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Study of genetic variation in *STAT5A* gene with Single Strand Conformation Polymorphism (SSCP) method in Rathi cattle

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Abstract
Total 160 lactating animals of Rathi cattle were selected to analyze for genetic variation in *STAT5A* gene. DNA was isolated from blood samples by kit method. Species- specific primers were constructed for exon-7, intron 9–10 and intron 15–exon 16 of *STAT5A* gene based on available sequences of *STAT5A* gene in the NCBI GenBank database. a polymerase chain reaction in a final reaction volume of 25µl were prepared for regions of *STAT5A* gene and after amplification fragments of 215, 224 and 379 bp of exon-7, intron 9–10 and intron 15–exon 16 region of *STAT5A* gene obtained, respectively. Genetic variation of all three regions of *STAT5A* gene was analyzed by SSCP method. In exon-7, two alleles (A, B) and three genotypic patterns (AA, AB and BB) were observed with 0.738, 0.262, 0.594, 0.288 and 0.118 frequencies, respectively. In intron 9–10, polymorphism was observed with two alleles and two genotypic patterns with 0.921, 0.069, 0.843 and 0.156 frequencies, respectively. In intron 15–exon 16 region of *STAT5A* gene polymorphism was observed by SSCP method in studied population with 0.937, 0.062 0.875 and 0.125 frequencies of two alleles (A, B) and two genotypic patterns, respectively. In studied Rathi cattle population polymorphism was observed in *STAT5A* gene. This can be useful in study of milk production traits in Rathi cattle.

Keywords: *STAT5A* gene, polymorphism, Rathi cattle, SSCP and PCR

Introduction
In livestock census 2019, the total number of cattle in the country is 192.49 million that indicate a 0.8 % higher value than the previous Census. Native milch breeds of cattle like Sahiwal, Tharparkar, Gir, Red Sindhi and Rathi produced more milk than other native cattle breeds. The indigenous cattle contribute 10% and non-descript cattle contribution 11% of total milk production in the country (BAHFS, 2019) [3]. Milk is characterized as secretion from mammary glands in mammals and it meets the complete requirement of infants (Fox, 2009) [13]. According to BAHFS (2019) [3], Rajasthan produced 12.6 % of total milk production and holds the second rank in India. Native milch cattle breed Rathi has breeding tract in Hanumangarh, Loonkaransar tehsil of Bikaner, Shriganganagar districts of the state (Anonymous, 2018) [1]. Rathi cattle contribute 0.83 percentages to the total population of indigenous cattle in India (Breed Survey, 2013) [6] even in dry conditions, Rathi animals have good potential for milk production. Dhaka *et al.* (2015) [10] observed Rathi cattle produce good lactation milk yield in the semi-arid region of Rajasthan. By selection of beneficial QTL increases the frequency of that allele in the population and production and performance can be magnified (Kumar *et al*., 2020) [17]. Phenotypic variations associated with QTLs are used as DNA-based markers. DNA-based markers could be used in a selection of favorable QTLs in marker-based selection (Eggen, 2012) [11]. *STAT5A* genes are located on bovine chromosome 19 and associated with some serum cytokines, mastitis and milk production traits (Bouwman *et al*., 2011; Usman *et al*., 2014) [5]. For improvement in these products and economic traits, selection could be based on heritability and genetic variations (Montaldo *et al*., 2010) [18]. Even same breed animals show variation in milk production and milk composition that depend on the variation in the genetic make-up of dairy animals (Pasha and Hayat, 2012) [19]. The present study was conducted to detect genetic variation in region of *STAT5A* genes through molecular marker approaches such as SSCP. Genes encoding STAT transcription factor are considered as key links in the gene networks constituting the hereditary component of milk productivity. *STAT5A* genes have effect on milk production traits and milk-fat composition (Selvaggi *et al*., 2017) [21].

Material and Methods

An overall 160 milking cows with a minimum of 120 days of lactation were selected for the present study from a pure breeding population of Rathi. About 2 ml of blood samples were collected. Genomic DNA from the whole blood sample was extracted through the spin column method as per standard method (Sambrook and Russell, 2001) [20]. The purity (OD ratio 260/280) and concentration (ng/µl) of extracted genomic DNA was determined by a Nanodrop spectrophotometer and quality through 0.8 % agarose electrophoresis to detect fragmentation. Primers were constructed based on available sequences of *STAT5A* gene in the NCBI GenBank database (Table 1). A 215, 224 and 379-bp region of *STAT5A* gene was amplified by polymerase chain reaction in a final reaction volume of 25 µl (Table 2). The gradient PCR programme was used to find out the appropriate annealing temperature that was further used for the amplification of all samples (Table 3).

Table 1: Primer sequences and expected fragment sizes of PCR products of selected genomic regions

Selected Region	Primer Sequences	GenBank Accession No.	Expected Fragment Length	References
STAT5A Exon-7 region	Forward 5'-CTG CAG GGC TGT TCT GAGAG-3'	AJ237937	215	Selvaggi *et al*. (2009) [9]
	Reverse 5'-TGG TAC CAG GACTGTGCACAT-3'			
STAT5A Intron 9-10	Forward 5'CCAGGGTGCATACAGGACAG3'	AJ237937.1	224	He *et al.* (2012) [15]
	Reverse 5'GCAGGTTACGAGGACTCAGG3'			
STAT5A Intron-15 Exon-16	Forward 5'CTTGGGAGAACCTAACATCACT3'	AJ237937.1	379	Flisikowski *et al*. (2004) [12]
	Reverse 5'AGACCTCATCCTTGGGCC3'			

Table 2: PCR reaction mixture used for amplification of genomic DNA exon-7 region of *STAT5A* gene

S. No.	Contents	Volume		
		Exon-7	intron-9 exon-10 intron-10	intron-15 exon-16
1.	5X PCR buffer	5 µl	5 µl	5 µl
2.	1.5mM MgCl2	2 µl	2 µl	3 µl
3.	10 Mm dNTP's mix	1 µl	1 µl	1 µl
4.	Forward primer 70pmol/µl	1.5 µl	1.5 µl	1 µl
5.	Reverse primer 70pmol/µl	1.5 µl	1.5 µl	1 µl
6.	Genomic DNA 25 ng/µl	4 µl	4.5 µl	4 µl
7.	Taq DNA polymerase 5U/µl	0.2 µl	0.25µl	0.2µl
8.	DNAase free water	9.8 µl	9.25 µl	9.8 µl

Table 3: PCR programming for amplification of regions of *STAT5A* gene

Steps	Temperature			Time			No. of Cycle
	exon-7	intron-9 exon-10 intron-10	intron-15 exon-16	exon-7	intron-9 exon-10 intron-10	intron-15 exon-16	
I. Initial Denaturation	95 ºC			5 min.			1cycle
II. Cycle							
(i). Denaturation	95 ºC			1 min			35 cycles
(i). Annealing	60.5ºC	60 ºC	59.5ºC	45 sec			
(ii). Synthesis	72ºC			1 min			
III. Final extension	72ºC			10 min			1cycle
IV. Hold	4ºC			4 min			1cycle

Detection of Genetic Variation

The genetic variation in the selected genomic regions of *STAT5A* gene was identified through SSCP method (Zhang, 2007) [24]. To detect mutations, SSCP analysis (Orita *et al*., 1989) was performed according to guidelines described by Hayashi and Yandell (1993) [14] with slight modifications.

Genetic analysis of studied population

The genetic structure of the studied population for gene and genotypic frequencies, observed heterozygosity (H_o), expected heterozygosity (H_E) and expected unbiased heterozygosity (H_E unbiased), an effective number of alleles and Nei's genetic distances were analyzed through POPGENE program (*ver*. 3.1) of Yeh (1997) [23]. The Hardy-Weinberg equilibrium for gene and genotypic frequencies was tested using chi-square ($\chi 2$) and maximum likelihood ratio test (G^2 test). A probability value of less than 0.05 was considered to be significant. The standard error of allelic frequency was calculated as [p (1-p)/2n] 1/2 where n is the sample size and p is the frequency of an allele (Spiess, 1989) [22].

Results

Polymorphism in *STAT5A* gene

Three SSCP bands patterns P1, P2 and P3 were observed for a 215-bp fragment of exon-7 of *STAT5A* gene in Rathi cattle (Fig 1). SSCP analysis denotes variation in 'A' allele locus of *STAT5A* gene exon-7 region and is suggestive of multiple allelic nature in Rathi cattle. Thus polymorphism contained in the 215-bp fragments of *STAT5A* gene was revealed through the SSCP method and these three patterns considered as AA, AB and BB in Rathi cattle. Two SSCP bands patterns P1 and P2 were observed for a 224-bp fragment of intron 9–10 of *STAT5A* gene in Rathi cattle (Fig 2) and are suggestive of multiple allelic nature of this region. Thus polymorphism

contained in the 224-bp fragments of the *STAT5A* gene was revealed through the SSCP method and this pattern considered as AA and AB in Rathi cattle. Two SSCP band patterns 'P1' and 'P2' were observed for the 379-bp fragment of intron-15 exon-16 of *STAT5A* gene in Rathi cattle (Fig 3) and variation in 'A' allele of this region of *STAT5A* gene was considered AA, AB and BB.

In Holstein dairy cows, Kiyici *et al*. (2020) [16] studied polymorphisms and found three types of genotype CC, CT and CT and two type of alleles T and C in *STAT5A* gene. In intron 9–10 of *STAT5A* gene polymorphism was investigated by PCR-SSCP method in Chinese Holstein cattle and three genotypes were observed AA, GG and AG (Bao *et al*., 2010) [4].

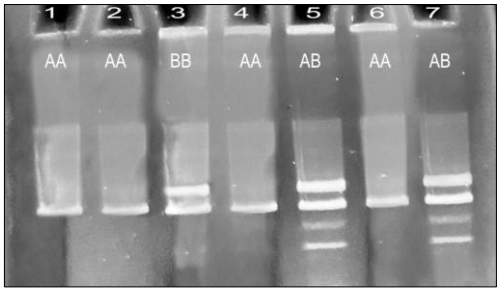

Fig 1: PCR-SSCP patterns of exon-7 of *STAT5A* gene in Rathi cattle

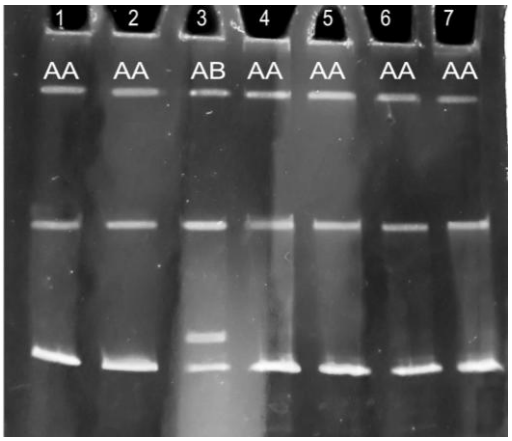

Fig 2: PCR-SSCP patterns of intron 9 of *STAT5A* gene in Rathi cattle

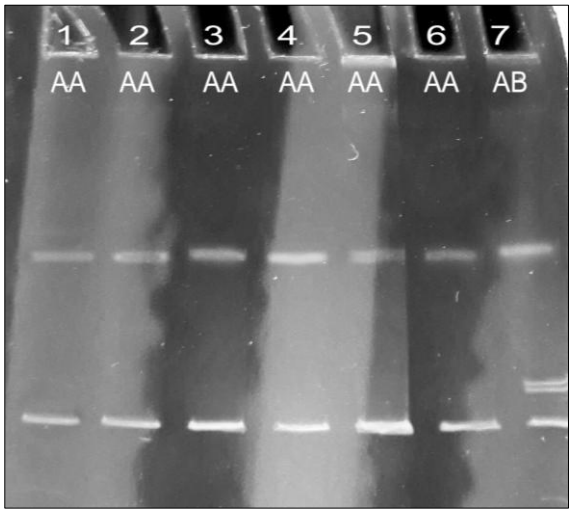

Fig 3: PCR-SSCP patterns of intron-15 exon-16 of *STAT5A* gene in Rathi cattle

Gene and genotypic frequency of *STAT5A* gene

The genetic structure of Rathi cattle in terms of gene and genotypic frequency of *STAT5A* gene, as detected through SSCP marker, are presented in Table 4.

Dario *et al*. (2009) [9] observed two allele- C and T; three genotypes- CC, CT, and CT in *STAT5A* gene in four cattle breeds. Arslan *et al*. (2015) [2] analyzed this region and found two types of alleles C and T with highest frequencies for *STAT5A*C were estimated for the Zavot and Turkish Grey breeds (0.86). In Turkish cattle breed, Cobanoglu *et al*. (2020) [8] detected higher gene frequency of 'C' allele than the 'G' allele for the *STAT5A* gene. In Jersey and Black-and-White cows, Brym *et al*. (2004) [7] investigated intron 9 and intron-15 exon-16 of *STAT5A* gene and found three SSCP patterns in both regions. In Holstein cows, He *et al*. (2012) [15] had analyzed population for intron 9 gene, two alleles and three genotypes A, G, AA, AG and GG were detected with 0.495, 0.505, 0.252, 0.486 and 0.262 frequencies, respectively. In Agerolese cows, Selvaggi *et al*. (2017) [21] revealed T and C allele and TT and TC genotype with 0.875, 0.125, 0.75 and 0.25 frequencies, respectively for intron-15 exon-16 of *STAT5A* gene.

Table 4: Gene and genotypic frequencies of *STAT5A* gene detected through SSCP analysis

Group	Genotypic Pattern			Gene frequency	
	P1	P2	P3	A	B
exon-7	0.594 (95)	0.288 (46)	0.118 (19)	0.738	0.262
intron 9–10	0.843 (135)	0.156 (25)		0.921	0.069
Intron 15–Exon 16	0.875 (140)	0.125 (20)		0.937	0.062

Note: Number in parenthesis is number of observations

Hardy-Weinberg equilibrium for regions of *STAT5A* gene

Both Chi-square and G-square likelihood test ratios revealed a highly significant departure of gene and genotypic frequency for variants of exon-7 of *STAT5A* gene from expected Hardy-Weinberg equilibrium (P≤0.05). However, G-square test value observed for the *STAT5A* exon-7 gene had a significant deviation from Hardy-Weinberg equilibrium (P≤0.05) which indicates that animals differ in their genotypic distribution to gene frequency (Table 5). Both Chi-square and G-square likelihood test ratios revealed the non-significant deviation of gene and genotypic frequency of intron 9-10 and intron-15 exon-16 of *STAT5A* gene from expected Hardy-Weinberg equilibrium (*P*> 0.05). This indicates that these animals similar in their genotypic distribution for gene frequency (Table 5).

Kmiec *et al*. (2010) revealed population of Holstein Friesian cattle was in a genetic equilibrium as per the Hardy-Weinberg law for exon-7 of *STAT5A* gene. In Chinese Holsteins, Bao *et al.* (2009) observed Hardy-Weinberg disequilibrium for genotypic frequencies of intron 9-10 and intron-15 exon-16 of *STAT5A* gene in Chinese Holsteins. Selvaggi *et al.* (2013) also reported the studied population of Jersey cattle was in Hardy-Weinberg equilibrium.

Table 5: Hardy-Weinberg equilibrium of *STAT5A* gene

Gene	Chi^2	p-value	Significance	G^2	p-value	Significance
Exon-7	8.984	0.002	*	8.363	0.003	*
intron 9–10	1.099	0.294	NS	2.036	0.153	NS
Intron 15–Exon 16	0.673	0.411	NS	1.267	0.260	NS

Note: * = significant (P≤0.05), NS= non –significant

Observed (H_O), expected heterozygosity (H_E), Polymorphism information content (PIC), Fixation Index (F_{IS}), Effective number of alleles (n_e) for regions of *STAT5A* gene

The observed genetic variation within the population (H_o) and expected heterozygosity (H_E) in indicates genetic diversity in Rathi cattle for the *STAT5A* gene (Table 6).Genetic divergence and inbreeding coefficient of *STAT5A* allele among Rathi animals were quantified by fixation indices or F-statistics. The medium PIC values of these regions of *STAT5A* gene indicate the presence of sufficient genetic variation at these loci (Table 6). The number of expected alleles and Shannon information index shows the genetic variation in *STAT5A* gene (Table 7). Similar results were observed by different works (Dario *et al*. (2009) [9], Cobanoglu *et al*. (2020) [8].

Table 6: Within-population heterozygosity estimates, PIC and F_{IS} values of Rathi cattle for *STAT5A* gene

Gene	Observed Heterozygosity (H_o)	Expected Heterozygosity (H_E)	Nei's unbiased Heterozygosity (H_e)	PIC	Fixation index (F_{IS})
Exon-7	0.287	0.376	0.375	0.372	0.233
intron 9–10	0.1562	0.1445	0.1440	0.1436	-0.0847
Intron 15–exon 16	0.1562	0.1445	0.1440	0.1436	-0.0667

Table 7: Observed and effective number of alleles and Shannon information index for *STAT5A* gene

Gene	Sample size of alleles (n)	Observed number of alleles (na)	Effective numbers of alleles (ne)	Shannon's Information Index (I)
Exon-7	320	2.000	1.600	0.562
intron 9–10	320	2.0000	1.1683	0.2742
Intron 15–Exon 16	320	2.0000	1.1683	0.2742

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