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Identification of allelic variants, differential expression, and in silico analysis of caprine serum lysozyme gene

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Abstract: Serum lysozyme gene empowers the immune system due to its bacteriolytic property targeting peptidoglycan layer of cell wall. Polymorphs of serum lysozyme gene are reported to be significantly associated with disease resistance traits. Two different gene fragments viz. fragment I (275 bp) and fragment II (230 bp) of serum lysozyme gene were amplified and screened by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) technique to identify genetic variation in two different goat breeds (Barbari and Marwari) of India. The serum lysozyme activity of different genotypes and mRNA expression across the breed was also estimated. In fragment I, four genotypes (AA, BB, AB, and CC) and three alleles (A, B, and C) were identified. The CC genotype (frequency-0.44) and B allele (frequency-0.46) was found to be prevalent in the population. In fragment II, six genotypes (AA, BB, AC, AD, AE, and FF) and six alleles (A, B, C, D, E, and F) were identified. The AD genotype (frequency-0.44) and A allele (frequency-0.53) were found to be prevalent in the population. A significant difference ($p \leq 0.05$) in mean serum lysozyme activity was found among different genotypes of fragment I in Marwari goat only. The genotypes of fragment II did not show any statistically significant effect on serum lysozyme activity. Relative quantification showed that nondescript animals had higher expression levels of serum lysozyme gene as compared to registered goat breeds. A single nucleotide variation was detected at 93rd position (G to A) of full-length coding sequence where guanine in Barbari goat was replaced by adenine in Black Bengal goat. The polymorphic nature of the serum lysozyme gene and its differential expression will help to establish the role of the lysozyme gene in the disease resistance mechanism.

Key words: Allele, genotype, goat, lysozyme, PCR-SSCP

1. Introduction

Goat plays a major role in the livelihood of Indian farmers by providing food security as well as a source of income to poor people. The incidence of disease increases the morbidity and mortality in goats causing significant economic loss. Hence, identifying the important indigenous goat breeds, which are more resistant to diseases for enhancing productivity is the need of the hour.

The immune-related genes involved in innate and adaptive immunity can play a pivotal role during the study of the genetic basis of disease occurrence. The lysozyme gene product contributes to innate immunity by cleaving the β-1, 4 linkages of bacterial peptidoglycan during phagocytosis [1,2]. Lysozyme is a ubiquitous bacteriolytic enzyme present in body fluids and tissues. The ruminants are not as efficient in lysozyme activity except at abomasum as compared to other species [3]. The serum lysozyme activity reflects the homeostatic expression of the reticulo-endothelial system (RES), which is an important component of the innate immune system [4]. Experimental findings have also produced evidence for lysozyme being an index of macrophage functional status [5]. The indirect immune-potentiating effect of lysozyme has been established beyond doubt, which suggests its role as a candidate gene for disease resistance [6]. Gene expression is considered the functional aspect of a gene. The expression of a gene is affected by multiple mechanisms including rearrangement, loss, and the effect of other environmental factors. Diversity of expression level gene (lysozyme gene in this study) will help to understand

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the variable immunity of individuals to different diseases. So, analysis in this regard will establish the mechanism of disease resistance and susceptibility of animals and improve the production performance of animals by using only resistant animals during future breeding.

Therefore, the present study was designed for the characterization of Indian goats. The genetic polymorphism of the lysozyme gene in Barbari and Marwari goats was identified by PCR-SSCP analysis followed by its association study with serum lysozyme level and somatic cell count. To predict the functional aspect of this gene, the full-length coding sequence of the serum lysozyme gene was cloned and sequenced followed by in silico analysis. The variation in the level of expression of the serum lysozyme gene was studied by quantitative real-time PCR. Therefore, a comprehensive analysis at all levels of the central dogma of serum lysozyme gene, i.e. DNA, RNA, and protein was established in this study.

2. Materials and methods

2.1. Experimental animals for collection of blood, serum, and milk samples

Total 200 animals, 100 from each breed of goat viz. Barbari (from ICAR-Central Institute for Research on Goats, Mathura, Uttar Pradesh) and Marwari (from Rajasthan Agricultural University, Bikaner, Rajasthan) were included for blood (5 mL with EDTA as an anticoagulant) and milk (1 mL) sample collection. The goats were maintained under a semiintensive management system with 6–7 h of grazing and feeding with seasonally available green fodder, supplemented with concentrate mixtures depending upon the status and age of the animals. Animals were usually housed separately according to their age, sex, physiological condition, and state of health. The Barbari and Marwari goats were selected as they belong to the different locality of India representing a diversified population. Hence, a comparative analysis became possible. For expression profile analysis, blood samples of five animals each from Barbari and Black Bengal (from ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh) goats were taken, which was compared with locally available nondescript goats. Since Marwari goats were available far away from the experimental center, Black Bengal goats were used instead. All the samples were collected as per the ethical guidelines of the institution after getting permission from the competent authority.

2.2. Isolation of genomic DNA and amplification of the serum lysozyme gene

Genomic DNA was isolated from the blood samples by the conventional extraction method [7]. The isolated DNA samples were checked for quality (Horizontal agarose gel electrophoresis- Genei, India), purity (UV-spectrophotometer- Eppendorf, Germany), and concentration (Nanodrop Qiagen, India). The $OD_{260/280}$ ratio should be 1.7 to 1.9. The genomic DNA concentration should be minimum 50–100 ng/µL. Two different DNA fragments of length 275 bp (fragment I) and 230 bp (fragment II) were amplified using the designed (by Oligoanalyser) primer P_1 (5'-CAA ATG GGA TGG AAT GAA-3') and P_2 (5'-CAA TAA AAC TGA AAG GAA AAA-3') for fragment I, P_3 (5'-AAT ACT TGG ATC TGT CTG T-3') and P_4 (5'-AAA ATG GGT TGA AGT AAA-3') for fragment II. The amplified PCR products were checked by 1.5% agarose gel electrophoresis.

2.3. Single strand confirmation polymorphism (SSCP) and nucleotide sequence analysis

Single nucleotide polymorphisms (SNPs) were screened in both the fragments using SSCP technique [8]. The PCR products were resolved on polyacrylamide gel and silver stained for visualization of bands [9, 10, 11]. The alleles were selected based on their differential SSCP patterns and then cloned into pGEM-T Easy cloning vector (Promega, USA). The frequency of serum lysozyme gene and genotypes were estimated [12].

2.4. Estimation of serum lysozyme level and somatic cell count

Serum lysozyme level was determined using the 'lysoplate' assay method with some modifications [13]. Lypolised *Micrococcus lysodeikticus* was used as the substrate. The standard curve was prepared with known lysozyme concentration (Sigma, USA), and the lysozyme level in the test serum sample was estimated. Somatic cell count (SCC) was done in the collected milk samples [13]. On a clean grease-free slide, milk sample (10µL) was added to a 1 cm^2 area. It was uniformly smeared and allowed to dry. One drop of Newman's Lampert stain was applied to the field. The slide was washed after 10 min, air dried, and observed under oil-immersion lens at 100×. Somatic cells were counted 3 times each in 10 fields and recorded. The association study of the serum lysozyme activity and somatic cell count with the identified SSCP genotypes was done by ANOVA analysis [14].

2.5. Cloning, sequencing, and analysis of serum lysozyme coding sequence (CDS)

Total RNA was isolated from the white blood cells, and cDNA was synthesized by reverse transcription-polymerase chain reaction (RT-PCR) using the gene specific forward primer (P_5 - 5'-ATG AAG GCT CTC ATT ATT CTG -3') and reverse primer (P_6 - 5'-TTA CAC TCC ACA ACC CTG AA -3') by RT-PCR Kit (MBI Fermentas, USA) as suggested by the manufacturer instructions. The amplified cDNA was checked in 1.2% horizontal agarose gel electrophoresis with standard DNA molecular weight marker (MBI Fermentas, USA), eluted by Gel Extraction kit (Qiagen, USA) and cloned into pGEM-T Easy cloning vector (Promega, USA). The nucleotide sequence was

determined by Sanger's dideoxy chain termination sequencing method in Automatic ABI Prism DNA sequencer and analyzed in BLAST (www.ncbi.nlm.nih.gov/BLAST) tool. The phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA) 7 tool.

To delineate the functional domains, the translated amino acid sequences were analyzed by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The translated protein sequences were analyzed by ProtParam online program (http://web.expasy.org/protparam/) to examine physicochemical properties *viz*. molecular weight, theoretical pI estimated half-life, instability index, and grand average of hydropathy (GRAVY). The three-dimensional structure of lysozyme protein was predicted using Modeller 9.19 (L1), Phyre2 (L2), Geno3D (L3), and Swiss Model (L4). The amino acid sequence of lysozyme in FASTA format was retrieved from NCBI (Accession no: ACX37695.1 for Barbari and ACX50258.1 for Black Bengal goat). The amino acid sequence was searched for a template. The best possible structure was predicted considering the DOPE (Discrete Optimized Protein Energy) score. The FASTA sequence of the caprine lysozyme protein was directly used as the input material for structure prediction in Phyre2, Geno3D, and Swiss Model. The predicted three-dimensional protein structures were used as inputs in RAMPAGE to obtain the Ramachandran Plots. The hydrophobicity plots analysis of the three-dimensional protein structures was done using Discovery Studio 3.5. The predicted three-dimensional structure was refined using RasMol. The ligand-receptor interaction was identified using Discovery Studio 3.5.

2.6. Differential expression analysis of serum lysozyme gene

The difference in the expression level of serum lysozyme gene in three groups viz. Barbari, Black Bengal, and nondescript goats (5 animals from each group) was studied using quantitative real-time PCR (Stratagene Mx3000p). The animals were healthy, free from any diseases, and above six months of age. The collection of fresh blood samples from Marwari goat was a difficult process since the animals were kept away from the research station. Therefore, instead of Marwari, Black Bengal was selected for expression analysis. The nondescript animals were included for a better understanding of expression level as a method of comparison. Two sets of intron spanning primers, one gene specific primer for serum lysozyme gene (P_7 - 5'-CTG GAT GTG TTT GGC CAG ATG-3', P_8 - 5'-CCA CCA GTC GCT ATT GAT TTG-3') with product length 112 bp and other for β–actin, (P_9 - 5'-ATC ATG AAG TGT GAC GTC GAC-3', P_{10} - 5'-CAG TGA TCT CTT TCT GCA TCC-3') as internal control with product length 112 bp were used for amplification. The cDNA was prepared using Brilliant SYBR Green real-time PCR master mix (Qiagen, USA) to quantify mRNA expression. Negative control having all ingredients except cDNA was also kept ascertaining contamination-free reaction set up. The threshold cycle (C_t) values for all the reactions were recorded. Fold change in the expression level of Barbari and Black Bengal goats was compared with nondescript goats after normalization with internal control [15].

Fold change in mRNA level in treatment group = $2^{\Delta\Delta Ct}$

Where,

$\Delta\Delta Ct = \Delta Ct$ of target – ΔCt of internal control

Where,

ΔCt of target =Ct of target gene in breed under study – Ct of target gene in other breed.

ΔCt of internal control = Ct of internal control gene of breed under study – Ct of internal control in other breed

All the statistical tests were done by one-way ANOVA followed by Tukey's honestly significant difference (HSD) as a multiple comparison test.

3. Results

3.1. DNA polymorphism and sequencing of serum lysozyme gene

The good quality genomic DNA was isolated from all the collected blood samples. The genomic DNA fragments I and II of serum lysozyme gene were amplified successfully in all the samples (Figure 1 and Figure 2). The PCR-SSCP analysis of fragment I (275 bp) revealed three genotypes (Figure 3) viz. AA (0.28), BB (0.28) and CC (0.44) in Barbari goats and four genotypes in Marwari goats (Figure 4) *viz*. AA (0.23), BB (0.27), AB (0.38) and CC (0.12). The frequency of AB and CC genotypes were highest in Marwari and Barbari goats, respectively. The gene frequency of alleles 'A', 'B' and 'C' were 0.280, 0.280 and 0.440 in Barbari goats and 0.425, 0.455, and 0.120 in Marwari goats, respectively. Few genotypes were completely missing from the studied population of goats.

The PCR-SSCP analysis of fragment II (230 bp) of serum lysozyme gene revealed four genotypes (Figure 5) viz. AA (0.23), BB (0.18), AC (0.15) and AD (0.44) in Barbari goats and 5 genotypes (Figure 6) *viz*. AA (0.16), BB (0.22), AD (0.27), AE (0.34) and AF (0.01) in Marwari goats. The homozygous genotypes *viz*. CC, DD, EE and FF could not be identified in any of the population. The allelic frequency of 'A' allele was the highest (0.525), followed by 'B' (0.180), 'C' (0.075), and 'D' (0.220) alleles in Barbari goats. The allelic frequency of 'A' allele was the highest (0.47), followed by 'B' (0.220), 'E' (0.170), 'D' (0.135), and 'F' (0.010) alleles in Marwari goats. After nucleotide sequence of these alleles (Table 1 and 2) were submitted to NCBI GenBank *viz*. GU014288 (Allele 'A'), GU014289 (Allele 'B') and GU014290 (Allele 'C') of fragment I whereas GU256264 (Allele 'A'), GU256264 (Allele 'B'), GU256265 (Allele 'C'), GU256266 (Allele 'D'), GU256267 (Allele 'E') and GU256268 (Allele 'F') of fragment II.

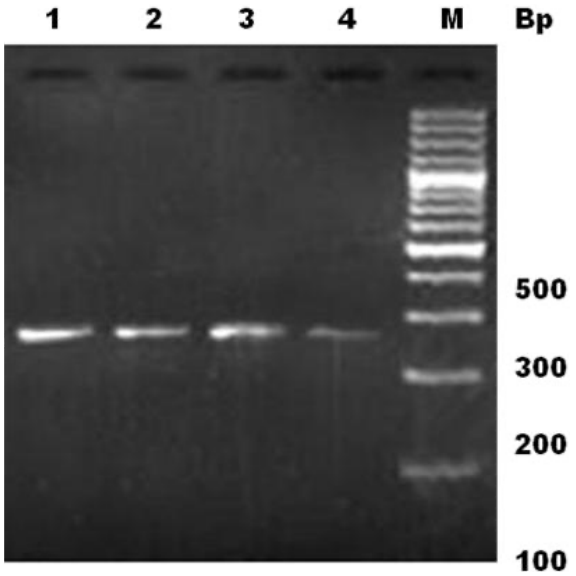

Figure 1. Amplification of the fragment I (275 bp) of serum lysozyme gene in goat Lane M: 100 bp DNA ladder. Lane 1-4: Amplified PCR product of serum lysozyme gene in Barbari and Marwari goats.

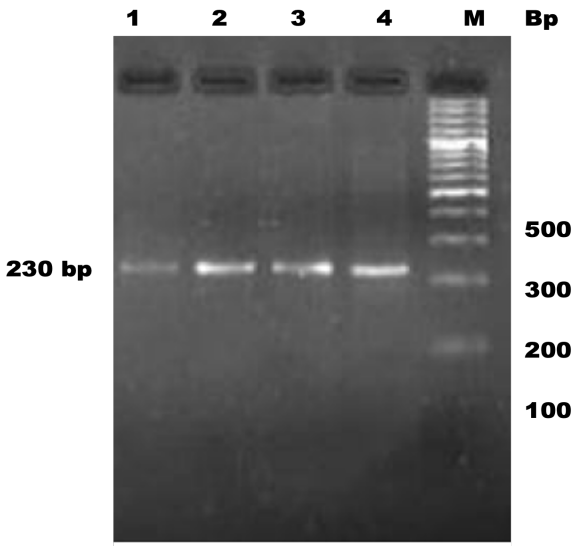

Figure 2. Amplification of the fragment II (230 bp) of serum lysozyme gene in goat Lane M: 100 bp DNA ladder. Lane 1-4: Amplified PCR product of serum lysozyme gene in Barbari and Marwari goats.

3.2. Serum lysozyme activity and milk somatic cell count in goat

The mean serum lysozyme activity in Marwari goats was higher than Barbari goats (Table 3). The genotype wise mean serum lysozyme activity was calculated for both the fragments in both the breeds (Table 4). Although

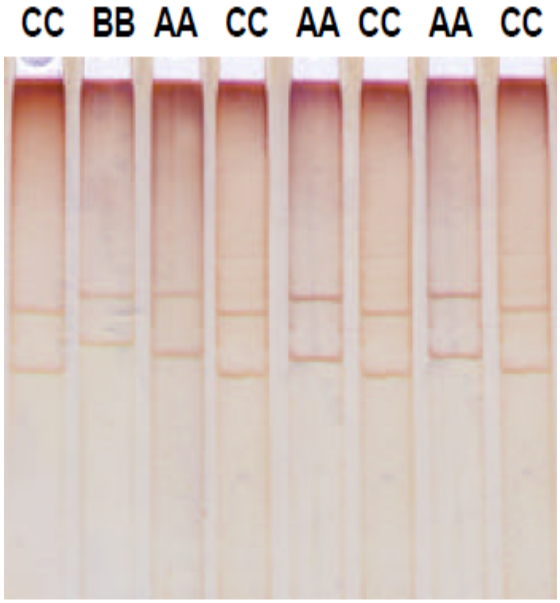

Figure 3. SSCP analysis showing different banding patterns of the fragment I of serum lysozyme gene in Barbari goat.

Figure 4. SSCP analysis showing different banding patterns of the fragment I of serum lysozyme gene in Marwari goat.

the mean values were apparently different in both the breeds for different genotypes of fragment I, a significant difference ($p \leq 0.05$) was found among different genotypes in Marwari goats only. In Marwari goats, BB genotype (2.54±0.29 µg/mL) had the highest mean serum lysozyme activity followed by the genotype AA (2.04±0.20 µg/mL), CC (1.88±0.28 µg/mL) and AB (1.62±0.15 µg/mL). Despite the difference in the mean serum lysozyme activity, the genotypes of fragment II did not show any statistically

Figure 5. SSCP analysis showing different banding patterns of the fragment II of serum lysozyme gene in Barbari goat.

Figure 6. SSCP analysis showing different banding patterns of the fragment II of serum lysozyme gene in Marwari goat.

significant effect on serum lysozyme activity in either of the breeds.

The mean somatic cell count in Barbari and Marwari goat milk was estimated (Table 5). The mean somatic cell count of milk in different genotypes was also calculated (Table 6). Although the mean somatic cell count varied in different genotypes, genotypes did not reflect any statistically significant effect on somatic cell count values in both the fragments.

Table 1. Allele-wise nucleotide variation in fragment I (275 bp) of serum lysozyme gene.

Allele	Nucleotide position		
	95th	159th	190th
Allele A	G	T	G
Allele B	G	C	G
Allele C	A	T	A

3.3. Cloning and nucleotide sequence analysis of serum lysozyme gene of goat

The quality and integrity of the isolated total RNA was found to be informative in formaldehyde agarose gel electrophoresis. The CDS of the lysozyme gene for both breeds was amplified and confirmed in agarose gel electrophoresis (Figure 7). The amplified products were eluted and cloned. The plasmid was isolated and subsequent plasmid PCR confirmed the presence of the desired 447 bp insert. The nucleotide sequences obtained from the cloned products were submitted to NCBI GenBank (Accession No: GQ889414 and GQ903681) and analyzed. The GC content was 46% in both Barbari and Black Bengal goats. A single nucleotide difference was detected at the 93rd position (G to A), where guanine in Barbari goat was replaced by adenine in Black Bengal goat. No amino acid change was detected in deduced amino acid sequences of lysozyme gene. So, the change in nucleotide sequence at the 93rd position of CDS can be considered as a silent type of mutation. The nucleotide sequences of serum lysozyme gene of goat and other species (Sheep-DQ480756, Cattle-U25810, Pig-NM214392 and Human-M19045) were compared using MEGA 7. The comparative sequence analysis of goat and cattle revealed the difference at 23 places whereas, the comparative analysis of sequence with sheep revealed differences only at 3 places. The percent similarity analysis showed that the nucleotide sequence of cDNA of Barbari goat has 99.8% similarity with the Black Bengal goat (Table 7). The CDS of both the breeds of goat were closer to the sequences of sheep followed by cattle, pig, and human. In the phylogenetic tree, it was observed that Barbari and Black Bengal goats formed a cluster and found closer with sheep, whereas cattle, human, and pig formed another cluster (Figure 8).

The CDS was translated *in silico* to get the polypeptide sequence of 147 amino acids which was compared with amino acid sequences of other species (Table 8 and 9). SMART analysis indicated that serum lysozyme protein had molecular weight 16.445 K Da, signal peptide (1–18) and motif having lysozyme activity (19-147). The theoretical pI 8.46 suggested the alkaline nature of the protein. The average estimated half-life is 30 hours in mammalian

Table 2. Allele-wise nucleotide variation in fragment II (230 bp) of serum lysozyme gene.

Allele	Nucleotide position											
	4th	59th	60th	62nd	63rd	64th	70th	78th	105th	123rd	225th	230th
Allele A	A	C	A	A	G	G	A	-	T	T	C	T
Allele B	A	C	A	A	G	G	A	-	T	A	C	A
Allele C	A	A	T	T	A	A	C	-	T	T	C	T
Allele D	A	C	A	A	G	G	A	A	-	T	C	T
Allele E	T	C	A	A	G	G	A	-	T	T	C	T
Allele F	A	C	A	A	G	G	A	-	T	T	A	T

Table 3. The mean serum lysozyme activity in goat breeds.

Breed	Serum lysozyme activity (µg/mL)	
	Mean ± SE	Range
Marwari	2.01 ± 0.12^{b}	0.42 to 5.92
Barbari	1.60 ± 0.08^{a}	0.46 to 4.41

Different superscript indicates significant difference at 5% level of significance.

Table 4. The mean lysozyme activity in different genotype of goats.

Fragments	Genotypes	Mean (± SE) lysozyme activity (µg/mL)	
		Marwari goat	Barbari goat
Fragment-I (275 bp)	AA	2.04 ± 0.20^{b}	1.62 ± 0.12
	BB	2.54 ± 0.29^{c}	1.66 ± 0.13
	AB	1.62 ± 0.15^{a}	-
	CC	1.88 ± 0.28^{b}	1.55 ± 0.16
Fragment-II (230 bp)	AA	1.95 ± 0.27	1.79 ± 0.21
	BB	1.65 ± 0.26	1.64 ± 0.18
	AC	-	1.45 ± 0.20
	AD	1.98 ± 0.21	1.51 ± 0.13
	AE	2.28 ± 0.21	-
	FF	2.62 ± 0.31	-

Different superscript indicates significant difference at 5% level of significance.

system. The instability index of 36.12 suggested it as a stable protein. The GRAVY index of –0.079 was indicative of a hydrophilic and soluble protein.

Table 5. The mean somatic cell counts in goats.

Breed	Somatic Cell Counts (10^5/ml)	
	Mean ± SE	Range
Barbari	1.90 ± 0.26^{a}	1.11 to 13.0
Marwari	2.60 ± 0.19^{b}	1.12 to 14.8

Different superscript indicates significant difference at 5% level of significance.

Table 6. Effect of the genotype on somatic cell count in goats.

Fragments	Genotypes	Mean (± SE) Somatic Cell Count (10^5/ml)	
		Barbari	Marwari
Fragment-I (275 bp)	**AA**	1.74 ± 0.23	2.31 ± 0.30
	BB	1.91 ± 0.20	3.29 ± 0.46
	AB	-	2.18 ± 0.18
	CC	2.06 ± 0.22	2.63 ± 0.56
Fragment-II (230 bp)	**AA**	2.63 ± 0.39	2.18 ± 0.31
	BB	1.71 ± 0.26	2.46 ± 0.35
	AC	1.10 ± 0.43	-
	AD	2.15 ± 0.21	3.04 ± 0.34
	AE	-	3.04 ± 0.35
	FF	-	2.23 ± 0.32

The three-dimensional structures were predicted by four different analysis tools (Figure 9). The 3LN2 chain A (Chain A, Crystal Structure of A Charge Engineered Human Lysozyme) was selected as the template in Modeller 9.19 based on the identity (85%) and query coverage (87%). The Ramachandran Plots (gives an exact distribution of the amino acid residues in the three different regions, i.e. favoured

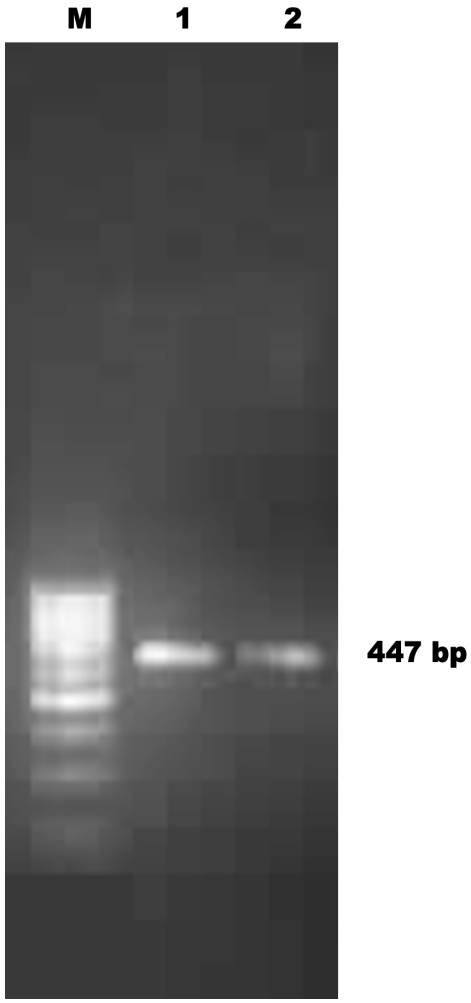

Figure 7. Amplified CDS of the serum lysozyme gene in goat. Lane M: 50 bp DNA ladder. Lane 1: Amplified CDS of the serum lysozyme gene in Black Bengal goat. Lane 2: Amplified CDS of the serum lysozyme gene in Barbari goat.

Table 7. Percentage identity and the divergence of the coding sequence of lysozyme gene.

Breed/ Species	Barbari	Black Bengal	Sheep	Cattle	Pig	Human
Barbari		99.8	99.1	95.1	81.7	88.4
Black Bengal	0.2		99.3	95.3	81.9	88.6
Sheep	0.9	0.7		94.4	81.1	87.8
Cattle	5.1	4.9	5.1		81.1	86.8
Pig	21.3	20.9	21.3	22.2		80.3
Human	12.8	12.5	12.8	14.7	23.1	

Up diagonal elements show percent identity and below diagonal elements shows percent divergence.

region, allowed region, and outlier region) of the predicted structures were made by RAMPAGE server. The L4 structure (by Swiss Model) with a maximum percentage of residues in the favoured region and no residues in the outlier region was accepted as the best predicted three-dimensional structure (Table 10). However, hydrophobicity plots revealed that all the four modeled structures had the required configuration in terms of their interaction with water, solvents, and other cellular molecules. The ligand-receptor interaction sites were located using Discovery Studio 3.5 (Figure 10).

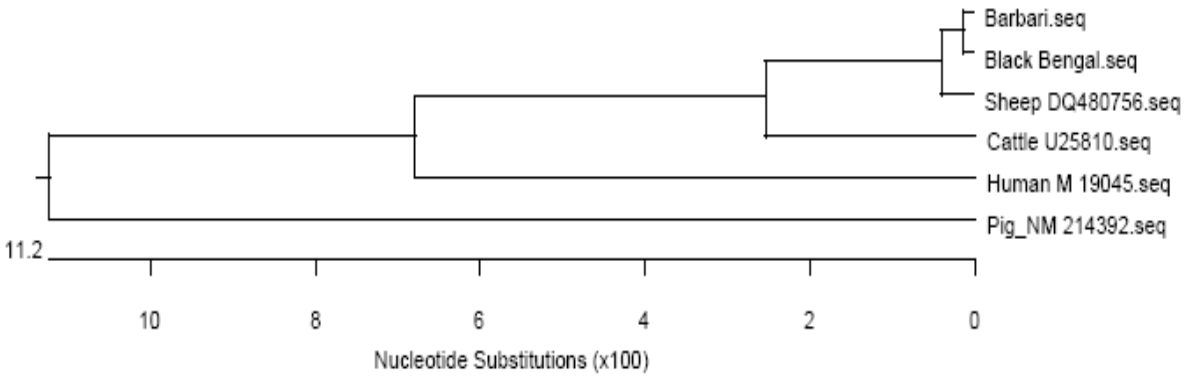

Figure 8. The phylogenetic relationship of different species considering nucleotide sequence of the lysozyme gene. The Black Bengal and Barbari goats are present in one cluster.

Table 8. Comparative amino acid sequence of the lysozyme gene in different livestock species.

	Amino Acid Position									
Breed/Species	5th	12th	18th	29th	37th	55th	65th	68th	97th	114th
Barbari	I	L	G	T	N	S	S	R	I	A
Black Bengal	I	L	G	T	N	S	S	R	I	A
Sheep	I	L	A	T	N	S	S	R	I	A
Cattle	L	F	G	S	G	N	A	Q	L	T

Table 9. Percentage identity and the divergence at amino acid level of lysozyme protein in different livestock species.

Breed/ Species	**Barbari**	**Black Bengal**	**Buffalo**	**Cattle**	**Sheep**	**Pig**	**Human**	**Mouse**
Barbari		99.8	81.5	82.0	99.1	77.9	87.7	76.7
Black Bengal	0.2		81.1	81.5	99.3	78.3	87.9	77.0
Buffalo	17.1	17.4		97.7	80.9	73.0	73.9	62.2
Cattle	17.1	17.4	2.3		81.3	73.2	74.3	62.2
Sheep	0.9	0.7	17.6	17.6		78.3	87.5	77.2
Pig	21.1	20.8	25.6	26.0	21.1		77.0	75.2
Human	12.1	11.8	24.9	24.8	12.0	23.1		75.8
Mouse	25.3	25.0	38.2	38.6	25.0	26.1	26.1	

Up diagonal elements show percent identity and below diagonal elements shows percent divergence.

3.4. Differential expression analysis of serum lysozyme gene

A significant difference was found in the expression level of the serum lysozyme gene among three goat populations (Figure 11). Nondescript animals showed higher expression levels as compared to animals of the other two well-defined breeds. In Barbari goats, mRNA level was downregulated by 11.31 folds as compared to nondescript goats, whereas, in Black Bengal goat, mRNA level was downregulated by 4.92 folds as compared to nondescript goats. After comparing the two well-defined breeds, it was found that Black Bengal goats have 2.3 folds higher expression levels of serum lysozyme gene as compared to Barbari goats.

4. Discussion

4.1. DNA polymorphism in serum lysozyme gene

PCR-SSCP analysis is a widely used screening method that allows the identification of different genomic variants in a large number of samples. SSCP analysis detects the sequence

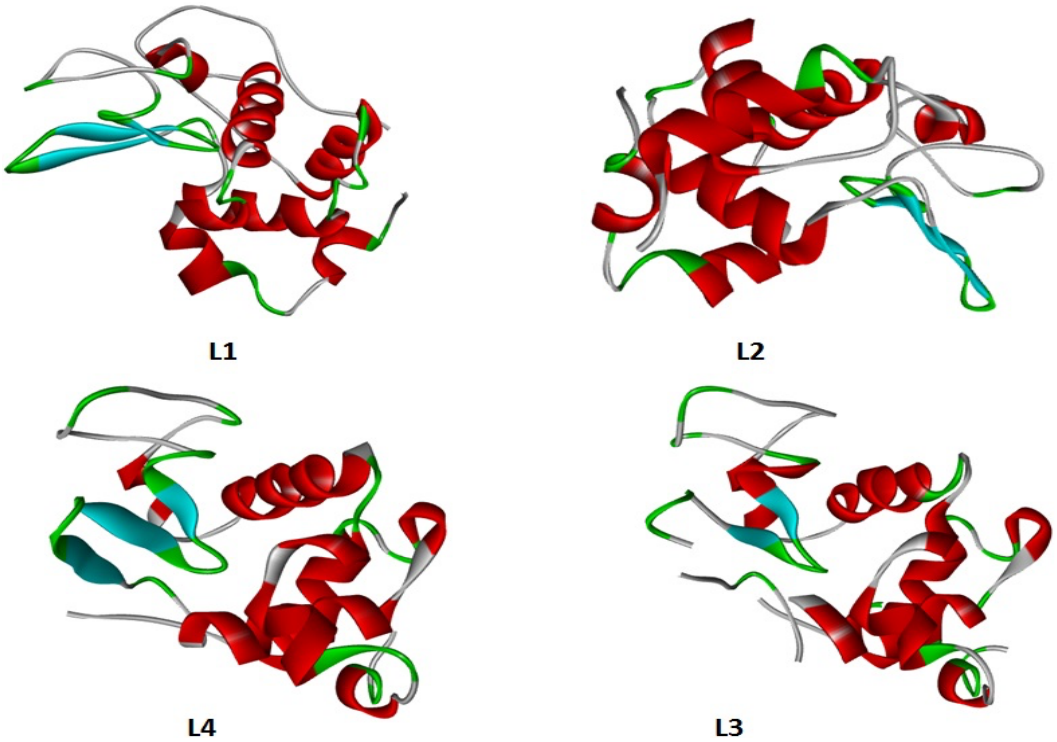

Figure 9. The three-dimensional protein structures generated using different tools, i.e. Modeller 9.19 (L1), Phyre2 (L2), Geno3D (L3) and Swiss Model (L4).

Table 10. Evaluation of the residues in Ramachandran plot prepared by RAMPAGE.

Protein Structure	Favoured Region (%)	Allowed Region (%)	Outlier Region (%)
L1	95.9%	4.1%	0.0%
L2	93.2%	4.8%	2.1%
L3	87.7%	9.8%	2.5%
L4	96.9%	3.1%	0.0%

variations through electrophoretic mobility differences. The absence of AB genotype in fragment I in Barbari goats can be considered as a marker for breed identification and characterization. In fragment II, the genotypes AE and AF could not be identified in Barbari goats, whereas AC genotype could not be identified in Marwari goats, which may be useful in breed characterization. Any report of SSCP analysis of serum lysozyme gene was lacking in goats. So, the comparative analysis could not be done.

4.2. Serum lysozyme activity and milk somatic cell count in goat

The mean lysozyme activity was lower in Barbari goat than in Marwari goat. Earlier, the mean serum lysozyme activity in Barbari goats was reported to be in a range from 0.048 to 3.236 µg/mL [16]. The mean serum lysozyme activity in goats of the present study was comparable with cattle i.e. 3.16 µg/ml and 2.26 µg/ml in Rathi and Tharparkar breeds, respectively but found to be much lower as compared to buffalo i.e. 27.35 µg/ml [17,18]. The statistical significant ($P<0.05$) effect of genotypes on serum lysozyme activity was only found in fragment I of Marwari goats. Lack of significant association in mean lysozyme activity and rest of the genotypes in both of the breeds may be attributed to insufficient sources of variation for statistical correction and nonsignificant contribution of variation at this particular locus to the total variance. Any statistically significant effect of the genotypes on somatic cell count of milk on goats could not be identified. The lack of significant association may be due to the absence of sufficient source of variations.

4.3. Cloning and nucleotide sequence analysis of serum lysozyme gene of goat

The mature peptide of goat serum lysozyme contains 14 strongly basic (K and R) and 11 strongly acidic (D and E) amino acid residues, so the isoelectric point of goat lysozyme was calculated to be 8.123. Earlier studies reported the molecular weight of bubaline lysozyme protein as 16 K Da [19,20]. However, the molecular weight of bovine lysozyme protein was reported as 18 K Da, which was higher than that of goat [21].

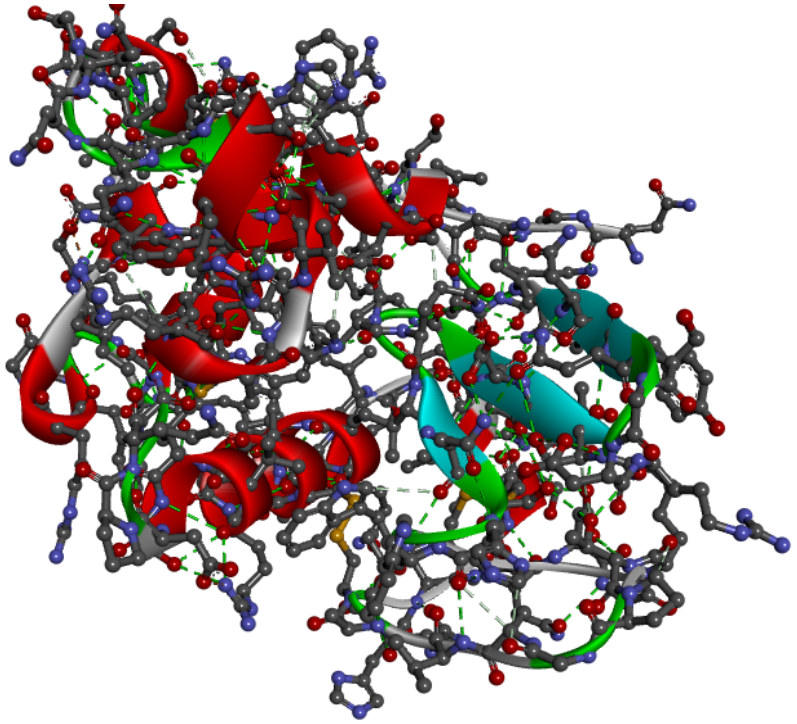

Figure 10. Serum lysozyme protein of goat showing the ligand receptor interaction sites.

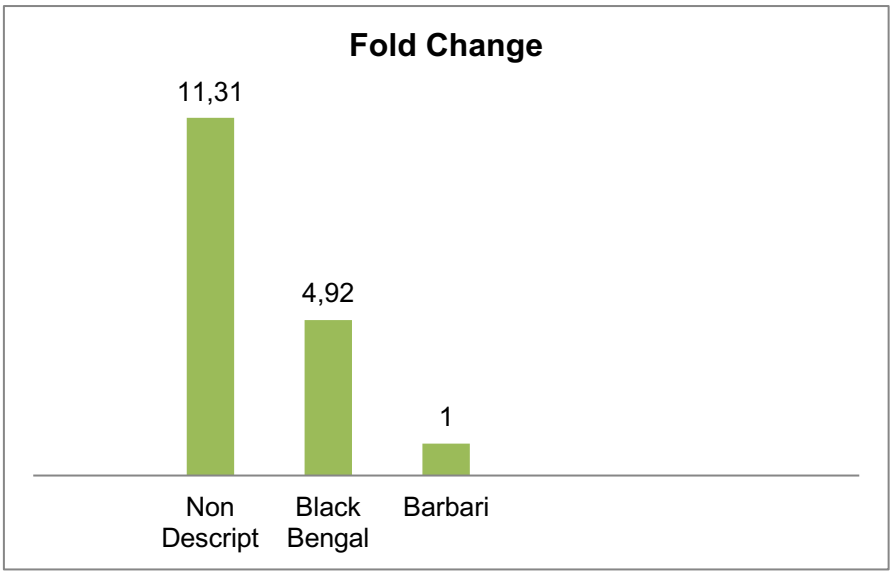

Figure 11. The comparative expression levels of lysozyme gene. The highest expression level is seen in non descript goats followed by Black Bengal and Barbari goats.

Modeller implements comparative protein structure modeling by satisfaction of spatial restraints [22]. Phyre2 is a bioinformatics tool to predict the protein structure with analysis of its functions and mutations [23]. Geno3D uses distance geometry, simulated annealing, and energy minimization algorithms to build three-dimensional protein models [24]. Swiss Model workspace is a web-based integrated service dedicated to protein structure homology modelling [25]. The three-dimensional protein structure was predicted using the above mentioned bioinformatics tools. The Ramachandran Plot presents allowed and disallowed regions for each amino acid residue. Disallowed zones in the Ramachandran Plot correspond to conformation distributions where steric

clashes make protein folding energetically very expensive for all residues [26]. The hydrophobicity plot determines the average hydrophobicity in a segment from the N terminal to the C terminal of the amino acid chain. These plots are useful in determining the hydrophobic interior portions of globular proteins and membrane-spanning regions of membrane-bound proteins [27]. Based upon Ramachandran Plot and hydrophobicity plot analysis all the four predicted protein structures were found to be satisfactory. However, the protein structure predicted by Swiss Model was found to be the most stable. Since it was the first report of predicted protein structure of goat lysozyme it could not be compared.

4.4. Differential expression analysis of serum lysozyme gene

Nondescript goats, maintained in the field condition, generally faced more challenge of infection than the goats from well-organized farm. This might be the reason for the elevated level of serum lysozyme gene expression in blood of nondescript goats. Higher expression level in Black Bengal goats as compared to Barbari goats is suggestive of its elevated strength of health potential.

5. Conclusion

The serum lysozyme gene was found to be polymorphic in Indian goats. The quantitative mRNA expression level of the serum lysozyme gene was variable in different goat populations causing their differential disease resistance potential. The functional aspects of the gene is more understood through amplification of full-length coding sequence and subsequent bioinformatics analysis of its protein structure.

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Conflict of interest

The authors declare that there is no conflict of interest.

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