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Differential Expression of Serum Lysozyme Allelic Variants in Muzaffarnagri Sheep

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ABSTRACT

Lysozyme degrades the bacterial cell walls and gives rise to degradation product that stimulates and activates the immune system. Several gram positive and gram negative bacteria were found to be susceptible to different degree of purified lysozyme. Variation in promoter region may regulate the expression of a particular gene. Hence, considering lysozyme gene a potential marker for general immune response, expression pattern of various genotypes on the basis of variations in promoter region is investigated in Muzaffarnagri sheep. A 268 bp fragment spanning partial promoter, exon 1 and partial intron 1 of serum lysozyme gene were amplified and sequenced. Sequencing revealed five genotypes AA, AB, AC, BB and CC and consequently three alleles A, B and C in Muzaffarnagri sheep. Differential expression study of various genotypes by real time pcr revealed significant difference ($P \leq 0.05$) in the serum lysozyme expression in animals having different genotypes. Animals having AA genotype showed higher expression of serum lysozyme than the animals having AB, AC and BB genotype.

Keywords: Lysozyme, genotype, expression, sheep

Lysozyme is a hydrolase enzyme, degrades the bacterial cell walls and gives rise to degradation product that stimulates and activates the immune system (Jolles and Jolles, 1984). Lysozyme is considered to be constituents of primitive unspecific defense mechanism associated with the monocyte macrophage system (Flemming, 1922). Antibacterial property of lysozyme is mediated through its direct bacteriolytic action as well as via stimulatory effect on macrophage phagocytic function. Several gram positive and gram negative bacteria were found to be susceptible to different degree of purified lysozyme from bovine and human milk (Vakil *et al.,* 1969). This enzyme usually functions in association with lactoferrin or immunogenic A. It is effective against *Escherichia coli* and causes lysis of some species of Salmonella. It can limit the migration of neutrophils into damaged tissue and functions as anti-inflammatory agent (Dayal *et al.,* 2012). Some workers suggested that it can be used as candidate gene for improvement of mastitis resistance (Seyfert *et al.,* 1996). Lysozyme gene in all mammalian species comprised of four exons and three introns. Lysozyme gene has been found to be polymorphic in various species including cattle, buffalo, goat and sheep (Maga *et al.,* 2006; Sahoo *et al*., 2010; Dayal *et al*., 2012).Variation in promoter region may regulate the expression of a particular gene. Hence, considering lysozyme gene a potential marker for general immune response as well as mastitis resistance, expression pattern of various genotypes on the basis of variations in promoter region needs to be studied. Hence present investigation was undertaken to study the differential expression of various genotypes of serum lysozyme gene in Muzaffarnagri sheep.

MATERIAL AND METHODS

Collecton of Blood sample

Blood samples were collected randomly from 75 Muzaffarnagri sheep of same sex and season of birth from the organized herds. Genomic DNA was extracted from 5 ml of blood by phenol-chloroform extraction method with slight modification (Dayal *et al*., 2005). 1-2 ml of blood was collected without adding anticoagulant for the isolation of serum for the evaluation of serum lysozyme activity. For cDNA construction, about 5 ml blood samples was collected in a DEPC treated sterile vial and transported in icebox.

Isolation of total RNA

Total RNA was isolated from white blood cells using TRIzol reagent (Sigma-Aldrich, Germany) and chloroform and subsequently precipitated using isopropanol. The extracted RNA was then treated with the RNase-Free DNase Set (Qiagen) for removal of genomic DNA. The quality of RNA was checked in 1.0% Formaldehyde Agarose gel.

PCR Amplification

A 268 bp fragment spanning partial promoter, exon 1 and partial intron 1of serum lysozyme gene were studied to identify the polymorphism expected to present at this loci. The primers used for amplification were designed from cattle whole gene sequence with laser gene software. Primers used for amplification were forward, 5'CCCAAACCAGTCACATAAGAAGGA3' and reverse 5'CTGGCTAACTATTTGAAAGGATGAA3'. PCR cycling conditions were standardized with different concentrations of $MgCl_2$, *Taq* polymerase, dNTPs and primers. PCR reaction is performed in a total volume of 25 µl with 100 ng of genomic DNA, 15 pmoles of each primer, 2.5 mM of $MgCl_2$, 200 µM of each dNTP, 1X PCR reaction buffer and 1 U of *taq* DNA polymerase. DNA was initially denatured for 5 min at 95°C, then 34 cycles of denaturation for 30's at 95°C, annealing for 45 sec at 50°C and extension for 60 sec at 72°C followed by final extension for 10 min at 72°C.

Single strand conformation polymorphism (SSCP)

Single strand conformation polymorphism was performed to identify various genotypes. A total volume of 2.5 µl of PCR product was properly mixed with 17.5 µl formamide dye (95% formamide, 0.025%xylen cyanol and 0.025% bromophenol blue and 4.5% 0.5 M EDTA). The mixture was denatured at 95°C for 5 minand snapped cool on ice for 15 min. Finally mixture was run on 10% native PAGE (50:1, acrylamide and bis-acrylamide) with 5% glycerol. The electrophoresis was performed at 4°C temperature for 14 h at 200V. After electrophoresis gel was stained with silver nitrate staining to visualize the banding patterns (Basam *et al.,* 1991).

Sequencing

PCR products belonging to different genotypes were run on 1% low melting agarose gel and the desired product was eluted from the gel using gel elution kit for purification. Purified PCR products were cloned by using TA cloning strategy in pGEMT easy vector. Cloned product was identified by blue white screening. Positive clones were sequenced by the automated dye-terminator cycle sequencing method.

cDNA synthesis

First strand of cDNA was synthesized from the individual RNA using Revet Aid H minus First Strand cDNA Synthesis Kit (Fermentas) as per the manufacturing protocol. Two pairs of primers, one for amplification of serum lysozyme

Table 1: Primers used for expression profiling

Gene	Primers	Sequence	Size (bp)
Serum lysozyme	P1	5'CTG GAT GTG TTT GGC CAG ATG3'	112
	P2	5'CCA CCA GTC GCT ATT GAT TTG3'	
β – actin	P3	5'ATC ATG AAG TGT GAC GTC GAC3'	112
	P4	5'CAG TGA TCT CTT TCT GCA TCC3'	

gene and other for amplification of β – actin gene which was used as internal control and have the same annealing temperatures as well as product size was designed on the basis of bovine and ovine sequences (Accession No. DQ 480756 and U 25810) available publically at NCBI with the help of laser gene software (Table 1).

Real time PCR

qPCR reactions were setup for differential expression of serum lysozyme gene. Brilliant® SYBR® Green QPCR master mix (Qiagen) was used for the preparation of the reaction mixture. All the reactions were run in STRATAGENE Q-CYCLER using MX 3000P software. Each sample was run in triplicate both for target gene as well as internal control. Various combinations of reaction chemicals were tried to optimize the concentration of each component. Real time PCR reaction is performed in a total volume of 25 µl with 100 ng of template cDNA, 0.2 µM of reverse and forward primer and 12.5 µl 2X SYBR Green master mix. A negative control, containing all the reaction components except the template was also made to check any contamination of foreign DNA in the reaction components. Reaction samples were incubated at 95°C for 15 min, initially for activation of Taq polymerase. It was followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. than one cycle was run at 95°C for 5 sec, 65°C for 1 min and 97°C for 1 min for melting curve analysis. Gene expression results were calculated using the $2^{-\wedge\wedge}C_t$ method (Livak and Schmittgen, 2001) with β actin used to normalize the data. Fold changes in gene expression between factors were determined as described (Livak and Schmittgen, 2001).

Statistical analysis

Gene and genotype frequencies were calculated by gene counting method described by Falconer and Mackay (1998). Sequence comparison was performed with Laser gene software. A general linear model incorporating factors likeage, sire, dam within sire and genotype as fixed effect were employed to estimate the effect of genotype on serum lysozyme expression.

RESULTS AND DISCUSSION

Alleles and Genotypes

SSCP revealed that serum lysozyme gene is polymorphic in Muzaffarnagri breed of sheep. Five genotypes AA, AB, AC, BB and CC and consequently three alleles A, B and C (Fig. 1) were documented. Their frequencies were calculated as 0.30 for AA, 0.17 for AB, 0.36 for AC, 0.13 for BB, and 0.04 for CC, genotypes and 0.560 for A, 0.220 for B and 0.220 for C, alleles.

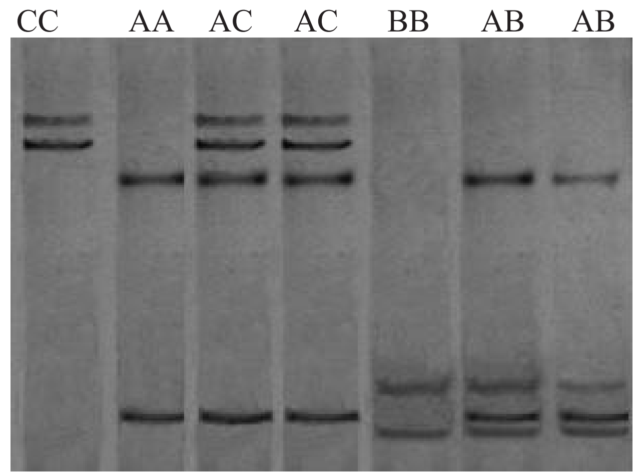

Fig. 1: SSCP genotypes of serum lysozyme gene (268 bp fragment)

DNA Sequencing

Sequence of all the alleles detected through sequencing is submitted to NCBI and accession numbers were obtained. Accession no. of various allelic variants of 268 bp fragment were GQ888734 (A allele), GQ888735 (B allele) and GQ888736 (C allele) Sequences of all the three allele A, B and C were aligned using MEGALIGN programme of DNASTAR software.

rom the alignment of the allelic variants (Fig 2), it was found that there were differences at 4 positions among the alleles, out of which one variation was found in the promoter region where as three variations were found in the intron 1. There was no variation in exon 1 region. B allele differs from A allele by having Thymine instead of Adenine at 247th position and Cytosine instead of Guanine at 260th position. C allele differs from A allele by having Guanine instead of Cytosine at 14th position and Thymine instead of Guanine at 262nd position. As there is no variation found in exonic region, amino acid sequence is identical for all the three alleles. Variation in promoter and intron regions is important as they might have an effect on regulation of serum lysozyme expression. Sahoo *et al.*

(2010) also reported 4 SNPs in the exonic region whereas 8 SNPs in intronic region in riverine buffalo. Pareek *et al.* (2003) also did not found any mutation in coding region however 2 SNPs were detected in intron 2 and 3 of the gene at 8603 and 9963 positions.

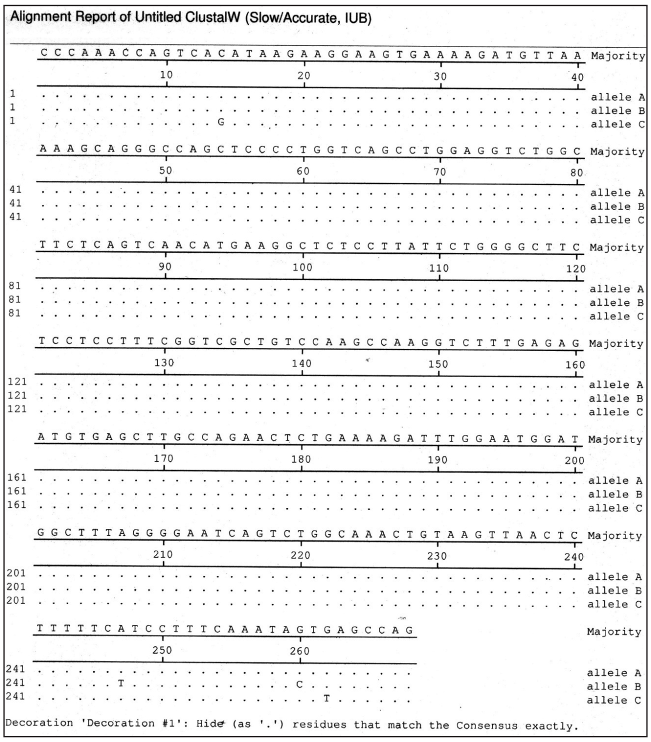

Fig. 2: Sequence alignment of allelic variants of 268 bp fragment of serum lysozyme gene

Differential expression by real time PCR

In the present investigation, the relative quantification of serum lysozyme in the different genotypes was studied on the basis of variations in the 268 bp fragment containing promoter region of Muzaffarnagri breed of sheep. Five genotypes viz AA, AB, AC, BB and CC were observed on the basis of this fragment in the Muzaffarnagri sheep. Animals having CC genotype was not included in the study due to their lower frequency. Real time PCR of each sample showed clear amplification plot and dissociation curve without any primer dimer formation. Present findings revealed significant difference ($P \leq 0.05$) in the serum lysozyme expression in animals having different genotypes (Table 2).

Table 2: Real time PCR result of serum lysozyme expression in term of ^CT and fold change

Genotypes	^Ct Value	Fold change with reference to AA Genotype
AA	3.27 ± 0.46^{a}	—
AB	5.95 ± 0.40^{c}	-6.38
AC	3.39 ± 0.46^{a}	-1.07
BB	5.40 ± 0.46^{b}	-4.36

Animals having AA genotype showed higher expression of serum lysozyme than the animals having AB, AC and BB genotype. With reference to animals having AA genotype it was observed that the level of expression in AB genotype was down regulated by 6.38 fold where as in AC genotype it was decreased by 1.07 fold. Animal having BB genotype showed decreased level of expression by 4.36 fold (Table 2). This could be due to the mutations in the B allele at 247th and 260th positions in the intron 1 where in thymine and cytosine (purine) residues are present; with respect to A and C alleles where adenine and guanine (pyrimidine) are present at these positions. These mutations may have a role in the regulation of serum lysozyme gene expression and can be used in future breeding programme for improvement of general disease resistance.

CONCLUSION

Lysozyme is a ubiquitous enzyme found in all major taxa of living organisms having diverse role starting from digestion to immune response. It is a potential marker for general immune response across the species. Hence, lysozyme genotype showing higher expression of serum lysozyme can be used in future breeding programme for improvement of general disease resistance traits in sheep.

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