Frequency of TLR4 AluI-based genotypes in goats with or without SCM

Genotype	Category of Animal		Total	Odds Ratio	95% CI
	Non-SCM	SCM			
AA	13	4	17	0.28*	0.81 to 0.99
AB	14	21	35	3.83*	1.37 to 10.69
BB	10	5	15	0.54	0.16 to 1.80

*Significant association was found, P<0.05

Table 3. Frequency of TLR4 AluI-based genotypes in Anglo-Nubian goats with or without SCM

Genotype	Category of Animal		Total	Odds Ratio	95% CI
	Non-SCM	SCM			
AA	6	2	8	0.08*	0.01 to 0.58
AB	3	13	16	13.0*	1.70 to 99.38

*Significant association has been found P<0.05

Table 4. Frequency of TLR4 AluI-based genotypes in Upgraded goats with or without SCM

Genotype	Category of Animal		Total	Odds Ratio	95% CI
	Non-SCM	SCM			
AA	5	1	6	0.32	0.03 to 3.60
AB	1	4	5	2.40*	1.69 to 341.01
BB	7	1	8	0.17	0.02 to 1.91

*Significant association has been found P<0.05

ratio result in goats with genotype AA was 0.28 with 95% confidence interval (CI) range of 0.81 (lower limit) to 0.99 (upper limit). Significant association was found. This means there was 0.28 times more likely that subclinical mastitis would occur, if the goat had genotype AA than those of other genotypes.

Frequency of subclinically mastitic animals in genotype AB was 21 compared to 14 in non-subclinically mastitic. Odds ratio analysis shows significant association for genotype AB, which was 3.83, thus there were 3.83 times more likely that subclinical mastitis would occur, if the animal had genotype AB than those of other genotypes. Genotype BB had frequency of 5 in subclinically mastitic compared to 10 in non-subclinically mastitic goats. Genotype BB had odds ratio of 0.54 in the occurrence of subclinical mastitis, but no significant association was found.

The frequency of TLR4 AluI-based genotype of Anglo-Nubian breed is presented in Table 3. The frequency of genotype AA with regard to occurrence of subclinical mastitis was 2 and non-occurrence was 6. Odds ratio for occurrence of subclinical mastitis in genotype AA is 0.08, thus, in Anglo-Nubian breed there is 0.08 times more likely that subclinical mastitis would occur, if the goat had genotype AA than with genotype AB. In genotype AB, frequency of occurrence of subclinical mastitis is 13 compared to 3 in non-occurrence. Odds ratio for genotype AB is 13.0 with significant association. Thus, there were 13.0 times more likely that in Anglo-Nubian breed, animal will suffer subclinical mastitis if it has genotype AB than those of genotype AA.

In Table 4, frequency of genotype AB with regard to occurrence of subclinical mastitis in Upgraded goats is 4 compared to 1 in the non-occurrence of subclinical mastitis. Odds ratio result for genotype AB is 2.40 with significant association. Therefore, there were 2.40 times more likely that in Upgraded goats, animal will suffer subclinical mastitis, if it has genotype AB than those of other genotypes. On the other hand, genotypes AA and BB had odds ratio of 0.32 and 0.17, respectively in the probability of occurrence of subclinical mastitis, but no significant association was found.

Frequency of TLR4 AluI-based genotype of Saanen and Native goats are not presented since there was no significant association found. This study in TLR4 using AluI enzyme showed a significant association using odds ratio. Analysis results in the frequency of occurrence or non-occurrence of subclinical mastitis in genotype AA in all breeds of goats and in Anglo-Nubian breed showed odds ratio of 0.28, and 0.08, respectively. Therefore, in all breeds of goats under study, and in Anglo-Nubian breed, likelihood of the animal with genotype AA to suffer from subclinical mastitis is 0.28 times greater in all breeds, and 0.08 times greater in Anglo-Nubian breed, respectively than those of other genotypes. On the other hand, genotype AB showed odds ratio results of 3.83, 13.0 and 2.40 in all breeds of goats, Anglo-Nubian, and Upgraded goats, respectively. This would indicate that in all breeds of goats, there is 3.83 times more likely that they will suffer subclinical mastitis; 13.0 times more probability in Anglo-Nubian breed, and 2.40 times more chances in Upgraded goats to suffer subclinical mastitis if they have genotype AB than those of other genotypes.

These results are in consonance with studies of Gupta et al. (2015) wherein allele A was associated with mastitis resistance, while allele B was associated with mastitis susceptibility in cattle. Although, their study was directed to T4CRBR2, which is part of exon 3, their findings reveal that polymorphism in T4CRBR2 induced C-T SNP at nucleotide 1,397 in exon 3 that led to disease resistance (Novák 2013). In addition, the study of (Gulhane and Sangwan, 2012) in water buffaloes using StyI enzyme showed that allele A was significantly more frequent in group of healthy buffaloes, thus, it was associated with resistance to mastitis. The said allele was found to have nucleotide substitution of cytosine to guanine at 217 nucleotide position in allele B, causing substitution of amino acid arginine by threonine.

In this study, polymorphism in locus 377 of the 494 bp segment of ligand-binding region can be recognized by AluI enzyme. Allele A can be identified by nucleotide substitution from G to C showing no cut in the fragment that indicates amino acid substitution of glutamic acid by aspartic acid.

Determination of genetic expression of **TLR4** *gene in goats with and without subclinical mastitis*

The recurring nature of subclinical mastitis has led to interest on genetic resistance to subclinical mastitis in dairy animals. Though the cause of the condition is multifactorial, resistance still rely on the ability of the immune system to combat the reoccurring infection. Vital immune effectors responsible for early detection and capture of the infectious agent is the TLR4.

Expression analysis of TLR4 gene in lactating goats were performed to give an idea on how these receptors are modulated by the body to confer resistance to infection such as sub-clinical mastitis. The optimized RT-qPCR assay was used to quantify the expression of TLR4 in goats. Figure 3 shows the optimized TLR4 relative expression analysis. Quantification cycles (Qc) of different samples and the gene amplification was validated by its characteristic melting curves showing only one peak.

The relative quantification of mRNA transcript of TLR4 in goat had been evaluated. The fold change is

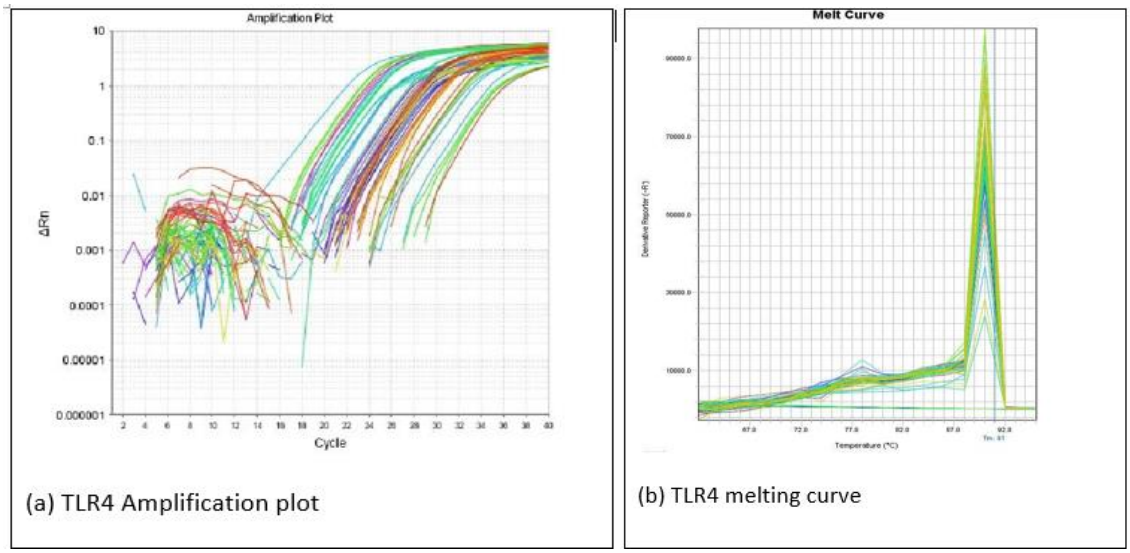

Figure 3. Amplification plot (a) and melt curve (b) of TLR4

the ratio of the normalized mean expression between the positive and negative with subclinical mastitis group. Results showed that TLR4 expression in non-subclinically mastitic goat was 1.40±2.22 fold or maximum expression of 3.63-fold. This was higher to the 0.26±0.39-fold or a maximum of 0.65-fold expression in subclinically mastitic animals. Statistical analysis showed significant difference with P-value of 0.04 ($p<0.05$) between the expression of TLR4 in animals with and without SCM.

This significant result suggests that increase in the expression of the TLR4 gene increased the protective function of the immunologic protein. TLR4 is a part of innate immune response. It is present in all cells and it enables recognition of foreign bodies (Takumi and Taro, 2014). Upon binding of specific lipopolysaccharides of microorganism, it results to a cascade of immune-regulatory process that enables capture and phagocytosis of microorganism. Increase expression of TLR4 protein in non-SCM animal could be attributed to the prompt protection from TLR4 (Vaure & Liu 2014).

The upregulation of the expression of the TLR4 can be explained by the stimulation of recognition of specific pathogen molecules present in bacterial agents. Lipopolysaccharide (LPS) from the cell walls of gram-negative organism are activators of TLR4 activity. TLR4 are receptors present on the surface of tissue macrophages, mast cells and dendritic cells that specifically stimulated by specific pathogen molecular patterns. Upon recognition of specific pathogen through TLR4, macrophages or mast cells would increase the synthesis and secretion of pro-inflammatory cytokines and lipid mediators initiating the inflammatory response through cytokines and chemokine expression (Takumi & Taro 2014; Kany et al. 2019). TLR stimulation of dendritic cells induces the initiation of adaptive immune response (Martin-Gayo & Yu, 2019; Takagi et al., 2016). Studies have shown the significant increase of gene expression of TLR4 in dairy herd with mastitis (Panigrahi et al. 2014).

In this study, the increase in TLR4 expression seen in goats could be attributed by the possible protection induced by the immunoreceptor against causative agent of subclinical mastitis. TLR4 expression was expected to increase in animals with infection ascribing to its role to stimulate host defense by mediating cytokine production and initiate adaptive immunity (Wakchaure et al. 2012). However, according to Zhuang et al. (2020), TLR4 expression is also dependent on the organism present. He observed a significantly lower TLR4 mRNA level in *Staphylococcus aureus* infected primary mammary epithelial cells compared to *E coli* infected cells.

One specific pathogen molecule recognized and that stimulates TLR4 is lipopolysaccharide. Lipopolysaccharide upregulation of TLR4 expression which have been seen in some animals may not be the same to other animals. Vaure and Liu, (2014) explained the phenomenon of LPS tolerance due loss of surface TLR4 expression and rapid induction of the negative feedback regulators of TLR4 pathway. Decrease in TLR4 stimulation could lead to delay in influx of polymorphonuclear neutrophils into the mammary gland after intra-mammary infection. This can result to decrease TLR4 function consequently leading to disease condition (Vangroenweghe et al. 2005)

CONCLUSION

Nucleotide sequences of Native, Upgraded, Anglo-Nubian and Saanen goats had high similarity to *C. hircus* and *O. aries* TLR4 gene sequences from NCBI GenBank. Nucleotide and amino acid sequences of

other ruminants such as cattle and water buffalo had lower similarity to goat nucleotide and amino acid sequences. Ligand-binding domain of TLR4 was highly polymorphic and polymorphisms were identified using restriction enzyme AluI. Significant association had been found in Genotype AA in TLR4 with low odds ratio values in all breeds, and in Anglo-Nubian; while genotype AB had high odds ratio values in all breeds, Anglo-Nubian and Upgraded crossbreds. Expression analysis showed an upregulation of TLR4 in goats without SCM, which suggest a prompt role in the protection of animal against possible infection. TLR4 are receptor proteins that are present in cells specially the macrophages, as part of innate mechanism of immunity, they can be swift in recognizing impending infection.

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