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Identification of Novel Milk Protein Gene Variants in Sahiwal Cattle Breed of Pakistan

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Abstract: This novel study was aimed at identification of new genetic variants in Sahiwal cattle breed of Pakistan and determined the effects of these variants on milk yield. Five major milk protein genes in Sahiwal cattle were analyzed and two single nucleotide polymorphisms identified through bi-directional sequencing. These include A to T in exon XI at position 11462 of the alpha s1 casein gene; resulting in a Glutamic Acid (GAA) to Aspartic acid (GAU) substitution at position 84 of alpha s1 casein protein and T to C change at position 8491 of the exon VII in beta-casein gene resulting in a Valine to Alanine substitution at position 197 of beta casein protein. Amplification Refractory Mutation System (ARMS) and SNaPshot genotyping protocols were optimized for genotyping new genetic variants. The genotypes in both the alpha-s1 casein and beta casein genes were found associated with milk yield but their influence was not statistically significant. However, the least square means of milk yield for TT genotypes of alpha s1 casein and of beta casein genes were higher compared to other genotypes.

Keywords: αs1-casein, β-casein, genetic variant, sahiwal cattle

INTRODUCTION

Top 10 dairy companies were turning over $114 billion in 2009 in worldwide agriculture and 84% of it was shared by dairy cows as a result there is great importance of the production potential of these animals (Whitley, 2010). Sahiwal cattle breed is one of the best dairy breeds of Pakistan and India due to its qualities of being heat tolerant and resistant to internal as well as external parasites. Cows produce 2270 kg of milk on average during one lactation period while also suckling a calf (Khan *et al*., 1999).

Milk protein genetic polymorphism provides the opportunity to estimate the genetic merit of sires and cows and practice selection independent of sex and age of animal; as a result many populations of dairy cattle were screened to determine the differences in various breeds or group of breeds (Gurcan, 2011; Bonfatti *et al*., 2010), calculate frequencies of these variants in different cattle breeds (Hamza *et al*., 2010) and investigate relationships between genetic variants and productive traits (Hristov *et al*., 2012).

With newly developed genotyping techniques based on DNA analysis, which include Amplification Refractory Mutation System (ARMS) and SNaPshot SNP genotyping it is now possible to include information on milk protein genotypes into selection programs. It could provide more accurate predictions of breeding values of animals to be selected and thus improve response to selection (Lara *et al*., 2002).

Five major milk protein genes including αs1-casein, β-casein, κ-casein, α-lactalbumin and β-lactoglobulin were screened through sequencing which resulted in identification of two new genetic variants in αs1-casein and β-casein genes reported here. Two genotyping techniques; amplification refractory mutation system and SNaPshot were optimized for large sets of sample size.

MATERIALS AND METHODS

Genomic DNA samples: Blood samples of 150 Sahiwal cattle breed were collected from Research Centre for Conservation of Sahiwal cattle (RCCSC, Khanewal) and 120 samples from Livestock Production Research Institute (LPRI, Okara). DNA was extracted using a standard protocol (Sambrook and Russell, 2001).

Phenotypic data and statistical analysis: Phenotypic data of the selected animals were collected which include animal's age, milking record, milk content records, calving record, sire and dam names. Direct counting was used to estimate allele and genotypic frequencies of milk proteins. The chi-square test was used to find association of different milk protein genotypes with milk yield. Statistical Program for Social Sciences (SPSS Version 15.0) by SPSS Inc, 2001 was used for one way Analysis of Variance.

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Table 1: Primer sequences

Marker	Primer sequences (5'-3')	Tm (°C)	Amplicon size
Primer sequences for sequencing			
X111S1 (a)	F-CCCAGGAATTTGTGGCTAAA	56	345
	R-GCTCATTCCCTGATACATTGG		
X7.2BCN (b)	F-GAGCCCTTTACTGAAAGCCAGA	58	395
	R-TCTCAGCTATGCTTATTTTGGAACC		
Primer sequences for ARMS genotyping			
X111S1A/T (c)	FO-AGGAAATTGTTCCCAATAGTGTTGAGGTGA	57	237
	RO-ATCAATACAACATTCTTGCTCATTCCCTGA		
	FI-TTTTTTAGCAGAAGCACATTCAAAAG<u>A</u>AT		162 (A allele)
	RI-CCAGGTAACGCTCAGAGGGCACA<u>C</u>CT		130 (T allele)
X7.2C T/C (d)	FO-TCTTGGATGCACCAGCCTCACCAGCCTC	67	384
	RO-TGGTGGAGGGGAGGTGAAGGTTCTCCAGC		
	FI-AGGCCTTTCTGCTGTACCAGGAGCC<u>G</u>GC		246 (C allele)
	RI-TAGGGAAGGGTCCCCGGACAGGACCGA<u>T</u>TA		196 (T allele)
Primer sequences for SNaPshot genotyping			
X111S1 (e)	Forward 1st round primer CCCAGGAATTTGTGGCTAAA	56	345
	Reverse 1st round primer GCTCATTCCCTGATACATTGG		
	Extension primer sequence CAGAAGCACATTCAAAAGGA		21
X7.2BCN (f)	Forward 1st round primer GAGCCCTTTACTGAAAGCCAGA	58	395
	Reverse 1st round primer TCTCAGCTATGCTTATTTTGGAACC		
	Extension primer sequence CTGTACCAGGAGCCTGT		18

(a): Sequencing primers for exon 11 of alpha s1 casein gene; (b): Sequencing primers for exon 7 of beta casein gene; (c): ARMS genotyping primer set for alpha s1 casein gene; (d): ARMS genotyping primer set for beta casein gene; (e): SNaPshot primer set for alpha s1 casein gene; (f): SNaPshot primer set for beta casein gene; FO: Forward outer; RO: Reverse outer; FI: Forward inner; RI: Reverse inner; bp: Base pair, Tm: Melting temperature

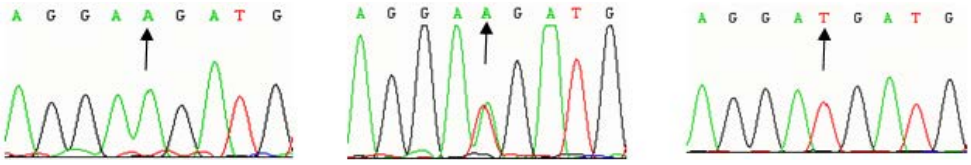
Lactation records obtained from dairy farms were adjusted, those greater than 150 and less than 305 days were fixed to 305 days values using dairy farms correction factors.

Primers design: Primers were designed using the bovine αs1-casein and β-casein gene sequences available at NCBI website. The exons XI and VII of αs1-casein and β-casein were amplified by PCR using primers X111S1F, X111S1R and X7.2BcnF X7.2BcnR for αs1-casein and β-casein respectively (Table 1). The tetra-primer ARMS-PCR procedure required the design of two outer primers to amplify a 200-500 bp fragment and two allele-specific inner primers (Ye *et al*., 2001). The inner primers were designed as described by Ye *et al*. (2001) following the rules to introduce a second deliberate mismatch at position -2 from the 3' end utilizing the web-based program accessible from http://www.cedar.genetics.soton.ac.uk/ public_html/primer1.html (Ye *et al*., 2001). Primers for SNaPshot SNP genotyping were designed using the website http://www.basic.northwestern.edu/biotools/oligocalc.html. The primers were tailored to immediately end 5' site before the target SNP (Table 1).

Genomic DNA amplification: PCR reaction (25 uL total volume) included 100 ng of bovine genomic DNA, 0.16 pmol of amplification primer (Table 1), 2.5U Taq DNA polymerase, 2 mM $MgCl_2$ and 100 uM dNTPs. Thermal profile included initial denaturation for 3 min at 94°C, followed by 35 cycles of 45 sec at 94°C, 45 sec at 56°C, 45 sec at 72°C and final extension for 10 min at 72°C using a GeneAmp PCR System 9700 (Applied Biosystems, CA). Annealing temperature for αs1- casein and β-casein was 56 and 58°C, respectively.

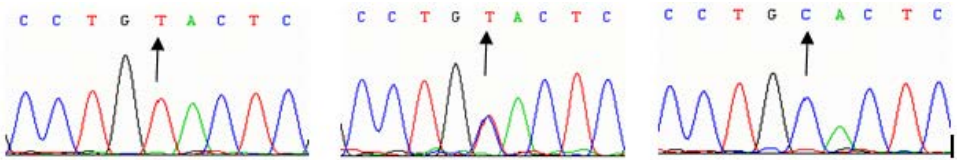
PCR amplification for sequencing: The PCR products were purified by Microcon (Millipore Corporation, MA) and sequenced using the Big Dye Terminator Cycle Sequencing v3.1 Ready Reaction kit on an ABI PRISM 3730 automated sequencer (Applied Biosystems, CA).

PCR amplification for ARMS: Amplification Refractory Mutation System (ARMS) of genotyping was used to type SNPs. This protocol involves two separate PCR reactions amplifying two different alleles of an SNP. First PCR reaction involves genomic DNA amplification (using conditions explained in section 2.4). Second PCR reaction was performed in a total volume of 10 uL containing 50 ng of template DNA, 0.4 pmol of each inner primer, 0.2 pmol of each outer primer, 200 uM dNTP, 1x PCR buffer (20 mm Tris-HCl pH 8.4, 50 mm KCl) and 1U Taq polymerase. Samples were overlaid with one drop of mineral oil and incubated for 2 min at 94°C, followed by 30 cycles of 30 sec denaturation (94°C), 30 sec annealing (50°C for



(a) AA (b) AT (c) TT

Fig. 1: Results of direct sequencing of a 345-bp fragment of the alpha s1-casein gene new variant, showing polymorphic site at position 11462 and sequence changes (indicated by arrows)



(a) TT (b) CT (c) CC

Fig. 2: Results of direct sequencing of a 498-bp fragment of the beta-casein gene new variant showing polymorphic site at position 8491 and sequence changes (indicated by arrows)

α-S1cn and 67°C for β-casein) and 30 sec extension (72°C) and an additional 5-min extension at 72°C at the end of the 30 cycles. Amplification products were resolved by electrophoresis in 3% agarose gels with ethidium-bromide.

PCR amplification for SNaPshot genotyping: The SNaPshot® Multiplex Kit a single-tube was used to interrogate SNPs at known locations using recommended protocol from Applied Biosystems, CA.

RESULTS AND DISCUSSION

Nineteen exons of αs1-casein gene were sequenced, bi-directionally. Sequencing of exon XI using primer X111S1 (Table 1) revealed a point mutation from A to T in exon XI (Fig. 1) at position 11462 of the sequence published by Koczan *et al*. (1991) (GenBank accession no. X59856). All three genotypes AA, AT and TT were found in the samples studied. A change of amino acid from Glutamic acid (E) to Aspartic acid (D) at position 84 of the protein was recorded by comparison of this amino acid sequence with that reported by Treweek *et al*. (2011). Total of 9 exons of β-casein gene were sequenced and sequencing of PCR product from exon VII with primer X7.2BCN (Table 1) showed a T to C point mutation (Fig. 2) at position 8491 of the sequence published by Mackinlay *et al*. (1965) (Acc. No. X14711). This mutation causes a Valine (GTA) to Alanine (GCA) substitution at position 197 of the protein. All three genotypes TT, CT and CC were found in samples screened.

ARMS SNP genotyping and SNaPshot genotyping assays were successfully applied to type two different

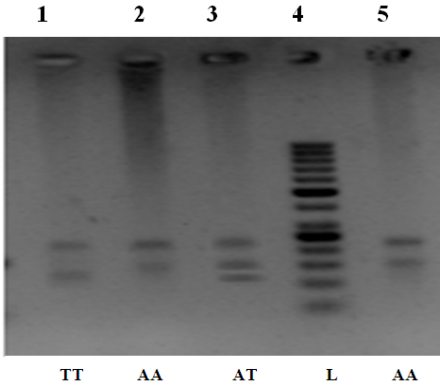


Fig. 3: ARMS for αS1-casein new variant
Lane 1: Showing TT genotype for this variant; Lane 2 and 5; Showing AA genotype; Lane 3: Showing AT genotype; L: 50 base pair DNA ladder in lane 4

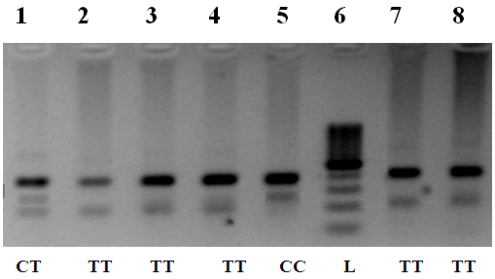
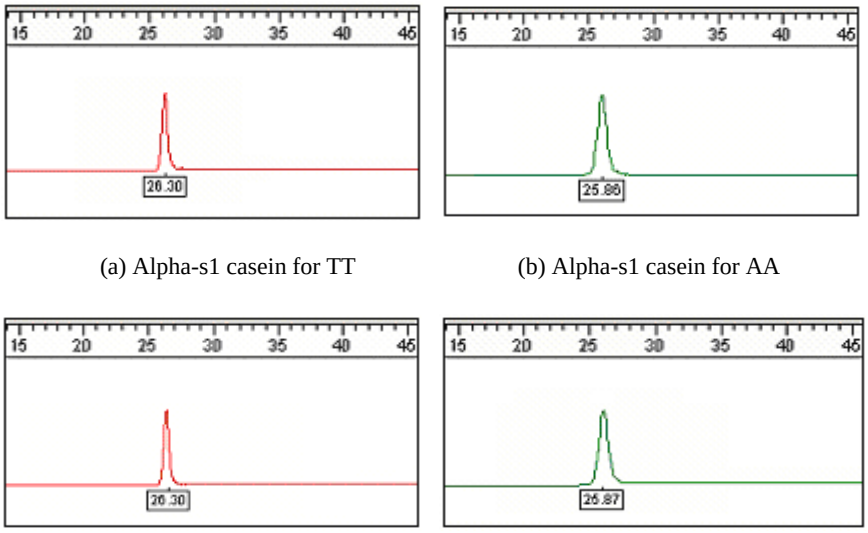


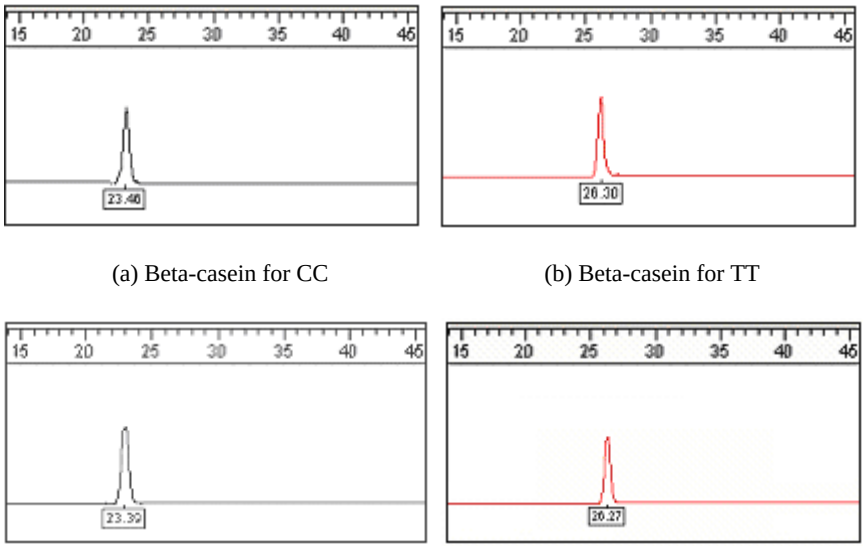
Fig. 4: ARMS for beta casein new variant
Lane 1: Showing CT genotype for beta casein variant; Lane 2, 3, 4, 7 and 8: Showing TT genotype; Lane 5: Showing CC genotype; L: 100 base pair DNA ladder in lane 6



(a) Alpha-s1 casein for TT (b) Alpha-s1 casein for AA

(c) Alpha-s1 casein for AT

Fig. 5: Primer extension based genotyping of the alpha S1 casein new variant in Sahiwal cattle, (a) TT homozygous genotype, (b) AA homozygous genotype, (c) AT heterozygous genotype



(a) Beta-casein for CC (b) Beta-casein for TT

(c) Beta-casein for CT

Fig. 6: Primer extension based genotyping of the beta-casein new variant in Sahiwal cattle, (a) CC homozygous genotype, (b) TT homozygous genotype, (c) CT heterozygous genotype

SNPs (Fig. 3 to 6) respectively. SNaPshot SNP genotyping is generally considered quite reliable for biallelic discrimination (Syvanen, 1999). Multiplexing can also be done that makes it cost efficient and high throughput. Tetra-primer ARMS-PCR technique does not involve post-PCR manipulations so it is a more rapid and lower cost technique that carries the potential of being a reproducible higher throughput method for SNP genotyping. In addition to that these techniques can be used not only for the selection of sires for artificial insemination but also for selection of immature cows and for pre-implantation embryos for embryo transfer thus saving the cost of raring an animal.

Table 2: Chi square test for association of genotypes with milk yield

Protein	Value	df	p-value
α-S1 casein	4.394E5	210	0.000
β-casein	5.116E5	260	0.000

df: Degree of freedom; p-value: 0.05

Table 3: Lactation record and observed milk protein genotypes

Protein	Genotype	LSM	S.D.	Significance
α-S1 casein	AA	1902.9	616.36567	0.228 NS
	AT	1999.8	663.46632	
	TT	2165.9	868.64267	
β-casein	TT	1998.9	730.49852	0.582 NS
	CT	1965.0	662.01829	
	CC	1625.7	505.84160	

LSM: Least square means of milk yield; S.D.: Standard deviation of milk yield; NS: Non-significant

The gene counting method was used to calculate allele frequencies for the total number of animals included in this study at the αs1-casein and β-casein. It was noted that new DNA variant at the αs1-casein locus (GenBank accession no. EF538766) (Shahla *et al*., 2007a) occurred at higher frequency 35% in Sahiwal cattle. The new allelic variant of β-casein (GenBank accession no. EF628290) (Shahla *et al*., 2007b) also had high frequency in Sahiwal cattle 13%.

Additionally, we identified two single base substitutions in exon VII of β casein (A to G variation at position 8093 and T to C change at 8261) but did not cause amino acid change.

Data on milk protein genotypes was statistically analyzed to find out association with milk yield. Chi square test was applied for association of genotypes with milk yield which showed quite significant association of genotypes with milk yield (Table 2). Although the results were not significant ($p>0.05$), animals with genotype TT of αs1-casein had higher least square means of milk yield than genotypes AA and AT. The least square means of milk yield for new β-casein TT genotype were higher than either CC or CT (Table 3).

Genetic polymorphism of milk proteins has great interest in animal breeding, due to its relationship with production traits, milk composition and milk quality (Treweek *et al*., 2011). However, before the genetic variability of milk proteins can be used in selection programs, it needs to be established that no unfavorable associations exist between the economically interesting milk protein alleles and other traits of importance for milk production. Also, the gene frequencies in the population of interest should be investigated because the prospects of improving a given trait depend on the frequency of the favorable allele (Lunden *et al*., 1997).

Two genotyping techniques were optimized for fast and accurate futuristic genotyping studies. Studies on large population size on new variants should be conducted to observe the positive influence of TT genotypes of αs1-casein and β-casein on milk yield as Least Square Means were high for these genotypes so that it can be incorporated in genetic improvement programs of Sahiwal cattle. Likewise, the negative influence of β-casein CC genotype should also be studied and animals carrying this genotype should be eliminated from breeding stock upon validation of studies.

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