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CONTENTS

Genetic variation of Beta-Lactoglobulin gene and its association with milk production in Sahiwal and crossbred cattle

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ABSTRACT: Beta Lactoglobulin (β-Lg) is a major whey protein found in ruminant milk. This protein is found in various polymorphic forms some of which have been reported to bear a significant association with certain superior economic traits. Therefore, the present investigation was undertaken to detect polymorphism in β-Lg at the genic level. A total of 145 animals belonging to Sahiwal and crossbred were covered under the present investigation. DNA was extracted from the blood and was used to amplify a 398 bp fragment of β-Lg gene using polymerase chain reaction. The amplified fragment was subsequently digested using *Hae* III restriction enzyme. The restriction pattern so obtained was analyzed using 4% (w/v) agarose gel electrophoresis and was then visualized under UV transilluminator after staining with ethidium bromide. Three types of restriction patterns were seen in cattle. Genotype AA showed three (162, 137 and 99 bp long) fragments, genotype BB showed five (113, 99, 89, 73 and 24 bp long) fragments, while AB showed all seven (162, 137, 113, 99, 89, 73 and 24 bp long) fragments. Comparison with available database suggests that two of the restriction sites (at position 89 and 275) are polymorphic i.e., found in B but absent in A. Based on these observations genotypic frequencies of AA, AB and BB were found to be 0.43, 0.26 and 0.31 in Sahiwal and 0.44, 0.36 and 0.20 in crossbred, respectively. The allele frequencies of A and B were calculated from the genotypic frequencies and were found to be 0.56 and 0.44 in Sahiwal and 0.62 and 0.38 in crossbred cattle. The Analysis of variance and Chi-square test revealed no significant association between genotype and three lactation average milk productions in Sahiwal and crossbred cattle. AA genotype was found to produce more milk compared to other genotypes in Sahiwal and BB in crossbred. From the present investigation it can be concluded that polymorphism of β-Lg gene occurs in Sahiwal breed of cattle and has no significant role in milk yield.

Key words: Beta-Lactoglobulin gene,crossbred, PCR-RFLP, polymorphism, Sahiwal

The Beta-Lactoglobulin (β-Lg) gene has a 4.7 kb transcriptional unit comprising of 6 introns and 7 exons. The regulatory region of β-Lg gene, which retained its stage specific expression in mammary epithelial cells, was found to be 0.8 kb long (Harris *et al.*, 1990).β-Lg is one such important structural gene, in which detection of polymorphism can have an immense implication. The protein product of this gene constitutes a major component of the milk protein fraction. Several biological functions have been attributed to this protein. It is believed to play major role in the transfer of retinol, adsorption of the free fatty acids, enhancing the activity of pharyngeal lipases in neonates of cattle, protection of ascorbic acid in gut and transfer of passive immunity in neonates. Further, some of the β-Lg variants have also been found to bear a significant association with milk production and composition traits. In this context, we decided to study polymorphism at the genic level in β-Lg gene employing PCR-RFLP technique, so that in the foreseeable future the polymorphism at β-Lg gene may be used as a genetic marker for milk composition and production traits. Keeping all these facts in mind, the present investigation was undertaken to study the genetic variation of β-Lg gene in Sahiwal and its crosses with exotic and association of different genotypes with milk production.

MATERIALS AND METHODS

The present investigation was carried out by randomly selected 70 Sahiwal and 75 crossbred cattle maintained at Instructional Dairy Farm (IDF), G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. About 10 ml of venous blood was collected from the jugular vein in a sterile eppendorf tube containing 0.5 M EDTA (O.5 µl/10

ml of blood) as an anticoagulant under sterile conditions. The blood samples were kept in deep freezer at -20°C. until the isolation of genomic DNA. Genomic DNA was isolated from the blood samples by Phenol: Chloroform extraction method as described by Sambrook and Russel (2001) with slight modifications and stored at -20°C till further use. Purity of extracted DNA was checked by UV spectrophotometric analysis as described by Sambrook and Russel (2001). The DNA samples exhibiting an (OD_{260} nm/OD_{280} nm) ratio between 1.7-1.9 were considered as pure and used for further analysis. The concentration of genomic DNA was calculated by using the following formula (Sambrook and Russel, 2001).

DNA concentration = [OD_{260} x dilution factor x 50]/1000 (µg/µl)

(1OD value at 260nm is equivalent to 50 µg dsDNA per ml).

The quality of the genomic DNA was checked by submarine agarose gel electrophoresis. For this purpose 0.8% agarose w/v suspension in 1X TBE buffer was made and 2µl of genomic DNA was mixed with 1µl of 6X gel loading dye. Electrophoresis in 1XTBE buffer for 1-2 hr at 3-5 volt/cm was performed and the gels were visualized under UV light for observing the bands of DNA. Only intact DNA samples, devoid of smearing, were considered to be of good quality and were utilized for further work.

Polymerase chain reaction amplification of candidate gene

Standard PCR protocols were employed to amplify a 398 bp fragment of β-Lg gene in cattle. The amplified fragment spanned a part of exon IV and intron IV of the β-Lg gene.

One pair of primer for amplification of exon I and exon II of β-Lg gene of the cattle was designed on the basis of bovine, caprine and ovine sequences available publicly at NCBI with the help of DNASIS MAX software (Hitachi Genetic System Miraibio Inc, USA). Sequence of primer designed were P1 (Forward)- CGAGAACAAAGTCCTTGTGCT and P2 (Reverse) – CCGGTAACAAAGGCTGTTAGA of 21 bpeachwith GC % 47.61 for both.

PCR was carried out in a final reaction volume of 25 µl which include 10X PCR assay buffer (1X) 2.5µl, $MgCl_2$ (25mM) 1.5 µl, forward and reverse primer (100 ng) 0.5 µl each, dNTP mix (0.5 mM) 0.5 µl, Taq DNA polymerase (1U) 0.2 µl and genomic DNA (80-100ng) 1.0 µl and autoclaved triple distilled water 18.3µl using Bio-Rad PCR machine. All amplification conditions included an initial denaturing step of 5 min at 94°C followed by 35 cycles of denaturation (94°C for 30 sec), annealing (58°C for 30 sec) and extension (72°C for 1 min). Duration of final extension was 10 min at 72°C. PCR amplification was confirmed by submarine agarose gel electrophoresis using 1.2% agarose w/v suspension in 1X TBE buffer.

Restriction enzyme digestion

The 398 bp long amplicons obtained after PCR were then treated with *Hae* III enzyme for RE digestion. 15µl of each PCR product was digested with *Hae*III enzyme. Master Mix containing 10 X assay buffer 1.5 µl and rescriction enzyme *Hae* III (5U/µl) 0.7 µlfor digestion of the amplified products was prepared on ice. The master mix was vortexed for proper mixing and spun for few seconds and then the master mix was distributed to each of the 0.2 ml PCR tube containing 15µl PCR product in it. A negative control consisting of only master mix was also taken to check contamination in the reaction components. The *Hae*III digestion of the PCR products was carried out by overnight incubation at 37°C in water bath. The reaction was stopped by adding a drop of 0.5 EDTA (pH 8.0). The *Hae*III digested PCR product was electrophoresed in 4% w/v agarose gel (low EEO) for 2-3 h at 50 V. About 18 µl of the digested product was mixed with 2 µl of 6 X gel loading dye were loaded into each well along with 100bp DNA ladder in a separate lane. After completion of gel electrophoresis the digested product were visualized by keeping the gel over UV transilluminator and documented by photography to detect the genotype of each sample.

Estimation of gene and genotype frequency

The genotypes were detected by seeing the PCR-RFLP pattern of each sample in the gels. The frequency of β-Lg gene and genotypes in each breed were estimated by standard procedure (Falconer and Mackey, 1996).

Effect of genotype on milk yield

Analysis of variance and Chi- square (χ^2) tests were done for both Sahiwal and crossbred animals showing polymorphism at β-Lg gene level, to find out the effect of genotype on milk yield (Snedecor and Cochran, 1994).

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RESULTS AND DISCUSSION

Allelic pattern

Depending on the restriction pattern obtained after R.E. digestion of amplified product, each individual was assigned a particular genotypic pattern under which it falls.

A thorough scrutiny of the available database, corresponding to the 398 bp fragment which was amplified for the present study, revealed the presence of 4 recognition sites (GG-CC) for *Hae*III enzyme in β-LgB while only 2 recognition sites in β-LgA. On the stretch of amplified products, these sites are present at 89th, 162th, 275thand 299thpositions in B and at 162thand 299th positions in A. The sites at position 89thand 275thare polymorphic and hence these were used to differentiate between the two β-Lg variants. One of these polymorphic sites (position 275th) was located in the intronic region while the other (position 89th) was in the exon and corresponds to the change of amino acid at 118th position.

Due to the presence or absence of recognition sites the amplicon was cut into different sized fragments (3 in A, 5 in Band 7 in AB). Each animal was assigned a specific genotype depending on its restriction fragment pattern. Genotypes AA having fragments 162 bp, 137bp and 99bp; BB having fragments 113bp, 99bp, 89bp, 73 bp and 24bp; and AB having fragments 162 bp, 137 bp, 113 bp, 99 bp, 89 bp, 73 bp and 24 bp(Figs. 1 and 2).

The 24 bp fragments could not be visualized in 'agarose gel electrophoresis' but it did not interfere

Table 1: Gene and genotype frequency of Sahiwal and crossbred cattle

	Genotype frequency			Gene frequency	
Breed	**AA**	**AB**	**BB**	**A**	**B**
Sahiwal	0.43 (n=30)	0.26 (n=18)	0.31 (n=22)	0.56 ±0.059	0.44 ±0.059
Crossbred	0.44 (n=33)	0.36 (n=27)	0.20 (n=15)	0.62 ±0.068	0.38 ±0.068

n = Number of Animals

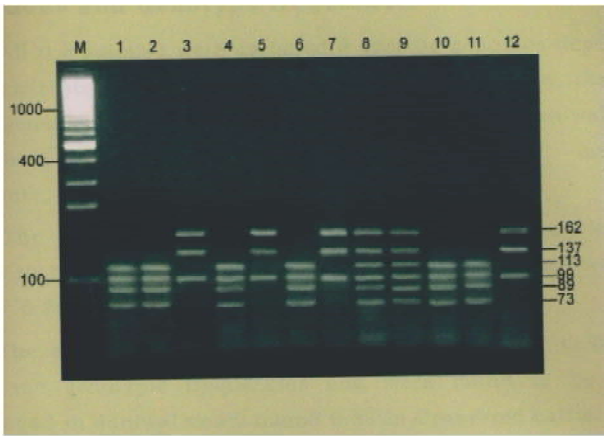

Fig. 1: Genotyping of Sahiwal breed of cattle
Lane M: Molecular size marker (100 bp DNA ladder)
Lane 3, 5, 7, 12: AA genotype
Lane 8, 9: AB genotype
Lane 1, 2, 4, 6, 10, 11: BB genotype

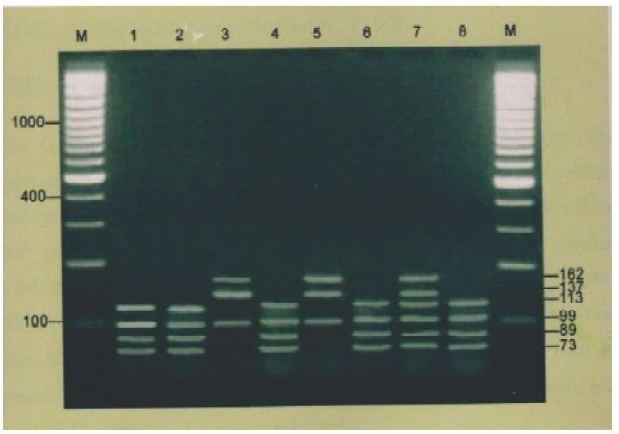

Fig. 2: Genotyping of crossbred cattle
Lane M: Molecular size marker (100 bp DNA ladder)
Lane 3, 5: AA genotype
Lane 7: AB genotype
Lane 1, 2, 4, 6, 8: BB genotype

Table 2: Average milk production per day of different genotypes in Sahiwal and crossbred cattle

Breed	Sahiwal			Crossbred		
Genotype	**AA**	**AB**	**BB**	**AA**	**AB**	**BB**
Average milk yield/day (in kg) in the 1st lactation	5.79±0.46 (n=30)	5.46±0.48 (n=18)	5.39±0.27 (n=22)	7.54±0.53 (n=33)	7.30±0.62 (n=27)	7.66±0.91 (n=15)
		4.63±0.42 (n=70)			5.16±0.28 (n=75)	
Average milk yield/day (in kg) in the 1st three lactations	5.51±0.98 (n=21)	4.70±0.53 (n=15)	5.43±1.10 (n=9)	8.73±0.75 (n=15)	9.32±0.74 (n=18)	10.80±0.39 (n=6)
		7.84±0.364 (n=45)			9.3±0.50 (n=39)	

n = Number of Animals; SE = Standard error

Table 3:Analysis of variance for 1st lactation yield and 1st three lactation milk yield of the β-Lg genotype in Sahiwal cattle

Breed	Stage of lactation	Sources of variation	Degree of freedom	Sum of squares	Mean sum of squares	F
Sahiwal	1st lactation milk yield	β-Lg genotypes	2	3.48	1.74	1.75^{NS}
		Error	67	67.268	1.004	
		Total	69	70.748		
	1stthree lactation milk yield	β-Lg genotypes	2	7.97	3.985	0.967^{NS}
		Error	42	168.504	4.012	
		Total	44	176.474		
Crossbred	1st lactation milk yield	β-Lg genotypes	2	3.02	1.51	1.75^{NS}
		Error	72	249.84	3.47	
		Total	74	252.86		
	1stthree lactation milk yield	β-Lg genotypes	2	6.10	3.05	0.43^{NS}
		Error	36	101.448	2.818	
		Total	38	107.548		

NS= Non-significant

Table 4:Chi-square (χ^2) analysis for the association of β-Lg genotype with high and low producing Sahiwal and crossbred cattle

Animal Group	Genotype	Sahiwal		χ^2	Crossbred		χ^2
		o	e		o	e	
High producing animals	AA	6	7.28	0.223^{NS}	5	5.72	0.090^{NS}
	AB	5	7.71	0.020^{NS}	5	5.28	0.021^{NS}
	BB	6	4.37	0.080^{NS}	3	4.68	0.061^{NS}
Low producing animals	AA	9	4.62	0.215^{NS}	6	4.32	0.098^{NS}
	AB	4	5.34	0.082^{NS}	4	2.60	0.023^{NS}
	BB	5	5.65	0.074^{NS}	2	2.40	0.066^{NS}
			Total	0.694^{NS}			0.359^{NS}

NS = Non-significant; o = observed value; e = expected value
Threshold level of high producing Sahiwal is 8 kg and crossbred cattle is 12 kg

in the identification of genotypes because of the presence of other easily recognizable allele specific fragments.

Polymorphism in β-Lg gene was detected in both Sahiwal and crossbred cattle(Figs. 1 and 2), which is in agreement with several other earlier reports (Medrano and Cordova, 1990; Chung *et al.,* 1998; Zhou *et al.,* 1996; Rachaganiet *al*., 2006; Arora *et al*., 2010).

Gene and genotype frequencies

All the animals were assigned a specific genotype depending on their restriction pattern. Based on this information, the gene and genotype frequencies were calculated for Sahiwal and crossbred cattle, using

the standard statistical methods (Falconerand Mackay, 1996) (Table 1). The AA genotype is predominant in both Sahiwal and crossbred cattle. The allelic frequencies of A and B alleles were calculated from the genotypic frequencies and were found to be 0.56 and 0.44 in Sahiwal and 0.62and 0.38 in crossbred cattle, which showed that A allele is found at a higher frequency than the B allele in almost all the breeds of cattle.

The calculated gene and genotype frequencies were found to be in close agreement to the results of earlier workers (Chung *et al.,* 1998*;* Zhou *et al.,* 1996; Schlee and Rottman, 1992). However, Singh*et al*. (2015) observed in β-Lg, that the genotypic frequency of BB was higher than that of AB and AA.

Effect of genotype on milk yield

In the present study, the polymorphism was detected in β-Lg gene and further analysis of variance and χ^2 test was employed to find out any significant association, if present, between the economic trait and β-Lg genotype in Sahiwal and crossbred cattle. Average values of milk production and composition traits (milk yield) along with S.E. were calculated separately, corresponding to different genotype of the same breed, to facilitate a better comparison among the animal (Table 2). AA genotype in Sahiwal and BB genotype crossbred cattle was found to produce comparatively more milk than the other genotypes (Table 2). However, the analysis of variance revealed a non-significant (P>0.05) association of genotype with average milk yield of first lactation and first three lactations (Table 3). Chi--square test for Goodness of Fit showed a non-significant (P>0.05) difference between expected and observed frequency of different alleles in each groups in Sahiwal and crossbred cattle (Table 4). These findings agree well with the earlier findings of Jairam and Nair (1983) and Arora *et al*. (2010). This indicates that there is no significant effect of β-Lg genotype with milk yield. However, the AB and BB genotypes of β-Lghad a significant ($p < 0.05$) effect on total milk yield and peak yield compared with AA (Singh *et al*., 2015).

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