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# Detection of Genetic Variations in the Toll-Like Receptors (TLRs) Gene Family of Tali Goat and Studying of Deleterious Single Nucleotide Polymorphisms (SNPs) that Potentially Cause Infectious Diseases

Research Article

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## ABSTRACT

Infectious diseases are highly prevalent diseases in farm animals such as goat that cause high economic losses. Toll-like receptors (TLRs) play a crucial role in the induction of innate immune responses, they can identify the invading pathogens. In this study, SNPs in TLR2, TLR3, TLR4, TLR6, TLR9, and TLR10 in Tali breed goat (a resistant breed) were identified and reported for the first time**.** A pooled DNA of 15 Tali goat, was sequenced and mapped to the goat reference sequence. Non-synonymous single nucleotide polymorphisms (nsSNPs) of this gene family of the Tali breed were compared with the retrieved available dataset of the Sanen breed goat (a susceptible breed). Identified SNPs were analyzed by orting intolerant from tolerant (SFIT), PolyPhen, Provean, and I- Mutant servers. The results obtained from various tools showed that the difference between deleterious nsSNPs of TLR3, TLR9, and TLR10 between Tali and Sanen goat may exhibit the greatest impact on protein stability. Additionally, the 3D structure of both Tali and Sanen breed forms of TLR3, TLR9, and TLR10 were modeled. The present study revealed the 5 deleterious nsSNPs in TLR3, TLR9, and TLR10 may affect the function of TLRs but didn't cause any significant change in the structures.

**KEY WORDS** *In silico* tools, infectious diseases, SNP, Tali breed goat, toll-like receptor.

## INTRODUCTION

Toll-like receptors (TLRs) are a group of proteins conserved during evolution that play a key role in the innate immune system. Several studies have shown the importance of these receptors in bacterial, viral, and fungal infections, autoimmune diseases, neurodegenerative diseases, and types of cancer. The innate immune system is the primary defense barrier of the host against invading pathogens. This system, preserved during evolution, is considered the ancient arm of the body's immune system from a phylogenetic point of view (Sameer and Nissar, 2021; Dabi *et al.* 2023). TLR genes have been identified in domestic animal species, and evidence gathered from several studies suggests an extensive role for TLR signaling in reproductive physiology. In females, TLRs play a role in the regulation of ovulation, fertilization, pregnancy, and childbirth, as well as in pathological conditions such as endometritis and mastitis.

In males, TLRs are involved in steroidogenesis and spermatogenesis.

The use of TLR agonists is also effective in treating some genital infections. In addition, gene polymorphisms in TLRs have been associated with mastitis, providing evidence that TLRs can potentially be used as markers in future breeding programs. Also, the broad roles of TLR in the physiology and pathology of human reproduction have been emphasized at different levels (Kannaki *et al*. 2011). Considering that Toll-like receptors are important sensors of various pathogens, their location, and abundance are related to the efficiency of the innate immune response. Therefore, TLRs play a key role in protecting animal hosts against microbial infections. The high similarity in the nucleotide and protein sequences of TLRs of humans, mice, and other mammals indicates the conserved role of these receptors in host defense against pathogens. In addition, different TLR ligands can be used as immune adjuvants, antiallergic agents, or immune protectors in domestic animal species. Hence, TLRs can be of critical importance for the development of future strategies in the field of veterinary medicine aimed at improving genetic resistance to infectious diseases, as well as in the development of drugs capable of enhancing or modulating the animal's immune response (Turin and Riva, 2008).

The first domestication of goats goes back to very distant times and to the highlands of western Iran (Lu, 2023). The Tali breed goat belongs to the tropical regions of southern Iran, which is characterized by high twinning capacity and resistance to diseases, external parasites, heat, humidity, and unsuitable pasture conditions (Rezvannejad *et al*. 2023).

TLRs are receptors with a transmembrane part that are usually expressed on guard cells such as macrophages and dendritic cells and recognize the protected structural molecules produced by microbes. During the last decade, the detailed mechanisms related to TLR signaling have been elucidated by various approaches including genetic, biochemical, structural, and cell biology and bioinformatics studies. It seems that TLR signaling is diverse and plays an important role in many aspects of the innate immune response to pathogens (O'Neill *et al*. 2013; Kawasaki and Kawai, 2014).

TLRs were identified for the first time in Drosophila embryos. Further studies showed that these receptors play a role in creating antifungal responses in adult flies. Later it was found that TLRs also exist in mammals. This finding led to the understanding of an important part of the development mechanism of the innate immune response (Takeda and Akira, 2005). From the total of 13 TLR genes identified to date (TLR1-13), 10 genes (TLR1-10) exist in cattle, sheep (Menzies and Ingham, 2006) and goat (Tirumurugaan *et al*. 2010).

Therefore, due to a lot of evidence about the effect of Toll-like receptors in the development of innate immunity, and resistance to viral, bacterial, and parasitic infections (Faria *et al*. 2012; Baral *et al*. 2014; Lester and Li, 2014; Ashour, 2015; Das *et al*. 2017).

Investigating genetic variations in Toll-like receptor genes in Tali goat and comparing those with the Sanen breed and species of mammals can provide very beneficial information about the cause of this breed's resistance to diseases.

## MATERIALS AND METHODS

### Animals

In this study, the pooled DNA sequence of Tali goat was used. This sequence has already been prepared in the following way (Rezvannejad *et al*. 2023). Blood samples were collected from 15 Tali goats in Hormozgan province (tropical region) of Iran. DNA was extracted from whole blood samples using a DNA extraction kit (Qiagen DNA, Hilden, Germany). Pooled DNA was used to generate paired-end libraries using the Illumina Sequencing Kit and the Illumina Genome AnalyzerIIx System at Novogene (http://www.novogene.com). In the next step, the reads were aligned with the goat reference genome "CHIR_2.0 (http://www.ncbi.nlm.nih.gov/assembly/GCA_000317765.2)" using BWA software, and the Picard-Tools-1 website (https://broadinstitute.github.io/picard) was used to remove duplicates. Moreover, the data on 10 Sanen goat breeds was collected from the National Center for Biotechnology Information (NCBI) website (http:// www.ncbi.nlm. nih.gov), which include genomic DNA accession numbers (ERR405774, ERR405775, ERR405776, ERR405777, ERR405778, SRR6246095, SRR6246096, SRR6246097, SRR6246098, and SRR6246100).

### Determining the genetic variations of the TLR genes using the VEP

Of the total of 13 TLR genes identified to date (TLR1-13), 10 genes (TLR1-10) are present in goat (Alim *et al*. 2016). The website Ensembl Variant Effect Predictor (McLaren *et al*. 2016; Howe *et al*. 2021) with the address (https://asia.ensembl.org/info/docs/tools/vep/index.html) was used for the identification of breed-specific genetic variants of the coding regions of goat TLR genes family. Genetic variations can provide information such as the location of genetic changes in the coding (exon) or non-coding (intron) regions of the genome, the lethality of these variants, production or deletion stop codon, or frameshift.

The detected nsSNPs were valued using various online bioinformatic tools (PROVEAN, PolyPhen-2, I-Mutant, and SIFT).

The Protein Variation Effect Analyzer (PROVEAN) is an online software that determines the effects of amino acid substitutions. PolyPhen-2 is an online bioinformatics database that predicts the effect of an amino acid substitution on the function and structure of the protein. I-Mutant is also a bioinformatic website used to predict protein stability. And Sorting Intolerant from Tolerant (SIFT) is a tool used to characterize missense variations. This tool determines the effect of amino acids on protein function.

A phylogenetic tree was obtained using the maximum-likelihood algorithm with the MEGA 7.0 package (https://www.megasoftware.net/).

**Determining and comparing the 3D structure of TLR receptors in reference, Tali, and Sanen goat by SWISS-MODEL web server**

SWISS-MODEL (https://swissmodel.expasy.org/) is a fully automatic protein structure determination server based on homology modeling, which is accessible through the Expacy web server (Bienert *et al*. 2017; Waterhouse *et al*. 2018). Considering that none of the reference goat TLR receptors have been determined, their third structure was determined with SWISS-MODEL. In addition, according to the genetic variations obtained for each Tali goat TLR gene, the sequence of Tali goat TLR receptors was determined and their 3D structure was determined using SWISS-MODEL. However, SWISS-MODEL provides several models for the desired sequence, the best model was selected based on GMQE (Global Model Quality Estimate) and QMEAND which are Global values. GMQE and QMEAND are Global indicating the quality of the built model and their values vary between 0 and 1. Higher numbers indicate that the favorite model has higher reliability.

GMQE depends on the coverage of the model and the target sequence. For example, if a model covers only half of the target sequence, it is very unlikely that it will have a GMQE higher than 0.5. While QMEAND investigates the quality of the model independently of the degree of overlap. PyMol and Swiss PDB-Viewer software were used to observe and compare the structure of TLR proteins of the reference goat, and the Tali breed goat.

## RESULTS AND DISCUSSION

**Genetic variations in TLR genes**

Whole genome sequencing data of the Tali breed with coverage 32x was called 606 million sequence reads. More than 99.4% of the generated sequence reads were mapped to the goat reference genome (CHIR_2.0), indicating high-quality sequences were obtained.

The genome size Tali breed was 2947.15Mb with a median GC % of 43%. The WGS analysis basic details have been published (Rezvannejad *et al*. 2023). Of the total of 13 TLR genes identified to date (TLR1-13), 10 genes (TLR1-10) are present in goat (Tirumurugaan *et al*. 2010) by database NCBI, it was found that TLR1, TLR6, and TLR10 genes are located on chromosome 6, TLR2 on chromosome 17, TLR3 on chromosome 27, TLR4 on chromosome 8, TLR5 on chromosome 16, TLR7 and TLR8 on chromosome X. and TLR9 is located on chromosome 22. The Tali and Sanen goat genome sequences was compared with the reference goat sequence using the Ensembl Variant Effect Predictor website to identify the genetic variations in the coding region of the goat TLR genes. Although, according to the database NCBI, all three genes TLR1, TLR6, and TLR10 are located on chromosome 6, only the results related to TLR6 and TLR10 can be seen on the VEP website, and TLR1 is not present in this chromosome, by this website. Based on the investigations with the help of the website, it was considered that TLR6 and TLR1 genes overlap. So, the Annotation system used in VEP could not identify and specify the TLR1 gene as a separate gene**.** To address this overlap, stringent filtering criteria were applied in the VEP pipeline, cross-referencing with the *Capra hircus* ARS1 genome assembly to improve SNP assignment accuracy. The overlap arises due to the close proximity and high sequence homology of TLR1 and TLR6 on chromosome 6, which complicates annotation. Also, considering that all of the goat X chromosome has not yet been sequenced, it is not possible to obtain the genetic variants of the TLR genes on this chromosome (TLR7 and TLR8). Therefore, in this research, only the results related to 7 goat TLR genes (TLR2-6 and TLR9-10) are presented (Table 1).

**Genetic variations identified on TLR family genes**

The study of genetic variations in the TLR family genes of the Tali goat shows that these genes have 2395 SNPs which 21.34% on downstream, 20.71% on upstream, 50.40% on intron, 0/42% on 5 prime UTR, and 7.10% on exon of genes were located.

Of 7.10% SNPs detected on exon genes, 3.63% were synonymous variants and 3.46% were missense variants compared to the reference goat (table 2). While, in Sanen breed 2976 SNPs were identified which 20.97% on downstream, 18.81% on upstream, 50.20% on the intron, 0/70% on 5 prime UTR, and 9.27% on exon of TLR genes were located. Of 9.27% SNPs detected on exon genes, 4.40% were synonymous variants and 4.87% were missense variants compared to the reference goat (Table 2).

**Table 1** Genomic and proteomic characterization of 7 genes of toll-like receptor (TLR) families in goat

| Protein type | Locus gene ID | Chromosome N. | Exons | Length (amino acids) | Accession No.[1] |
|---|---|---|---|---|---|
| TLR2 | 100860747 | 17 | 3 | 790 | A0A452G4X3 |
| TLR3 | 102182757 | 27 | 6 | 918 | A0A452ESC2 |
| TLR4 | 100860955 | 8 | 3 | 841 | A0A452F552 |
| TLR5 | 100860844 | 10 | 7 | 871 | A0A452DT45 |
| TLR6 | 100860932 | 6 | 4 | 966 | A0A452FN74 |
| TLR9 | 100860819 | 22 | 1 | 1036 | A0A452FY88 |
| TLR10 | 102190865 | 6 | 2 | 812 | A0A452E236 |

[1] Accession numbers from UniProt.

**Table 2** Identified SNPs on toll-like receptor (TLR) family genes by ensembl variant effect predictor server in Tali and Sanen breeds

| Gene name | Breed | 5 prime UTR variant | Downstream gene variant | Intron variant | Upstream gene variant | Synonymous variant | Missense variant | Stop gained |
|---|---|---|---|---|---|---|---|---|
| TLR2 | Tali | 10 | 71 | 316 | 125 | 14 | 9 | 0 |
| TLR2 | Sanen | 12 | 117 | 375 | 134 | 24 | 19 | 0 |
| TLR3 | Tali | 0 | 41 | 626 | 40 | 10 | 11 | 0 |
| TLR3 | Sanen | 0 | 49 | 748 | 65 | 23 | 29 | 0 |
| TLR4 | Tali | 7 | 80 | 107 | 75 | 11 | 18 | 0 |
| TLR4 | Sanen | 9 | 93 | 119 | 81 | 19 | 28 | 0 |
| TLR5 | Tali | 0 | 88 | 0 | 109 | 15 | 15 | 1 |
| TLR5 | Sanen | 0 | 93 | 0 | 126 | 15 | 16 | 1 |
| TLR6 | Tali | 0 | 97 | 225 | 25 | 10 | 15 | 0 |
| TLR6 | Sanen | 0 | 107 | 243 | 31 | 12 | 19 | 0 |
| TLR9 | Tali | 0 | 46 | 5 | 32 | 17 | 2 | 0 |
| TLR9 | Sanen | 0 | 64 | 9 | 37 | 18 | 4 | 0 |
| TLR10 | Tali | 0 | 88 | 0 | 80 | 11 | 14 | 0 |
| TLR10 | Sanen | 0 | 101 | 0 | 86 | 20 | 30 | 0 |

UTR: untranslated Region; 5′ UTR variant: variant in the 5′ untranslated region; Downstream/upstream gene variant: variant located outside the coding sequence, downstream or upstream of the gene; Synonymous variant: nucleotide change without amino acid change; Missense variant: change leading to a different amino acid and Stop gained: variant introducing a premature stop codon.

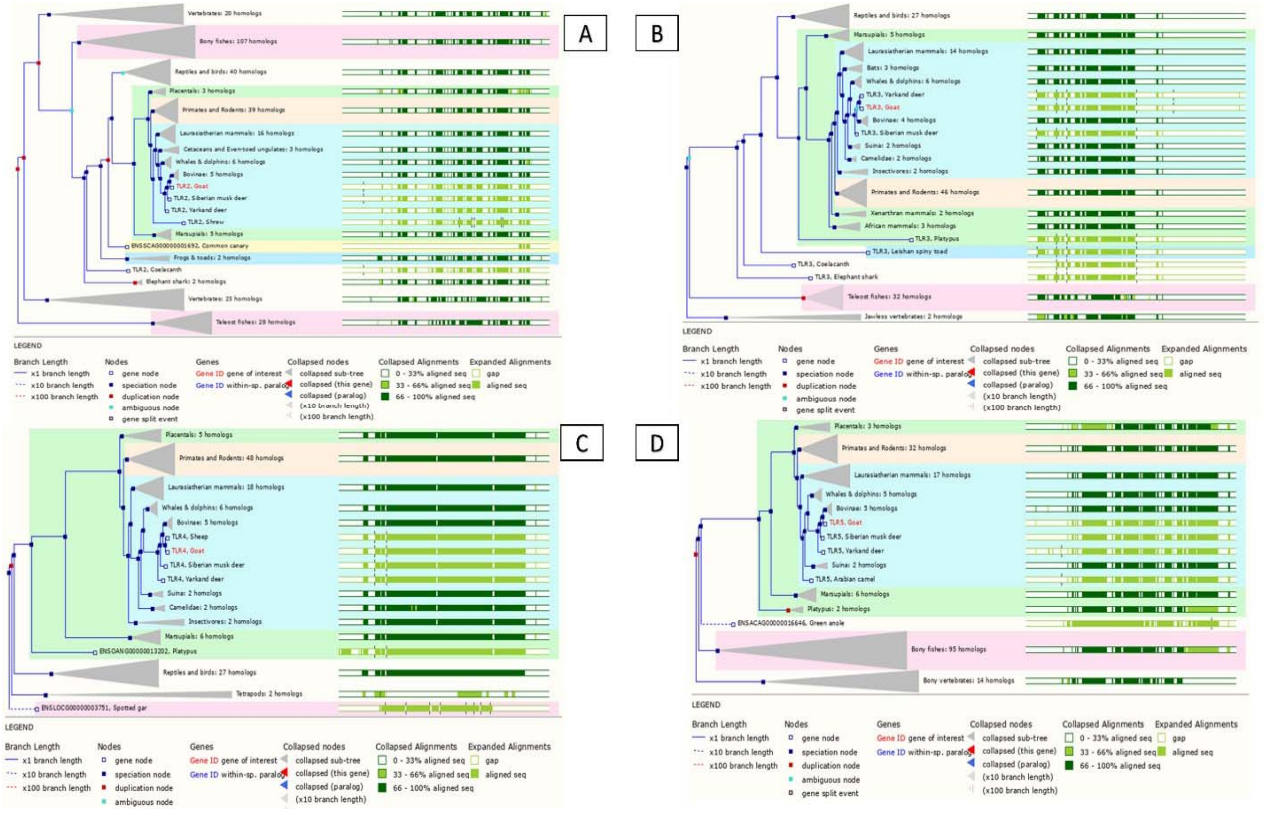


**Figure 1** Phylogenetic tree A) TLR2, B) TLR3, C) TLR4, and D) TLR5 in goat (*Capra hircus*)

**Phylogenetic analysis of TLRs family gene in goat**

A phylogenetic tree was realized based on similarities and differences between the TLR family gene sequences of goats with other mammalian species. The phylogenetic tree of goat TLR2, TLR5, TLR6, and TLR10 (*Capra hircus*) shows that this gene is more closely related to the cow gene (Figures 1 and 2).

These genes are also related to the TLR genes of Siberian musk deer and Yarkand deer. The phylogenetic tree of goat TLR3 (Figure 1) shows that this gene is very closely related to its equivalent gene in Yarkand deer. In later stages, this gene is closely related to the genes of Siberian musk deer and cattle. The phylogenetic tree of goat TLR4, and TLR9 is shown in Figures 1 and 2. As can be seen, these genes are more closely related to the TLR4, and TLR9 genes of sheep. In later stages, these genes are related to the TLR4, and TLR9 genes of cattle, Siberian musk deer, and Yarkand deer. Green bars are areas with 33-66% sequencing alignment, white areas show 0-33% aligned sequence and black bars indicate 66-100% aligned sequence.

**Effect of genetic variations on the structure of 3D TLR receptors**

The results obtained from determining the structure of TLR receptors in reference goat and Tali goat and the effect of SNPs identified in the previous step on the structure of these proteins in Tali goat are presented. As mentioned, the structure of these proteins was determined by SWISS-MODEL website and the best model was selected based on GMQE (Global Model Quality Estimate) and QMEAND is Global values. The reference goat TLR2 protein (Goat-TLR2) has 790 amino acids. Considering that Tali goat TLR2 only in 9 bases of this protein is different, the pattern used, the sequence similarity, and the degree of overlap of both proteins are completely similar. The best model constructed by the SWISS-MODEL for Tali goat TLR2 protein is shown in Figure 3.

The reference TLR3 receptor (Goat-TLR3) with Uniprot code A0A452ESC2 has 918 amino acids. Tali Goat-TLR3 is different from reference goat TLR3 by 11 bases. Comparing the structure of two proteins shows that their overall structure is similar. However, there are differences in some parts of the structure. The best model made by SWISS-MODEL for Tali Goat-TLR3 protein is presented in Fig. 3.

Goat TLR4 protein (*Capra hircus*) with Uniprot code A0A452F552 consists of 841 amino acid bases. The TLR4 of the Tali breed goat is different from goat TLR4 by 18 bases. Comparing the structure of two proteins shows that their overall structure is similar.

However, slight differences are observed in some parts of the structure. The best model made by SWISS-MODEL for Tali goat TLR4 protein is shown in Figure 3.

TLR5 protein of *Capra hircus* with Uniprot code A0A452DT45, structure has not been determined yet. The template used (3j0a.1.A) is human (*Homo sapiens*) TLR5 (Zhou *et al*. 2012). According to the model used (3j0a), the model made by SWISS-MODEL for both reference goat TLR5 and Tali goat proteins is in the form of an asymmetric dimer. This protein has 871 amino acids in reference goat. TLR5 of Tali goat is different from this protein by 15 amino acids.

Comparing the structure of reference goat TLR5 and Tali goat TLR5 proteins using PyMol software shows that although the overall structure of these two proteins is similar, differences in their structure can be seen. The best model made by SWISS-MODEL for Tali goatTLR5 protein is shown in Figure 3.

The 3D structure of the goat TLR6 protein (*Capra hircus*) with the A0A452FN74 code Uniprot has not been determined yet. This protein has 966 amino acid bases. TLR6 from the Tali breed differs from reference goat TLR6 by 15 amino acids. The best model made by the SWISS-MODEL web server for Tali goat TLR6 protein is shown in Fig. 3.

The reference goat TLR9 with Uniprot code A0A452FY88 has not been structure determined yet. This protein consists of 1036 amino acids. Also, the TLR9 of Tali goat differs from the reference goat TLR9 by only 2 amino acids. According to the model used for modeling (5y3m), the model provided by SWISS-MODEL is a homodimer for both reference goat TLR9 and Tali goat TLR9 proteins. Reference and Tali goat's third structure are also completely identical. Figure 3 shows the best model made by SWISS-MODEL for Tali goat TLR9 protein.

The 3D structure of goat TLR10 (*Capra hircus*) with Uniprot code A0A452E236 has not yet been determined. This protein has 812 amino acids. Also, TLR10 of Tali differs from reference goat TLR10 by 14 amino acids. Comparing the structure of two proteins shows that their overall structure is similar.

However, there are differences in some parts of the structure. The best model provided by SWISS-MODEL for Tali goat TLR10 protein is shown in Figure 3.

**Validation of missense genetic variations**

Various computational tools evaluate the deleterious effects of SNPs based on several different parameters. By combining the results of different tools, it is possible to grade more accurately.

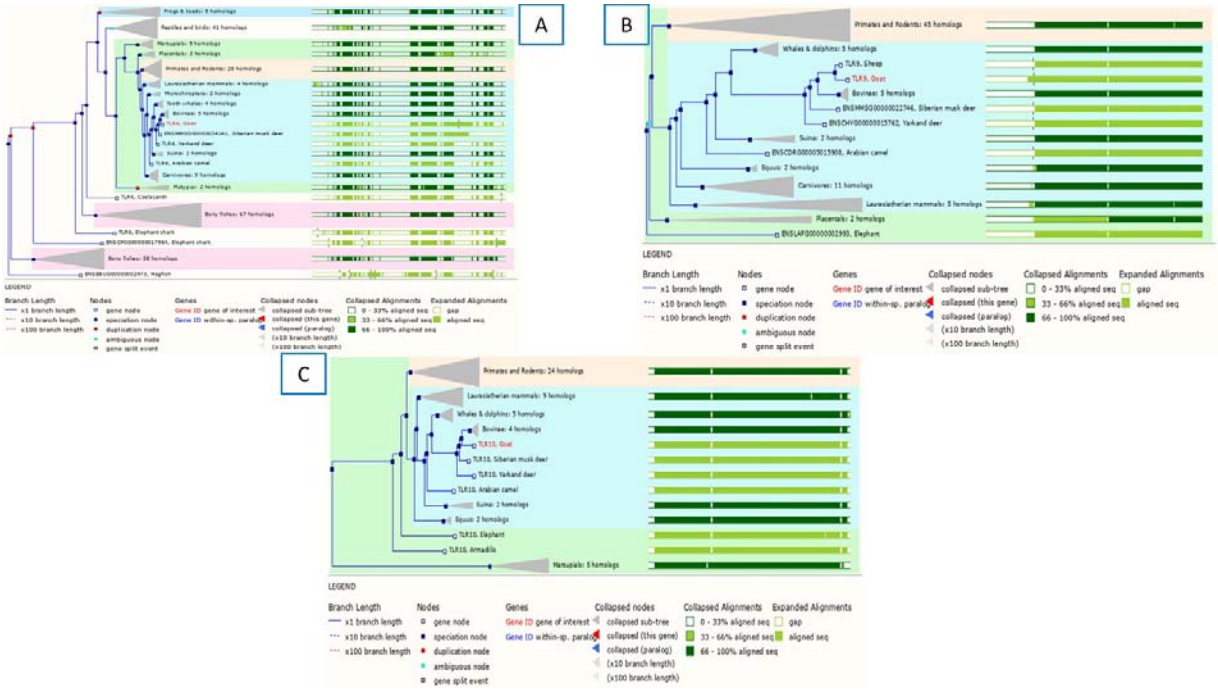


**Figure 2** Phylogenetic tree A) TLR6, B) TLR9, and C) TLR10 in goat (*Capra hircus*)

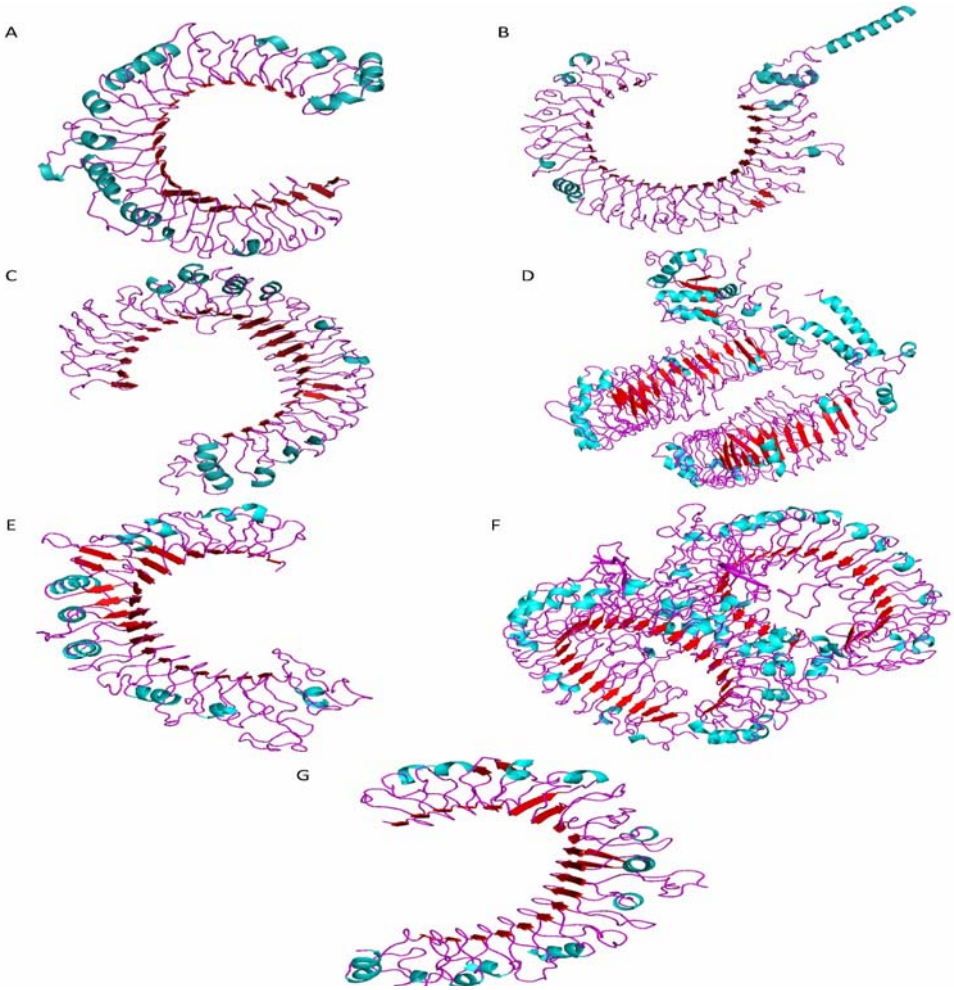


**Figure 3** 3D structure of A) TLR2, B) TLR3, C) TLR4, D) TLR5, E) TLR6, F) TLR9, and G) TLR10 in Tali goat by SWISS-MODEL website

For this purpose, in the present research, the accuracy of prioritizing candidate deleterious SNPs was increased by a combination of in silico tools such as SIFT, PROVEAN, and polyPhen-2. The PROVEAN tool recognizes non-synonymous genetic variants that are functionally important. PROVEAN scores of ≤−2.5 reflected a deleterious effect on an amino acid change, whereas scores of >−2.5 indicated a neutral effect. The SIFT score for each substitution has a range of 0.0 to 1.0.

The substituted amino acid with a score ≥0.05 is predicted as tolerated but <0.05 has a damaging effect. The position-specific independent count (PSIC) scores in polyPhen-2 show the difference between the 2 variants (wild-type amino acid and mutant amino acid). The amino acid substitutions with a score less than 0.5 are predicted as benign, with a score from 0.5 to 1.0 is predicted as damaging. All detected differentially missense SNPs between the Sanen and Tali breeds were studied by deleterious SNPs prediction in silico algorithms.

Missense SNPs were classified as deleterious which were considered as deleterious at least two servers out of three algorithmic tools (Table 3). Ten genetic variations of the Sanen breed have these characteristics comparison to the Tali breed. There are 3 deleterious SNPs on TLR2, 2 deleterious SNPs on TLR10 and TLR3, and 1 deleterious SNP on TLR4, TLR6, and TLR9.

I-Mutant technique helped to predict the effect of ns SNP on the stability of a protein. 10 nsSNPs which were been found submitted to the I-Mutant server to predict their free energy change and IR values (table 3). nsSNPs that reduce the stability of protein may cause major changes in the protein. 7 deleterious nsSNPs identified caused a significant decrease in protein stability, indicating probably caused major changes in the proteins in the Sanen breed.

Deletions, substitutions, and insertions, collectively known as genetic variations, can alter the function or structure of a gene's product, which may predispose an organism to disease resistance or susceptibility. Predicting the mechanisms of genetic changes helps researchers identify genes associated with diseases. Several computational works were performed to determine the deleterious SNPs. The effects of possible mutations in TLR genes, which have a vital role in many inflammatory diseases, were investigated in two goat breeds with disease-susceptible and disease-resistant characteristics.

Seven TLRs genes such as TLR2, TLR3, TLR4, TLR5, TLR6, TLR9, and LTR10 are used for the analysis. Given the high productivity of Saanen goats, understanding these deleterious SNPs is critical for breeding programs aimed at enhancing disease resistance in this economically important breed.

A missense genetic variant, which is also called missense mutation, is a type of genetic variant that changes the desired amino acid into another amino acid as a result of changing a base pair. This type of genetic variant can change the function of a protein. Synonymous change is a type of codon change that does not cause any change in the coded amino acid type. Previously, it was thought that this type of genetic variant does not affect the characteristics of the synthesized protein. But today there is evidence that shows that these seemingly silent variants have a very specific effect on the expression and function of the desired protein (Edwards *et al*. 2012). Only 1 genetic variant in both breeds was detected such as a stop-gained variant on TLR5. The stop gained is a type of mutation that leads to the creation of a stop codon and, thus, the premature termination of the protein translation process.

One of the families of pattern recognition receptors (PRRs) are the Toll-like receptors (TLRs) which play an essential role in immune responses, especially pathogen distinction by the extracellular matrix and have evolutionarily conserved (Fitzgerald and Kagan, 2020; Duan *et al*. 2022; Chen *et al*. 2024). TLRs have a major ability to recognize pathogen-associated molecular patterns (PAMPs), conserved structures of pathogens, or even damage caused by pathogens in the host, so they play an important role in the body's first line of defense against pathogens (Jeong and Lee, 2011; Nie *et al*. 2018; Behzadi *et al*. 2021; Kumar *et al*. 2023). Genetic polymorphism studies have increased our understanding of the performance of many traits such as immunity to disease. the global researchers have used to decipher disease resistance in some animals (Pighetti and Elliott, 2011).

Non-synonymous SNPs that produce variants not only alter the 3D structure of the protein, but also affect its function and stability, and may also form deleterious phenotypes (Jacob *et al*. 2020). Alim *et al.* (2016) showed that goat TLRs are associated with the responses to gastrointestinal nematode infection, including *H. contortus*, and thus influence the immune response to pathogens. polymorphisms identified in TLRs might affect the structure and or function of TLRs. Bhavaniramya *et al.* (2019) studied the effect of the deleterious SNPs in some TLR family proteins on mastitis in dairy cattle. It showed that deleterious SNPs may affect the structure and function of TLR2 between mutant and native form.

5 SNPid rs3469732021, rs684740222, rs682390992, rs680318088, and rs3469731149 on TLR3, TLR9, and TLR10 genes showed the significant deleterious effects which may affect the protein structure and function between Tali and Sanen breed, the other various SNPs were not predicted to be deleterious for the protein.

**Table 3** The difference between Missense SNPs analyzed in studied toll-like receptor (TLR) genes by SIFT, PROVEAN, PolyPhen-2, and I-Mutant servers in Tali and Sanen breeds

| Breed | Gene name | SNPrsID | Substitution | SIFT prediction | PROVEAN prediction | PolyPhen-2 | I-Mutant |
|---|---|---|---|---|---|---|---|
| Sanen | TLR2 | rs3469730402 | G59S | Tolerated 0.52 | Deleterious | Probably damaging 1 | Increase Stability |
| | TLR2 | rs3469731845 | D231A | Tolerated 0.58 | Deleterious | Probably damaging 0.65 | Increase Stability |
| | TLR2 | rs3469730185 | R580Q | Tolerated 1 | Deleterious | Possibly damaging 1 | Decrease Stability |
| | TLR3 | rs3469732021 | R332W | Deleterious 0 | Deleterious | Probably damaging 0.99 | Decrease Stability |
| | TLR3 | rs684740222 | I347T | Deleterious 0 | Deleterious | Probably damaging 1 | Decrease Stability |
| | TLR4 | rs3469731078 | D373Y | Tolerated 1 | Deleterious | Probably damaging 0.95 | Increase Stability |
| | TLR6 | rs674550684 | S70P | Deleterious 0.04 | Deleterious | Benign 0.02 | Decrease Stability |
| | TLR9 | rs682390992 | H282R | Deleterious 0.02 | Deleterious | Probably damaging 0.99 | Decrease Stability |
| | TLR10 | rs680318088 | L615P | Deleterious 0.4 | Deleterious | Probably damaging 0.98 | Decrease Stability |
| | TLR10 | rs3469731149 | P733R | Deleterious 0 | Deleterious | Probably damaging 1 | Decrease Stability |

The 5 different nsSNPs are identified as deleterious nsSNP from three TLR genes showed the major mutations at the position R332W and I347T on TLR3 protein, H282R on TLR9, and L615P, and P733R on TLR10 protein. At TLR3 protein, in position 332 amino acid Arginine (polar basic amino acid, positive charge, and hydrophilic) in Tali goat was changed to Tryptophan (aromatic amino acid, uncharged, and hydrophobic) in Sanen goat, and at position 347, Isoleucine (non-polar, and hydrophobic) was changed to Threonine amino acid (polar, un-charged, and hydrophilic). In TLR9, at position 282 amino acid Histidine (polar basic amino acid, positive charge, and hydrophilic) in Tali goat was changed to Arginine (polar basic amino acid, positive charge, and hydrophilic) in Sanen goat. And in TLR10 protein at position 615 amino acid leucine (non-polar, and hydrophobic) in the Tali goat was changed to Proline amino acid (non-polar, and hydrophobic). And at position 733 Proline amino acid (non-polar, and hydrophobic) in the Tali goat was changed to Arginine (polar basic amino acid, positive charge, and hydrophilic) in the Sanen goat.

This study has several limitations that warrant consideration. The use of pooled DNA sequencing, while cost-effective and suitable for population-level analyses, may mask individual genetic variations, such as low-frequency alleles or heterozygous states. Future studies should incorporate individual-level sequencing to capture a more granular view of SNP distribution. Additionally, the absence of TLR7 and TLR8 data due to incomplete sequencing of the X chromosome limits the comprehensiveness of our TLR gene analysis. Efforts to improve X chromosome assembly in the goat genome reference will be critical for addressing this gap. Finally, while our in silico predictions are robust, they remain hypothetical without experimental validation, as discussed above. These limitations highlight areas for refinement in subsequent research to enhance the applicability of our findings.

## CONCLUSION

In overall, results showed there are significant deleterious SNPs on TLR3, TLR9, and TLR10 in the Sanen goat while there aren't in the Tali goat. The effects which may affect the protein function but didn't cause any significant change in the structures. The 5 deleterious nsSNPs are identified as deleterious nsSNP from three different TLR genes which there are in the Sanen goat. The final findings of this research indicated that the position R332W and I347T muta-

tions in TLR3 protein, and P733R on TLR10 protein may be higher-risk nsSNPs that can affect TLR3 and TLR10 function. Future research should focus on functional assays to validate these findings and explore their implications for veterinary medicine and breeding programs. To address the overlap between TLR1 and TLR6 on chromosome 6 in the VEP analysis, we applied stringent filtering criteria in the VEP pipeline, cross-referencing with the Capra hircus ARS1 genome assembly to enhance SNP assignment accuracy. This overlap arises due to the close proximity and high sequence homology of these genes, which complicates annotation. Future studies could employ targeted sequencing to further resolve these regions. Additionally, experimental validation, such as protein expression assays (e.g., Western blotting or ELISA) to quantify TLR protein levels or functional assays (e.g., ligand-binding or cytokine induction studies), is recommended to confirm the functional impacts of the identified SNPs.

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