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Genetic polymorphism of serum lysozyme gene and its association with serum lysozyme activity in indigenous breeds of sheep

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ABSTRACT

Lysozyme acts as an important antimicrobial component in serum and body fluids. Polymorphs of serum lysozyme gene are reported to be significantly associated with disease resistance traits. Therefore, the present study was undertaken to detect polymorphism in serum lysozyme at the genetic level and its association with mean serum lysozyme activity in indigenous breeds of sheep. A total of 275 animals of same sex and season of birth belonging to 3 breeds of indigenous sheep, viz. Chokla, Malpura, and Muzaffarnagri were used in the study. Genomic DNA was extracted from blood samples of the contributing animals and used to amplify 275bp fragment (partial intron 2, exon 3 and partial intron 3) of serum lysozyme gene by polymerase chain reaction. Subsequently, the SSCP study was carried out to identify different allelic patterns and genotypes of the animal included in the study. Seven genotypes AA, AB, AC, AD, AE, AF and DD and consequently 6 alleles namely A, B, C, D,E and F of varied sizes were found in different breeds of sheep. All the 6 alleles were present in Chokla and Malpura whereas only 4 alleles A, C, D and F were present in Muzaffarnagri breed of sheep. As allele was pre dominant in all the 3 breeds of sheep. Nucleotide sequencing of samples showing different genotypic pattern showed differences at nucleotide level between different alleles. Different alleles showed variations at 8 places. Genotypes showed significant ($P \leq 0.05$) associations with mean serum lysozyme activity in Malpura and Muzaffarnagri breed of sheep.

Key word: Association, Lysozyme, Polymorphism, Sequencing, Sheep

Lysozyme, a hydrolase enzyme, is an important antimicrobial component in serum and body fluids. The lysozymes are 1, 4, β-N-acetyl muramidase that enzymatically degrade a glycosidic linkage between C-1 of N-acetyl muramic acid and C-4 of N-acetylglucosamine in the bacterial peptidoglycan component of cell wall. This enzyme usually functions in association with lactoferrin or immunogenic A. Lysozyme is effective against *Escherichia coli* in concert with IgA. It causes lysis of some species of *Salmonella* in association with ascorbate and peroxide. In addition, it can limit the migration of neutrophils into damaged tissue and functions as anti-inflammatory agent. Seyfert *et al.* (1996) has suggested that lysozyme gene can be used as candidate gene for improvement of mastitis resistance. Hence, considering lysozyme gene as a potential marker for general immune response as well as mastitis resistance, this gene needs to be characterized. Despite its tremendous potential, very little work has been done so far in ruminants, except scattered information of its recombinant product in cattle and goat (Maga *et al.* 2006) and polymorphism study in cattle (Pareek *et al.* 2003, Pursinowska *et al.* 2003) and buffalo (Sahoo 2010). So far few reports are available for genetic characterization and polymorphism of serum lysozyme gene in any breed of indigenous sheep. Hence, the present investigation was undertaken to identify the allelic variants of lysozyme gene in indigenous breeds of sheep and to study their association with serum lysozyme activity in sheep.

MATERIALS AND METHODS

Blood samples were collected randomly from 275 sheep of 3 breeds namely Chokla (100), Malpura (100) and

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Muzaffarnagri (75) 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. Blood, 1–2 ml was collected without adding anticoagulant for the isolation of serum for the evaluation of serum lysozyme activity. The serum was extracted after clotting of blood then stored at –70°C till further analysis.

A 275 bp fragment spanning partial intron 2, exon 3 and partial intron 3 of serum lysozyme gene were analyzed for detection of polymorphism expected to be 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′CAAATGGGATGGAATGAA3′ and reverse 5′CAATAAAACTGAAAGGAAAAA3′. PCR cycling conditions were standardized with different concentrations of $MgCl_2$, *Taq* polymerase, dNTPs and primers. PCR reaction is performed in in a total volume of 25 µl with 100 ng of genomic DNA, 15 pmoles of each primer, 2 mM of $MgCl_2$, 100 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 44°C and extension for 60 sec at 72°C followed by final extension for 10 min at 72°C.

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 min and snapped cool on ice for 15 min. Finally mixture was run on 12% native PAGE (50:1, acrylamide and bis-acrylamide) with 5% glycerol. The electrophoresis was performed at 4°C temperature for 18 h at 200V. After electrophoresis gel was stained with silver nitrate staining to visualize the banding patterns.

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. The 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.

Serum lysozyme activity in each animal was determined by using 'lysoplate' assay method (Lie *et al*. 1986).

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 like age, sire, dam within sire and genotype as fixed effect and chi squire test were employed to estimate the effect of genotype on serum lysozyme activity.

RESULTS AND DISCUSSION

All the 3 breeds, viz. Chokla, Malpura and Muzaffarnagri

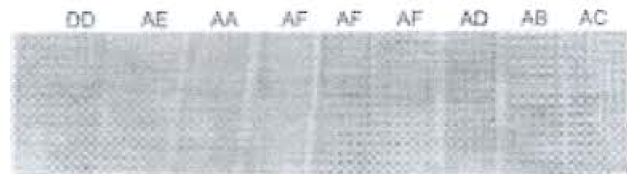

Fig 1. SSCP pattern of amplified serum lysozyme gene fragment

were found polymorphic for this locus (Fig. 1). Breed wise genotype and gene frequencies are shown in Table 1.

In Chokla sheep, 6 genotypes such as AA, AB, AC, AD, AE and AF were documented. At this locus, 6 alleles namely A, B, C, D, E and F were observed. In Malpura, 6 genotypes namely AA, AB, AC, AD, AE and AF were observed and consequently, 6 alleles viz. A, B, C D, E and F were found. In Muzaffarnagri sheep, 5 genotypes AA, AC, AD, AF, and DD and 4 alleles A, C, D and F were observed. AB genotype is the predominant genotype in Chokla (0.27) and Malpura (0.30) breeds of sheep whereas AC genotype is predominant in Muzaffarnagri breed (0.36) of sheep. Consequently it was calculated that A allele was predominant allele in all the 3 breeds of sheep. Other genotypes found to be frequent were AC in Chokla, AC and AD in Malpura and AA and AD in Muzaffarnagri breeds of sheep. Muzaffarnagri breed lacked B and E alleles. This information can be used for studying the characteristics and evolutionary relationship of these breeds of sheep. Frequency of heterozygotes is much more than the homozygotes. It might be due to natural selection favouring these genotypes or these genotypes may be artificially selected along with other characters, which were favored by artificial selection. Sahoo (2007) also reported polymorphism at this locus with 3 alleles in Murrah breed of buffalo.

In Malpura and Muzaffarnagri sheep, genotype showed significant effect ($P \leq 0.05$) on serum lysozyme activity. Though, there were differences in the lysozyme activity among different genotypes of Chokla sheep, these differences

Table 1. Genotype and gene frequency

Breeds	Genotype						
	AA	AB	AC	AD	AE	AF	DD
Chokla (N=100)	0.170 (n=17)	0.270 (n=27)	0.240 (n=24)	O.170 (n=17)	0.07 (n=7)	0.08 (n=8)	-
Malpura (N=100)	0.13 (n=13)	0.30 (n=30)	0.23 (n=23)	0.23 (n=23)	0.05 (n=5)	0.06 (n=6)	-
Muzaffarnagri (N=75)	0.27 (n=27)	-	0.36 (n=18)	0.24 (n=7)	- (n=3)	0.09 (n=20)	0.04
	Alleles						
	A	B	C	D	E	F	
Chokla	0.585	0.135	0.120	0.085	0.035	0.040	
Malpura	0.565	0.150	0.115	0.115	0.025	0.030	
Muzaffarnagri	0.613	-	0.180	0.160	-	0.047	

Table 2. Nucleotide differences between different alleles

Allele	Position							
	34	36	86	201	249	257	258	259
A	T	A	T	T	T	-	-	-
B	T	**G**	**C**	T	T	T	-	-
C	T	A	T	**C**	T	T	T	-
D	T	A	T	T	T	T	T	-
E	T	A	T	T	T	T	T	T
F	**C**	A	T	T	**G**	T	T	T

were found to be statistically nonsignificant. Animal having AE genotypes had highest serum lysozyme activity (2.71±0.26μg/ml) whereas animal having AF genotype had lowest serum lysozyme activity (1.59±0.29μg/ml) in Malpura sheep. Animals having AE genotype had 69% more mean serum lysozyme activity then the animals having AF genotype. The order of performance for serum lysozyme activity was AE>AB, AC, AD>AA>AF. In Muzaffarnagri sheep animals having AD genotype had highest serum lysozyme activity (3.31±0.29μg/ml) whereas animals having AA genotype has lowest serum lysozyme activity (2.03±0.28μg/ml). Animals having AD genotype had 63% more mean serum lysozyme activity than the animals having AA genotype. The order of performance for serum lysozyme activity was AD, DD>AF >AA, AC. Sotirov *et al.* (2005) also reported an interspecies or breed variation in lysozyme activity in swine, cattle and sheep. Sahoo (2010) had also reported the variation in serum lysozyme activity in different genotypes of Murrah buffalo. But, this difference was statistically nonsignificant.

Nucleotide sequencing revealed that various alleles identified are of different size. Size of A (Acc. No. GQ860956), B (Acc. No. GQ860957), C (Acc. No. GQ860958), D (Acc. No. GQ860959), E (Acc. No. GQ860960), and F (Acc. No. GQ860961) alleles are 272, 273, 274, 274, 275 and 275 bp, respectively. The allele wise nucleotide differences are presented in Table 2.

There were differences at 8 positions among the alleles, out of which 1 substitution was in exonic region whereas 3 substitutions were at partial intron 2 region and 1 substitution and 3 additions were at partial intron 3 region (Table 2). Although there was no variation in amino acid sequences of all the alleles, silent mutation in allele C is important since this mutated site may be relatively more prone to future mutation or indirectly affects the process of transcription and translation during the expression of protein/polypeptide (Dayal *et al.* 2009). Sahoo *et al.* (2010) also reported 4 SNPs in the exonic region whereas 8 SNPs in intronic region for this fragment in riverine buffalo. Pareek *et al.* (2003) 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. Analysis of this study confirmed that serum lysozyme gene is polymorphic in Indian breed of sheep and had functional association with serum lysozyme activity that can be included as indicator for disease resistance traits in breeding programme.

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