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Characterization of Exon 4 Region of AAT Gene and Its Association with Milk Production Traits in Sahiwal and Karan Fries Cattle

Alok Kumar Yadav* and Anupama Mukherjee

Division of Animal Genetics and Breeding, ICAR-National Dairy Research Institute, Karnal-132001, Haryana, INDIA

*Corresponding Author: alokvet1000@gmail.com

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Abstract

The present study involved records on milk production and milk constituents of 100 Sahiwal and 115 Karan Fries cattle collected from 2004 to 2016 from Division of Animal Genetics and Breeding, ICAR-National Dairy Research Institute, Karnal (Haryana, India). The parameters studied were First lactation 305 days Milk Yield (FL305DMY), First lactation Total Milk Yield (FLTMY), First lactation 305 days Protein Yield (FL305DPY), First lactation 305 days Fat Yield (FL305DFY) and First lactation 305 days SNF Yield (FL305DSNFY). Jugular blood samples were obtained for all the selected animals and DNA was extracted. PCR-RFLP analysis of PCR products was carried out using R saI for 100 Sahiwal and 115 Karan Fries cattle. The product 370 bp had three genotypes AA (370), AB (370+267+103 bp), and BB (267+103 bp) having genotype frequencies 0.20, 0.50, 0.30 and gene frequencies 0.45 (A) and 0.55 (B) in Sahiwal, and genotype frequencies 0.54, 0.24, 0.22 and gene frequencies 0.66 (A) and 0.34 (B), respectively, in Karan Fries cattle. These genotypes were highly significant for FL305DMY, FLTMY, FL305DSNFY and FL305DPY. AB genotypes were superior for all traits. Further, the regression equations (R2 values) derived were highly significant for FL305DPY in Sahiwal and FL305DMY, FLTMY, FL305DSNFY and FL305DPY for Karan Fries.

Keywords: AAT gene, Milk Constituents traits, Sahiwal and Karan Fries cattle.

Introduction

India has a rich genetic diversity in cattle with 53 recognized breeds. The country's total cattle population is 192.49 million, out of which the indigenous cattle population comprises 142.11 million (Livestock Census, 2019). Milk is an important source of essential nutrients for calves and a key raw material for human food (Reinhardt *et al.*, 2012). India ranks first in the world in terms of milk production with production of 198.4 million tons in 2019-20 (BAHS, 2019). Sahiwal is the best dairy breed in the Indian subcontinent. It is a comparatively heavy breed with a symmetrical body and loose skin (Nivsarkar *et al.*, 2000). The Karan Fries breed has evolved from crossbreeding between Tharparkar and Holstein Friesian at the ICAR-National Dairy Research Institute, Karnal, Haryana. The breed has 50 % inheritance from Friesian. The breed carries black patches and sometimes is completely dark with white patches on the forehead and the switch of the poll. The udder is also dark with white patches in teats. The animals are extremely docile and very good-yielders.

Alpha 1-antitrypsin (AAT), a strong protease inhibitor, also known as α-1-protease inhibitor (α1PI), belongs to the superfamily of serpins or serine proteinase inhibitors. AAT is a glycoprotein with a molecular mass of 52 kDa which forms a sodium dodecyl sulfate (SDS) staple complex with elastase (Carrell *et al.*, 1982). The bovine AAT gene consists of five exons, spanning about 9 kb of genomic DNA and encoding a 416-AA protein. Protein degradation in bovine milk affects the quality of dairy products. AAT can protect vulnerable elastic tissues from degradation by neutrophil elastase. Thus, the greater the AAT, the better the quality of milk and milk products. The objective of this study was to see the association of polymorphism at the exon 4 region of the AAT gene and milk production traits in Sahiwal and Karan Fries cattle.

Material and Methods

Experimental Animals and Genomic DNA Isolation

The data for the present study pertained to various milk production and milk constituents' traits were collected from history sheets and milk constituents' registers, data on milk production and milk constituents' records of 100 Sahiwal and 115 Karan Fries cattle spread over a period of 13 years from 2004 to 2016 were collected from Animal Genetics and Breeding division of ICAR-National Dairy Research Institute, Karnal, Haryana. Blood samples were collected from the selected population. About 10 mL of venous blood was collected aseptically from the jugular vein of the animals in a 15 mL polypropylene centrifuge tube under sterile conditions using 0.5 mL of EDTA as an anticoagulant. The tube was shaken gently to facilitate thorough mixing of blood with the anticoagulant. The tubes containing blood samples were transported to the laboratory as soon as possible in an icebox containing ice packs and were stored in the refrigerator at –20°C temperature until the isolation of DNA was done. The phenol extraction method as described by Sambrook and Russell (2001) was used for isolation of genomic DNA. Horizontal submarine agarose gel electrophoresis was used to check the quality of genomic DNA. The purity of genomic DNA was checked by spectrophotometer. The 6 µL of genomic DNA of each sample was dissolved in 294 µL of triple distilled water and spectrophotometer readings at OD_{260} and OD_{280} were recorded against 300µl double distilled water as a blank. Genomic DNA samples showing the OD ratio in the range of 1.7 to 1.9 were used further in the study.

Amplification of Targeted Region of AAT Gene

Only good-quality genomic DNA was used for the amplification of the exon 4 region of the AAT gene (370 bp) by polymerase chain reaction under optimized conditions.

F: 5'- ACACCCCAGATCTCCAGGAG-3'
R: 5'-TTGGACACCTTCAGAGGCTG-3'

Primers (F) and (R) were used to amplify the exon 4 region of the AAT gene, which were designed to amplify genes using Primer 3 software (http://www.primer3.ut.ee) (Untergrasser *et al*., 2012) and gene sequence available at NCBI database (http://www.ncbi.nlm.nih.gov). The primers designed were checked for specificity by BLAST (version 1.2.0). Primers were designed and synthesized from Sigma Aldrich Chemicals Pvt. Ltd (USA), the best amplification of the desired fragment was taken for further analysis. The standard programme for PCR reaction mix (25 µL) included 10X buffer 2.5 µL, 15 mM $MgCl_2$ 1.5 µL, d NTP's 200 µM 0.5 µL, F-primer 30 pm 3.0 µL, R-primer 30

pm 3.0 µL, Taq 0.2 µL, Template 100 ng 1.0 µL, and distilled water 14.3 µL.

Polymerase Chain Reaction Amplification of Exon 4 Region of AAT Gene

Various annealing temperatures were tried for PCR amplification of the AAT gene. A total volume of 25 µL for each sample was used to set up the PCR reactions. The set of primers was used to amplify target regions of the AAT gene in Sahiwal and Karan Fries cattle. The best results were obtained when amplification was performed in a PCR thermal cycler (Eppendorf Germany) programmed for 32 cycles with an initial denaturation at 94°C for 05 minutes, denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 30 seconds with a final extension at 72°C for 10 minutes.

Agarose Gel Electrophoresis of Polymerase Chain Reaction Product

The amplified product was checked for quality and quantity by agarose gel electrophoresis as described by Sambrook and Russell (2001).

A total of 5µL of amplified PCR product of each sample was mixed with 1µL of 6X gel loading dye buffer from each tube. The samples were loaded on 2% agarose gel containing ethidium bromide (1% solution @ 5µL /100mL) along with 100bp DNA ladder (O'GeneRuler™-Fermentas) at a constant voltage of 70V for 45 minutes in 0.5X TBE buffer. The amplified PCR product on agarose gel was visualized as a single compact band of different primer sizes under a UV transilluminator and documented by photographing through the gel documentation system (Bio-Rad, USA) (Plate 01).

Statistical Analysis of PCR-RFLP Data

The analysis was carried out with appropriate statistical methods using software in the computer center of the institute under the following headings:

Restricted Maximum Likelihood Method (REML)

Estimation of Breeding Value

The single-trait animal model was considered for the estimation of breeding value using WOMBAT software (Meyer, 2010). The following animal model was considered:

$$Y_{ijk} = X\, b_i + Z\, u_j + e_{ijk}$$

where,

Y_{ijk} = k^{th} observation of j^{th} random effect of i^{th} fixed effect
b_i = Vector of observation of fixed effect
X = Incidence matrix of fixed effect
u_j = Vector of additive genetic effect (animal effect)
Z = Incidence matrix of random effect
e_{ijk}= Vector of residual errors

Association Estimation

Based on the adjusted records, about milk yield and its constituents on Sahiwal and Karan Fries cattle maintained at ICAR-NDRI, Karnal, regression analysis was carried out to identify SNPs contributing significantly to the variation in milk and its constituents.

$$Y_{ijk}=a +b_iSNP_i+ b_jSNP_j+\ldots..b_nSNP_n+ e_{ijk}$$

where,

Y_{ijk} = Adjusted observation on k^{th}animal of i^{th} ,j^{th}….$_n^{th}$ SNPs

A = Intercept

$b_{i \ldots n}$ = Partial regression coefficient for SNPs considered

$SNP_{i,j...n}$ = Effect of SNPs taken as independent variable

e_{ijk} = Random error NID (0, σ^2_e)

Effect of Genotypes on Breeding Value

The relative contribution of Genotypes to the breeding value of the animal for milk yield and milk constituents was assessed using the following model:

$Y_{ij} = \mu + G_i + e_{ij}$
Where,
Y_{ij}= Breeding value of j^{th} animal of i^{th} genotype
μ= Overall mean
G_i= Effect of i^{th} genotype (SNPs/ haplotypes)
e_{ij} = Residual error NID (0, σ^2_e)

Results

PCR-Restriction Fragment Patterns and Genotyping At Exon 4 of AAT Gene (370 bp) in Sahiwal Animals

PCR-RFLP analysis of PCR products was carried out using *R saI* for 100 Sahiwal animals. The 370 bp had three genotypes AA (370), AB (370+267+103 bp), and BB (267+103 bp) having genotype frequencies 0.20, 0.50, 0.30, and gene frequencies were 0.45 (A), and 0.55 (B) (Plate 02). These genotypes were highly significant for FL305DPY and non-significant for FL305DMY, FLTMY, FL305DFY and FL305DSNFY. The mean±SE of AA genotype for FL305DMY, FLTMY, FL305DFY, FL305DSNFY, FL305DPY were 1838.5(Kg)±15.6, 2041.3(Kg)±15.8, 100.3(Kg)±0.66, 155.15(Kg)±0.17 and 42.86(Kg)±0.07, respectively and for AB genotype were 1797.52(Kg)±9.84, 2005.95(Kg)±9.99, 100.48(Kg)±0.42, 154.78(Kg)±0.10, 44.44(Kg)±0.04, respectively and for BB genotypes were 1811.5(Kg)±12.7, 1998.5(Kg)±12.9, 100.12(Kg)±0.54, 154.89(Kg)±0.14, 43.69(Kg)±0.05, respectively. AB genotype was superior for, FL305DFY and FL305DPY traits. AA genotype was superior for FL305DMY, FLTMY and FL305DSNFY (Table 1 and 2).

Table 1: ANOVA for Genotype effect of Exon 4 of AAT gene in Sahiwal Animals

Source	df	SS	MSS	F-value
FL305DMY				
Genotype	2	24090	12045	2.49
Error	97	469711	4842	
Total	99	493801		
FLTMY				
Genotype	2	24354	12177	2.44
Error	97	484001	4990	
Total	99	508355		
FL305DFY				
Genotype	2	2.47	1.23	0.14
Error	97	854.61	8.81	
Total	99	857.09		
FL305DSNFY				
Genotype	2	1.88	0.94	1.61
Error	97	57.00	0.58	
Total	99	58.89		
FL305DPY				
Genotype	2	37.15	18.57	183.95**
Error	97	9.79	0.10	
Total	99	46.95		

** *(p<0.01)*

Table 2: Least Square Mean and Standard Error for milk production traits in Sahiwal and Karan Fries cattle

Production Traits	Genotype	Sahiwal Cattle (n=100)		Karan Fries Cattle (n=115)	
		N	Mean (Kg)±SE	N	Mean (Kg)±SE
First Lactation 305 Days Milk Yield (FL305DMY)	AA	20	1838.5±15.60	62	3521.74±5.25[b]
	AB	50	1797.52±9.84	28	3607.30±7.81[a]
	BB	30	1811.5±12.70	25	3433.37±8.26[c]
Full Lactation Total Milk Yield (FLTMY)	AA	20	2041.3±15.80	62	4541.50±5.25[b]
	AB	50	2005.95±9.99	28	4627.06±7.81[a]
	BB	30	1998.5±12.90	25	4453.13±8.26[c]
First Lactation 305 Days Fat Yield (FL305DFY)	AA	20	100.31±6.64	62	137.00±4.99[a]
	AB	50	100.48±4.20	28	143.74±7.42[a]
	BB	30	100.12±5.42	25	122.51±7.85[b]
First Lactation 305 Days SNF Yield (FL305DSNFY)	AA	20	155.15±17.10	62	278.14±5.60[a]
	AB	50	154.78±10.80	28	278.88±8.40[a]
	BB	30	154.89±14.00	25	277.26±8.90[b]
First Lactation 305 Days Protein Yield (FL305DPY)	AA	20	42.86±7.11	62	113.37±4.60[a]
	AB	50	44.44±4.49	28	114.30±6.90[b]
	BB	30	43.69±5.80	25	112.42±7.30[c]

Regression Equation

The significance of the association of SNP with different performance traits was estimated by constructing the regression equation and the best fit equation for each of them was as under:

FL305DMY = 1815.82 + 22.7 SNP_AA – 18.31 SNP_AB – 4.4 SNP_BB (R^2=4.88)
FLTMY = 2015.25 + 26.1 SNP_AA – 9.30 SNP_AB – 16.8 SNP_BB (R^2=4.79)
FL305DFY = 100.30 + 0.3 SNP_AA + 0.17 SNP_AB – 0.18 SNP_BB (R^2=0.29)
FL305DSNFY = 154.94.5 + 0. 20 SNP_AA – 0.15 SNP_AB -0.0 5 SNP_BB (R^2=3.21)
FL305DPY = 43.67 – 0.80 SNP_AA + 0.77 SNP_AB +0.0 2 SNP_BB (R^2=79.14)

PCR-Restriction Fragment Patterns and Genotyping at Exon 4 of AAT Gene (370 bp) in Karan Fries Animals

PCR-RFLP analysis of PCR products was carried out using reported *R saI* for 115 Karan Fries animals. The 370bp had three genotypes AA (370), AB (370+267+103bp), and BB (267+103bp) having genotype frequencies 0.54, 0.24, 0.22 and gene frequencies 0.66 (A) and 0.34 (B) (Plate 03). These genotypes were highly significant for FL305DMY, FLTMY, FL305DSNFY, and FL305DPY and non-significant for FL305 DFY. These genotypes were highly significant for FL305DMY, FLTMY, FL305DSNFY, and FL305DPY and non-significant for FL305 DFY. The mean±SE of AA genotype for FL305DMY, FLTMY, FL305DFY, FL305DSNFY, FL305DPY were 3521.74(Kg)±5.25, 4541.50(Kg)±5.25, 130.00(Kg)±4.99, 278.14(Kg)±0.05 and 113.37(Kg)±0.04, respectively and for AB genotype were 3607.30(Kg)±7.81, 4627.06(Kg)±7.81, 143.74(Kg)±7.42, 278.88(Kg)±0.08, 114.30(Kg)±0.06, respectively and for BB genotype were 3433.37(Kg)±8.26, 4453.13(Kg)±8.26, 122.51(Kg)±7.85, 277.26(Kg)±0.08, 112.42(Kg)±0.07, respectively. AB genotypes were superior for all traits (Tables 2 and 3).

Regression Equation

The significance of association of SNP with different performance traits were estimated by constructing the regression equation and the best fit equation for each of them was as under:

FL305DMY = 3520.81 + 0.94 SNP_AA + 86.49 SNP_AB – 87.43 SNP_BB (R^2=67.66)
FLTMY = 4540.57 + 0.94 SNP_AA + 86.49 SNP_AB – 87.43 SNP_BB (R^2=67.66)
FL305DFY = 134.42 + 2.58 SNP_AA + 9.32 SNP_AB – 11.90 SNP_BB (R^2=3.49)
FL305DSNFY = 278.10 + 4.53 SNP_AA + 0.78 SNP_AB – 0.83 SNP_BB (R^2=61.24)
FL305DPY = 113.36 + 0.66 SNP_AA + 0.93 SNP_AB – 0.94 SNP_BB (R^2=75.93)

Table 03: ANOVA for Genotype effect of Exon4 of AAT gene in Karan Fries animals

Source	df	SS	MSS	F-value
FL305D MY				
Genotype	2	399871	199935	117.5**
Error	112	191141	1707	
Total	114	591011		
FLTMY				
Genotype	2	399871	199935	117.5**
Error	112	191141	1707	
Total	114	591011		
FL305DFY				
Genotype	2	6256.20	3128.10	2.03
Error	112	17275.91	1542.49	
Total	114	179015.81		
FL305DSNFY				
Genotype	2	34.87	17.43	88.47**
Error	112	22.07	0.19	
Total	114	56.95		
FL305DPY				
Genotype	2	47.05	23.52	176.64**
Error	112	14.91	0.13	
Total	114	61.97		

*** (p<0.01)*

Discussion

PCR-Restriction Fragment Patterns and Genotyping at Exon 4 of AAT Gene (370 bp) in Sahiwal Cattle

The result is in agreement with Li *et al*. (2010) in Chinese Holstein reported significant effect for milk fat percentage, milk protein percentage, and 305-day milk yield. They concluded that AAT is a potential candidate gene influencing milk production traits and could be implemented in breeding programmes to improve the production performance of Chinese Holstein cattle.

The result is also in agreement with the findings of Kheiripour *et al.* (2014) in Holstein dairy cows where the cows of the AB genotype had higher milk fat percentage than those of genotype AA. Cows with the AB genotype showed 0.07% higher fat percent and 0.02% higher protein percent as compared to the AA genotype. It was reported that substituting A alleles with B alleles resulted in an increase of milk fat by 0.07 percent and it was concluded that the association value could be implemented as a marker in breeding programmes for these traits.

The result is also in agreement with Yadav and Mukherjee (2019) in Sahiwal and Karan Fries Cattle where the AB genotype was superior for FL305DMY, FLTDMY, FL305DSNFY traits, and the AA genotype was superior for FL305DFY and BB genotype was superior for FL305DPY trait.

PCR-Restriction Fragment Patterns and Genotyping at Exon 4 of AAT Gene (370 bp) in Karan Fries Animals

The result is in line with the findings of Li *et al*. (2010) in Chinese Holstein who reported a significant effect of genotype on milk fat percentage, milk protein percentage, and 305-day milk yield. They concluded that AAT is a potential candidate gene influencing milk production traits

The result is also in agreement with Kheiripour *et al.* (2014) in Holstein dairy cows who reported that the cows of AB genotype had higher milk fat percentage than those of genotype AA. It was concluded that the association value could be implemented as a marker in breeding programmes for these traits.

The result is also in agreement with Yadav and Mukherjee (2019) in Sahiwal and Karan Fries Cattle where the AB genotype was superior for FL305DMY, FLTDMY, FL305DSNFY traits, and the AA genotype was superior for FL305DFY and BB genotype was superior for FL305DPY trait.

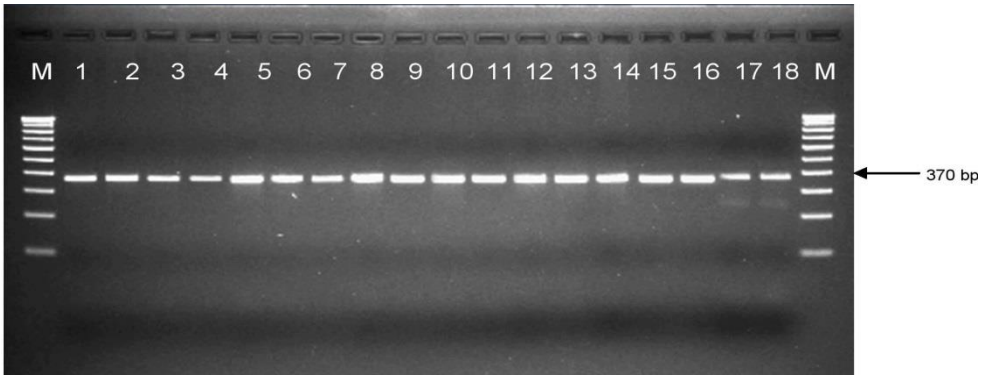

Plate 1: Amplified PCR product of 370bp of AAT Gene electrophoresed on 2% agarose in Sahiwal Cattle and Karan Fries Cattle **[***M: 100bp DNA ladder; Lanes: 1-18 are amplified PCR product*)**]**

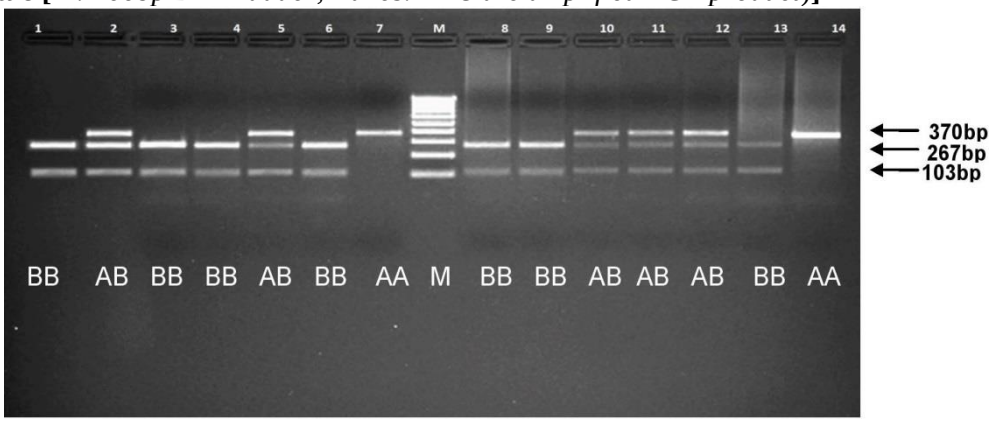

Plate 2: Gel Electrophoresis with 2 % Agrose gel stained with Ethidium Bromide showing PCR-RFLP pattern of AAT gene digested with *Rsa* l in Sahiwal *(M - 100bp DNA ladder; Lanes - AB= (370+267+103); BB= (267+103); AA= (370)*

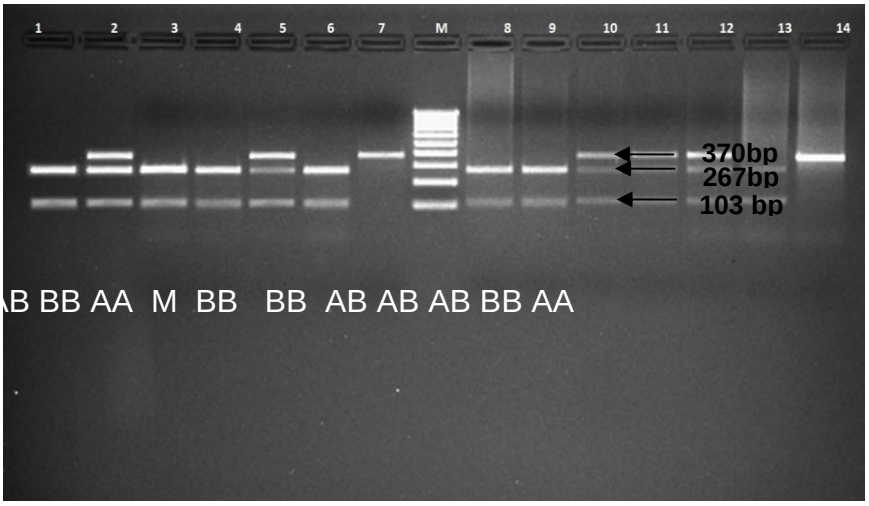

Plate 3: Gel Electrophoresis with 2 % Agrose gel stained with Ethidium Bromide showing PCR-RFLP pattern of AAT gene digested with *Rsa* l in Karan Fries [M: 100bp DNA ladder; Lanes: AB = (370+267+103); BB= (267+103); AA= (370)]

Conclusions

In the present study, the 370 bp product obtained in 100 Sahiwal and 115 Karan Fries cattle had three genotypes AA (370), AB (370+267+103 bp), BB (267+103 bp) having genotype frequency of 0.20, 0.50, 0.30 and gene

frequency 0.45 (A) and 0.55 (B) in Sahiwal and 0.54, 0.24, 0.22 and 0.66 (A) and 0.34 (B) for genotype and gene frequency, respectively in Karan Fries cattle. These genotypes were highly significant for FL305DPY and non-significant for FL305DMY, FLTMY, FL305 DFY, and FL305DSNFY but in Karan Fries, these genotypes were highly significant for FL305DMY, FLTMY, FL305DSNFY, FL305DPY and non-significant for FL305DFY. It is concluded that the heterozygous genotype of AAT genes may be applied as a potential genetic marker for milk production in Karan Fries cattle after validation on a large population. The influence of genotype for 370 bp amplicon size was highly significant for FL305DPY traits in both breeds.

Data Availability Statement

The data records on milk production and milk constituents of Sahiwal cattle and Karan Fries cattle were collected from Animal Genetics and Breeding division of ICAR-NDRI Karnal Haryana, India.

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Contribution by Authors

AKY: has made substantial contributions to conception and design, acquisition of data, performed statistical analysis of data and interpretation of data of the results and has been involved in drafting the manuscript or revising it critically for important intellectual content; AM: has given final approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interests

There is no conflict of interest.

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