

Nucleotide sequencing and DNA polymorphism studies of beta-lactoglobulin gene in native Saudi goat breeds in relation to milk yield

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ABSTRACT: β -Lactoglobulin (β -LG) is the dominant non-casein whey protein found in milk of bovine and of most ruminants. The amino acid sequence of β -LG along with its 3-dimensional structure illustrates linkage with the lipocalin superfamily. Preliminary studies in goats indicated that milk yield can be influenced by polymorphism in genes coding for whey proteins. The aim of this study is to identify and evaluate the incidence of functional polymorphisms in the exonic and intronic portions of β -LG gene in native Saudi goat breeds (Ardi, Habsi and Harri). Blood samples were collected from 300 animals (100 for each breed) and genomic DNA was extracted using QIAamp DNA extraction kit. A fragment of the β -LG gene from exon 7 to 3' flanking region was amplified with pairs of specific primers. Subsequent digestion with *Sac II* restriction endonuclease revealed two alleles (*A* and *B*) and three different banding patterns or genotypes, i.e. *AA*, *AB*, and *BB*. The statistical analysis showed that β -LG *AA* genotype had higher milk yield than β -LG *AB* and β -LG *BB* genotypes. Nucleotide sequencing of the selected β -LG fragments was done and submitted to GenBank NCBI (Accession Nos. KJ544248, KJ588275, KJ588276, KJ783455, KJ783456, KJ874959, and KP269078). Two already established SNPs in exon 7 (+4601 and +4603) and one fresh SNP in the 3' UTR region were detected in the β -LG fragments with designated *AA* genotype. The exonic SNPs, i.e. +4601 (G/A) and +4603 (G/C), were found within the *Sac II* restriction site and accountable for generating the *AA* genotypic patterns. Hence, the allele characterized by the substitution G>A has been sub-designated as *AA*^A, while the one characterized by the substitution G>C as *AA*^C. The polymorphisms in exon 7 did not produce any amino acid substitution.

Keywords: β -LG; Saudi goats; functional polymorphism; PCR-RFLP; genotyping

INTRODUCTION

Domestic goat (*Capra hircus*) is among the first domesticated livestock species and hence integral to animal husbandry. Goats are extensively reared at a global scale particularly in the developing world and serve as a vital resource of meat, milk, fibre,

and pelts (Zeder and Hesse 2000; MacHugh and Bradley 2001; Qureshi et al. 2014). Variable milk yields have been recorded among different goat breeds and within breeds as well. Researchers have established that milk yield can be influenced by polymorphisms in genes coding for whey proteins (Kumar et al. 2006; El-Hanafy et al. 2010).

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β -Lactoglobulin (β -LG) is the dominant non-casein whey protein present in milk of bovine and of most ruminants. It has widely been accepted to be absent in humans, though there are indications of minor presence (Hambræus and Lonnerdal 2003). The amino acid sequence of β -LG along with its 3-dimensional structure reveals association with the lipocalin superfamily (Kontopidis et al. 2004). Its ability to bind hydrophobic bioactive substances and amphiphilic molecules ranges from hexane to palmitic acid to retinol to vitamin D. Studies have revealed β -LG-pectin complexes as molecular nano-vehicles for delivering hydrophobic nutraceuticals such as ω -3 polyunsaturated fatty acids and vitamin D (Wang et al. 1997; Kontopidis et al. 2004; Zimet and Livney 2009; Ron et al. 2010; Cui et al. 2012). Other biological activities of β -LG include enzyme regulation, neonatal acquisition of passive immunity, a source of bioactive peptides, and antimicrobial activity against mastitis-causing bacteria. It is actively employed in the food industry for numerous characteristics (Kontopidis et al. 2004; Cui et al. 2012).

Milk proteins show genetic polymorphism due to nucleotide sequence substitution or deletion, various degrees of glycosylation and phosphorylation. To date, 14 β -LG variants in *Bos* genus (*B. taurus*, *B. javanicus*, and *B. grunniens*) have been identified at the protein and DNA levels (Caroli et al. 2009; Cui et al. 2012). Investigations have also revealed the polymorphic nature of β -LG in sheep with three genetic variants (A, B, and C). Possible relationship between β -LG polymorphism and yield, composition, and cheese-making ability of milk has been widely studied in different sheep varieties (Arora et al. 2010). Preliminary investigations have reported novel SNPs in the goat β -LG gene (Pena et al. 2000; Kumar et al. 2006; Jain et al. 2012).

Ardi, Habsi, and Harri are the three distinct Saudi Arabian goat breeds holding a special place in the regional agribusiness economy, though lack detailed genetic characterization. As the regional climatic conditions are quite harsh, livestock here has evolved to survive in extreme arid environment. Ardi and Harri goats share high genetic similarity (73.5%) and are wide-spread compared to Habsi animals. Harri breed is reared for its high milk productivity while Ardi lacks behind in terms of yield but is quite famous among the desert dwellers owing to its persistent milking

ability (Sabir et al. 2012, 2013). Limited information is available regarding Habsi goats. According to traditional breeders, Habsi is far more productive in terms of milk production and statistics from this study significantly support this notion. Earlier regional studies exploring β -LG polymorphism in goats have found novel genetic variants (Kumar et al. 2006; El-Hanafy et al. 2010). As no preliminary data is available regarding β -LG polymorphism in relation to milk production in Saudi breeds, the current investigation concentrated on analyzing β -LG polymorphism at the DNA level and analyzing potential associations with utility traits like milk yield.

MATERIAL AND METHODS

Experimental animals. Blood samples from Ardi and Harri goats ($n = 100$ for each breed) were collected from private farms located in the suburbs of Jeddah province and Riyadh city, while samples belonging to Habsi breed ($n = 100$) were obtained from two farms located near Al-Qunfudhah village, South Jeddah. All animals were female goats in their first to second season of lactation with an average age of 1–1.5 years.

Milk recording and statistical analysis. Animals were raised under extensive husbandry conditions and were fed on hay (*ad libitum*) and concentrated feed mixture (14% crude protein (CP)) according to their requirements. Daily recording of milk yield was initiated following 4 days of kidding. Milk yield was recorded 3 days per week during the first 16 weeks of lactation and average daily and weekly yield were then estimated. To document daily milk yield, female goats were separated from their kids for 9 h and afterwards hand milked and yield subsequently recorded. Total milk yield for each studied breed was statistically analyzed by one way ANOVA and the variations were tested by *F*-test at significance level ($P < 0.05$). Genotypes were determined by direct counting of restriction fragments observed in the gel. The frequency of different genotypes was calculated using the standard procedure given by Falconer and Mackay (1996):

Genotype frequency = n of individuals of a particular genotype / total n of individuals of all genotypes

Blood collection and DNA isolation. Around 10 ml of blood was collected from each goat using 0.5 ml ethylene diamine tetra acetate (EDTA; 0.5M, pH = 8) as an anticoagulant. The samples

Table 1. Primers (forward and reverse) utilized for the amplification of exon 7 to 3' UTR region of the β -LG gene

Primer	Primer sequence (5'–3')	Molecular weight	T _m (°C)	Length (mer)	GC content (%)	Size of amplicon (bp)
Primer (F)	CGGGAGCCTTGCCCCCTCTG	6126	69	20	75.0	427
Primer (R)	CCTTTGTCGAGTTTGGGTGT	6161	58	20	50.0	

T_m = primer melting temperature

were transported to the animal genetics laboratory and kept at –20°C until the isolation of genomic DNA. Genomic DNA was extracted using the QIAamp DNA extraction kit.

PCR-RFLP. A region of the β -LG gene spanning over exon 7' to 3' flanking area was amplified by employing a set of forward (5'CGGGAGCCTTG-GCCCCCTCTG3') and reverse (5'CCTTTGTC-GAGTTTGGGTGT3') primers (Table 1) (Kumar et al. 2006). Amplification was achieved through a pre-programmed MultiGene™ Thermal Cycler (Labnet International Inc., New Jersey, USA) in 25 μ l reaction mixture (reaction recipe in Table 2). While formulating final recipe in 200 μ l PCR tube, 23 μ l of master mix and 2 μ l of genomic DNA were blended in each animal specific reaction (amplification profile in Table 3). PCR amplicons were digested overnight at 37°C with 10 Units of *Sac II* restriction endonuclease. Restriction enzyme, 10 \times reaction-buffer, and autoclaved triple distilled water (TDW) were mixed for all the mandatory reactions in a 1.5 ml micro-centrifuge tube to design a master mix which was then dispensed into labelled 200 μ l PCR tubes. Following digestion, the samples were resolved in 3% (w/v) agarose gel in 1 \times TAE buffer stained with ethidium bromide for distinguishing genotypes.

Sequencing analysis. Selected β -LG fragments with designated genotypes were sequenced on both strands with the same set of primers used earlier

for PCR amplification following Sanger's dideoxy chain termination method (Sanger et al. 1977). The sequences of different genotypes were analyzed using the ClustalW sub-programme of the BioEdit Sequence Alignment Editor (BioEdit Version 7.2.5, 2013) to generate sequence alignment report.

RESULTS AND DISCUSSION

PCR-RFLP analysis. The 427 bp β -LG amplicons were digested with *Sac II* to establish polymorphic sites within the coding region of the gene. *Sac II* with a recognition site (5'...CCGC↓GG...3'/3'...GG↑CGCC...5') revealed two alleles (*A* and *B*) with three different restriction patterns or genotypes (Figure 1). The β -LG *AB* genotype had two restriction sites and generated three bands, i.e. 427 bp, 349 bp, and 78 bp. The β -LG *BB* genotype with only one restriction site revealed two bands of sizes 349 bp and 78 bp. An undigested product of size 427 bp termed as β -LG *AA* (*AA^A* and *AA^C*; see nucleotide sequence comparison) genotype was also obtained. These variable banding patterns also recognized the polymorphic site produced due to a single nucleotide substitution at position +4601 (Pena et al. 2000). The distribution of genotypic and allelic frequencies is presented in Table 4. The frequency of *A* allele was found to be lower compared to that of the *B* allele in all the

Table 2. PCR recipe

Reaction components	Quantity (1 \times)
dNTP Mix	0.5 μ l (200 μ M)
Forward primer (10 pmol)	1.0 μ l
Reverse primer (10 pmol)	1.0 μ l
10 \times buffer Complete with MgCl ₂	2.5 μ l
Taq DNA polymerase	0.5 μ l (1 U)
Autoclaved TDW	16.5 μ l
Genomic DNA	2.0 μ l (100 ng)
Total volume	25 μ l

TDW = triple distilled water

Table 3. Thermal Cycler parameters

- | |
|--|
| (1) Initial cycle at 95°C for 5 min
Then 35 cycles of steps containing: |
| (2) Denaturation at 95°C for 30 s
Annealing at 64°C for 1 min
Extension at 72°C for 90 s |
| (3) Final extension at 72°C for 5 min |

Amplified products were visualized through agarose gel electrophoresis (2%)

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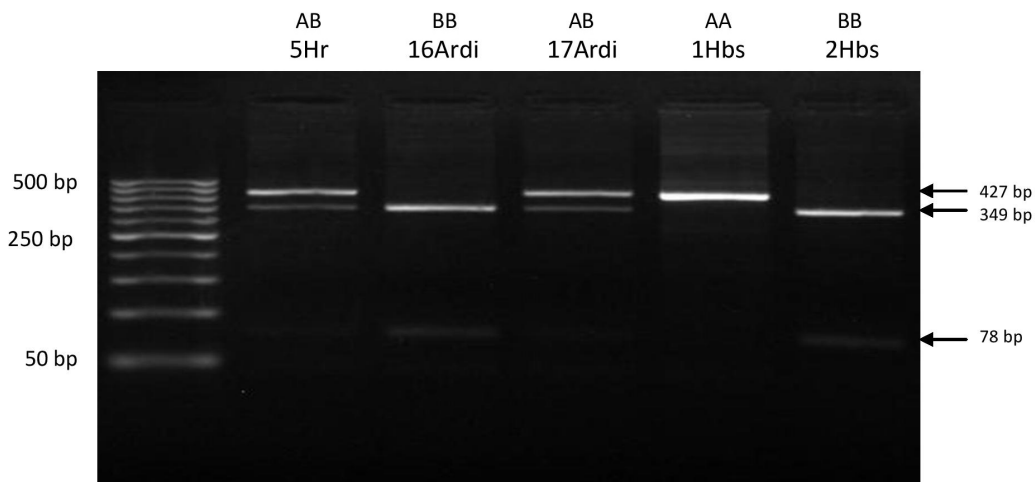


Figure 1. RFLP electrophoretic patterns of β -LG amplicons generated by *Sac II* digestion

Enzymatic digestion resulted in different restriction patterns or genotypes across the studied goat breeds. Lane 1: 50 bp DNA ladder, lanes 2–6: 5Harri, 16Ardi, 17Ardi, 1Habsi and 2Habsi

studied breeds, and in close agreement to the data presented earlier by Kumar et al. (2006) in Indian goats. The distribution patterns of β -LG genotypes exhibited that 48% of all the investigated animals showed as homozygous *BB*, 38% as heterozygous *AB*, and only 13% as homozygous *AA*.

Preliminary data has been presented in goats regarding β -LG genotypic variants and associated disparities in milk production traits (Kumar et al. 2006; El-Hanafy et al. 2010), though, no conclusive evidence is available regarding similar affects in sheep and cattle (Botaro et al. 2008; Kawecka and Radko 2011). As obvious from Table 4, Habsi breed with the highest presence of *A* allele showed significantly higher milk yield (138.26 ± 1.26 l) than Ardi (132.11 ± 1.08 l) and Habsi (131.32 ± 1.08 l) breeds ($P < 0.05$). Table 5 depicts maximum recorded values for milk yield at 16 weeks of lactation in the β -LG *AA* genotype, i.e. 146.82 ± 0.21 l in Ardi, 152.75 ± 0.25 l in Habsi, and 146.97 ± 0.25 l in Harri breed ($P < 0.05$) compared to β -LG *AB* genotype, where estimated yields stood at

141.01 ± 0.9 l in Ardi, 143.48 ± 0.65 l in Habsi, and 141.20 ± 0.92 l in Harri breed ($P < 0.05$). Minimal figures were obtained in the β -LG *BB* genotype, i.e. 122.99 ± 0.65 l in Ardi, 123.06 ± 0.81 l in Habsi, and 123.01 ± 0.62 l in Harri breed ($P < 0.05$). The results presented in this study are in close agreement with the data generated by Kumar et al. (2006) in Indian goats and El-Hanafy et al. (2010) in Egyptian goat breeds.

Nucleotide sequence comparison. Nucleotide sequencing of the selected β -LG fragments based on genotypic data was completed and following successful BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>) submitted to GenBank NCBI (Accession Nos. KJ544248, KJ588275, KJ588276, KJ783455, KJ783456, KJ874959, and KP269078). Comparison with already published sequence of the goat β -LG gene (NCBI Accession No. Z33881) via ClustalW revealed complete similarity except SNPs at specific points within fragments designated as *AA* genotype (NCBI Accession Nos. KJ544248, KJ783456, KP269078, and KJ874959) (Figure 2).

Table 4. Genotypic and allelic frequencies following *Sac II* enzymatic digestion of the amplified β -LG fragments and estimated milk yields for the studied goat breeds

Breed	<i>n</i>	Genotypic frequency			Allelic frequency		Milk yield (kg/16 weeks) (mean \pm SE)
		<i>AA</i>	<i>AB</i>	<i>BB</i>	<i>A</i>	<i>B</i>	
Ardi	100	0.08	0.4	0.52	0.28	0.72	132.11 ± 1.08^a
Habsi	100	0.23	0.41	0.36	0.43	0.57	138.26 ± 1.26^b
Harri	100	0.09	0.34	0.57	0.26	0.74	131.32 ± 1.08^a

^{a,b} $P < 0.05$

		10	20	30	40	50	
Z33881	1	CGGGAGCCTTGGCCCTCTGGGGACAGACGACGTCACCCCGCCTCCCC					50
KJ544248	1					50
KJ783456	1					50
KP269078	1					50
KJ874959	1					50
		60	70	80	90	100	
Z33881	51	ATCAGGGGACAGGAGGGACCGGGACCGGGTACCTCTCTGGGACCC					100
KJ544248	51		A			100
KJ783456	51		A			100
KP269078	51		A			100
KJ874959	51		C			100
		110	120	130	140	150	
Z33881	101	AGGCCCTCCAGGCCCTCCTGTGGCCTCCTGCTCGGGGCGCTCCTCCT					150
KJ544248	101					150
KJ783456	101					150
KP269078	101					150
KJ874959	101					150
		160	170	180	190	200	
Z33881	151	TCAGCAATAAAGGCATAAACCTGTGCTCTCCCTTCTGAGTCTTTCCTGGA					200
KJ544248	151					200
KJ783456	151					200
KP269078	151					200
KJ874959	151					200
		210	220	230	240	250	
Z33881	201	CAACGGGCAGGGGTGGAGAAGGCCCGGCACAGGGTGGGAGTGGTCTGG					250
KJ544248	201					250
KJ783456	201					250
KP269078	201					250
KJ874959	201					250
		260	270	280	290	300	
Z33881	251	CTCAGAGGATGACAGCGGGCTGGGATCCAGGGCTGTCATCACAGTCT					300
KJ544248	251					300
KJ783456	251					300
KP269078	251					300
KJ874959	251					300
		310	320	330	340	350	
Z33881	301	TGTGACATCTGGGGCCACACACATCACTGTGGCTCTTTGAAACTTTCA					350
KJ544248	301					350
KJ783456	301					350
KP269078	301					350
KJ874959	301					350
		360	370	380	390	400	
Z33881	351	GGAAACCAGGGAGGGACTCAGCAGAGATATCTGCCAGTTACCTTGGAGTGT					400
KJ544248	351			A		400
KJ783456	351			A		400
KP269078	351			A		400
KJ874959	351			A		400
		410	420				
Z33881	401	TCAGTCAACACCCAAACTCGACAAAGG					427
KJ544248	401					427
KJ783456	401					427
KP269078	401					427
KJ874959	401					427

Figure 2. Nucleotide sequence comparison of the β -LG fragments (NCBI Accession Nos. KJ544248, KJ783456, KP269078, and KJ874959) with GenBank reference sequence of the goat β -LG gene (NCBI Accession No. Z33881)

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Table 5. Milk yield (kg/16 weeks) for designated genotypes within the studied goat breeds

Genotype	Milk yield (kg/16 weeks)		
	Ardi	Habsi	Harri
AA	146.82 ± 0.21 ^a	152.75 ± 0.25 ^a	146.97 ± 0.25 ^a
AB	141.01 ± 0.9 ^b	143.48 ± 0.65 ^b	141.20 ± 0.92 ^b
BB	122.99 ± 0.65 ^c	123.06 ± 0.81 ^c	123.01 ± 0.62 ^c

^{a-c}*P* < 0.05

SNPs at position 79 (+4601) (G/A) (NCBI Accession Nos. KJ544248, KJ783456, and KP269078) and 81 (+4603) (G/C) (NCBI Accession No. KJ874959) were found in exon 7 and one at position 390 (+4912) in the flanking 3' UTR region (C/A) (NCBI Accession No. KJ544248, KJ783456, KP269078, and KJ874959). The exonic SNPs, i.e. +4601 (G/A) and +4603 (G/C), were present within the *Sac II* restriction site and accountable for generating the AA genotypic patterns. As a consequence, the allele characterized by the substitution G>A has been sub-designated as AA^A, while the one characterized by the substitution G>C as AA^C. Frequency of the AA^A allele in the analyzed sequences was 0.925 while that of AA^C allele was 0.075. No simultaneous existence was recorded for these exonic SNPs in any of the examined sequences. The +4601 (7E+83) nucleotide substitution has been established earlier in Spanish and French goats by Pena et al. (2000). The incidence of the tandemly repeated 10 bp (CCAGGCCCT) sequence, specific to ruminants only, has also been recorded (NCBI Accession Nos. KJ544248, KJ783456, KP269078, and KJ874959). Pena et al. (2000) named this polymorphic site as S₂I₂ variant due to the presence of the I₂ allele. However, an additional duplication of the inserted 10 bp sequence (the S₂I₃ variant) has not been recognized in any of the studied animals. Kumar et al. (2006) examined these alterations at both DNA and protein levels in Indian breeds and reported that β-LG AA genotype had a higher milk yield than β-LG AB genotype. The +4603 (G/C) nucleotide substitution has been recognized by Jain et al. (2012) in Indian goat breeds, though they did not elucidate associations, if any, with the utility traits. Two similar polymorphic sites (+4601 and +4603) in the exonic region of Saudi goats imply a selection pressure within this coding region. The polymorphisms in the coding region did not produce any amino acid change in the protein. The +4912 nucleotide substitution within the 3' UTR

region is a novel SNP. The 3' non-coding region has been established to control stabilizing factors on the mRNA (Bellasco and Brawerman 1993). Exon 7 comprises most of the 3' UTR region on the β-LG mRNA (Folch et al. 1994). Hence, intronic SNPs can influence regulatory switches controlling translation of the β-LG transcript.

CONCLUSION

The exon 7 to 3' flanking region of β-LG gene of *C. hircus* was analyzed to detect novel SNPs and to present a baseline data for Saudi goats. Two already annotated SNPs in exon 7 (Pena et al. 2000; Kumar et al. 2006; Jain et al. 2012) and one fresh SNP within the 3' UTR region have been detected. Based on this first hand data, further analyses are warranted as exploring such mutations for the phenotypic variation of milk yield could provide a means for improving this trait on commercial scale. SNPs within non-coding regions can also influence gene expression by controlling important regulatory switches. Thus, the identification of variations/variants across the entire gene in the existing gene pool is imperative.

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