Identification of Single Nucleotide Polymorphisms in the Nicastrese Goat and Sardinia Sheep Mannose-Binding Lectin

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Abstract

This study was undertaken to detect polymorphisms in the goat and sheep mannose-binding lectin encoding gene (MBL2) and to explore allelic variability of this gene in these two species. The analysis and comparison of the sequences obtained from sheep showed 13 polymorphic sites, six in the promoter and seven in exon 1, four of which were of the missense type. In the goats, 12 polymorphic sites were detected, five intronic, five in the promoter, and one exonic. The exon site was responsible for an amino acid change. Mutations detected at the MBL2 locus in the sheep are of particular interest, being potentially responsible for the alterations of gene expression. A population survey involved 102 ewes of the Sardinian breed and 218 goats of the Nicastrese breed, all reared in southern Italy.

Keywords

MBL2; Goat; Sheep; Polymorphism; DNA

Introduction

Mannose-binding lectin (MBL) has recently emerged as an important inborn host defense molecule. A liver-derived, collagen-like serum protein with its ability to activate the complement system during the acute phase of infection makes MBL important in the host's innate defense against microorganisms (Kilpatrick 2002). Most mammalian species have two forms (A and C) ofMBL, encoded by the MBL1 and MBL2 genes, respectively (Kozutsumi et al. 1981; Mogues et al. 1996; Storgaard et al. 1996; Oka et al. 1998; Sastry et al. 1991). In humans and ruminants, however, MBL is represented only by MBL2 (Holmskov et al. 1993). The protein-encoding region of MBL2 consists of four exons interrupted by three introns (Taylor et al. 1989).

The promoter sequence contains several consensus elements, which are believed to regulate the vast majority of gene expression (Arai et al. 1993). Genetically determined MBL deficiency is common and results in an increased risk of infection in a variety of clinical settings. MBL2 function is directly associated with its serum concentration, which is determined by the interplay between promoter and structural gene mutations (Madsen et al. 1995; Ju¨liger et al. 2000). In humans, exon 1 of the MBL2 gene contains four alleles (A, B, C, and D). Additional polymorphisms have also been described at nucleotide -550 (H, L), -221 (X, Y), and +4 (P, Q) of the promoter (Madsen et al. 1998). In humans, certain MBL2 genotypes represent a risk factor for bacterial (Hibberd et al. 1999; Koch et al. 2001), fungal (Eisen and Minchinton 2003), and viral (Thio et al. 2005; Zhang et al. 2005) infections. In the river buffalo species (Bubalus bubalis), several polymorphisms have been detected in the promoter region as well as in the exon 1 (Capparelli et al. 2008), similar to the human gene. Those authors report that such mutations are associated with Brucella abortus resistance. Furthermore, several SNPs have been detected in the pig Sus scrofa domestica (Lillie et al. 2006). On the other hand, no information is known concerning polymorphisms in the sheep and goat.

The present study reports on the identification and characterization of a high degree of variability at the MBL2 locus in these two species.

Materials and Methods

DNA Samples

Genomic DNA was extracted from blood leukocytes of 102 Sardinia sheep and 218 Nicastrese goats reared in the province of Catanzaro (Italy) according to the method of Goossens and Kan (1981).

Primer Design

All primers for amplification, sequencing, and genotyping were designed by means of DNAsis-Pro software (Hitachi), using the complete sequence of the goat (EMBL acc. no. AM933377) and sheep (AM933378) MBL2 gene as templates (Table 1).

PCR Conditions and DNA Sequencing

DNA regions of the MBL2 gene spanning the nucleotides from -961 to +3554 of three Nicastrese goats and -978 to +197 of 20 Sardinian sheep were amplified using a Gene Amp PCR System 2400 (Perkin Elmer). A typical 50 ll reaction mix comprised 100 ng genomic DNA, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 3 mM MgCl2, 200 nmol each primer, dNTPs each at 400 µM, 2.5 U Taq DNA Polymerase (Promega, Madison, WI), and 0.04% BSA. The amplification programs consisted of initial denaturation at 97°C for 2 min, annealing of primers at 46–62°C for 45 s, and an extension step at 72°C for 2 min; then 30 cycles of denaturation at 94°C for 45 s, annealing at 46–62°C for 45 s, and extension at 72°C for 2 min (except for the final extension of 10 min). Before the nucleotide sequencing, PCR products were purified with QIAquick columns (Qiagen). Nucleotide sequencing was carried out according to the dideoxynucleotide chaintermination technique (Sanger et al. 1977) using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) and an ABI Prism 377-18 (Applied Biosystems, Foster City, CA) nucleotide sequencer.

Genotyping at the Goat MBL2 Locus

In order to genotype the 218 individual samples of goat for the g.1662G>A and g.1853T>G mutations, two methods were established: one based on ACRS-PCR and the other on *Hinf*I PCR-RFLP (Table 2). For the former, the forward primer was modified by substituting C>T in position 17, providing a restriction site for the *Hsp92*II (CATG!) endonuclease that would include the mutation. The 25 µl reaction mix for each PCR product comprised 100 ng genomic DNA, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 3 mM MgCl2, 5 pmol each primer, dNTPs each at 400 µM, 2.5 U Taq DNA Polymerase (Promega, Madison, WI), and 0.04% BSA.

Genotyping of the same goat DNA samples for the three mutations in the promoter region (g.20G>A, g.160G>A, g.900C>A) was performed at the KBiosciences laboratory (Herts, UK, http://www.kbioscience.co.uk).

Genotyping at the Sheep MBL2 Locus

For genotyping the g.1030G?A mutation of 120 individual DNA samples of ewes of Sardinian sheep, a method based on the HphI endonuclease was devised (Table 2). The reaction mix was the same as described for the goat locus.

Digestion and Electrophoresis Condition

Digestion of 17 µl of PCR amplification product in each goat and sheep was performed using 10 U of the specific endonuclease for 5 h at 37°C, as suggested by the supplier's directions. PCR and digestion products were analyzed directly by electrophoresis in 3% TBE agarose gel (Bio-Rad) in 1X TBE buffer and stained with ethidium bromide.

Results and Discussion

Polymorphism Detection in the Sheep

Given the importance of the polymorphisms observed at the first exon and in the promoter in humans as well as in river buffalo, attention was focused on the analysis of such regions in the sheep gene. The DNA tract including the first exon and 977 bp of the promoter of the MBL2 gene was sequenced using, as template, the genomic DNA of 20 ewes of the Sardinia breed, randomly chosen, reared in the province of Catanzaro, Italy, and on the basis of the sequences available in EMBL (acc. no. AM933378).

Analysis and comparison of the sequences showed a total of 13 polymorphic sites. In particular, the sequence comparison relative to the first exon showed seven mutations, only five of which are responsible for amino acid changes. Two mutations (AM933378: g.1000A>C and g.1002A>G) concerned the first and third nucleotides of the fifth codon of the leader peptide. Of the four expected combinations, only three were observed: ACA (p.Thr5), ACG (p.Thr5), and CCG (p.Pro5), the latter being responsible for an amino acid change. The third mutation concerned the first nucleotide of the seventh exon of the leader peptide (g.1006C>T), being responsible for the p.Leu7Phe aa change. Two other mutations, g.1030G?A at the first nucleotide of codon 15 and g.1073C>G at the second nucleotide of codon 29, are responsible for the p.Val15Met and pThr29Ser aa changes. The remaining two mutations, observed at the first exon, are of the silent type. The first involves the third nucleotide of the second codon of the leader peptide, g.993G>A (p.=), and the second is at codon 16 of the mature

protein, g.1092C>T (p.=). In the investigated subjects, none of the observed mutations was found to be associated with any others.

Some of these might be of particular interest. It has already been reported that variations in the amino acid sequence of the leader peptide might influence a correct protein synthesis (Kozak 2005). The initiation of translation in multicellular eukaryotes is known to be affected by aspects of mRNA structure, including the m7G cap, the primary sequence or the context surrounding the AUG codon, the position of the AUG codon (i.e., whether it is the first AUG in the message or not), the leader

length, and the secondary structures, both upstream and downstream from the AUG codon (Kozak 1991). Therefore, A-rich or A/U-rich sequences downstream of the starting codon probably stimulate translation by inhibiting the formation of secondary structures, and it has been demonstrated that mutations of such codons might compromise the translation process. In the present study, the two mutations observed at the coding sequences of the leader peptide of the sheep MBL2 gene (g.1000A>C; g.1002A>G) would seem to concern at least one such codon: ACA (p.Thr5)>ACG (p.Thr5)>CCG (p.Pro5). It is, therefore, reasonable to hypothesize that these two mutations might have roles in a differential translational efficiency of the MBL2 gene in the sheep. Furthermore, from the analysis of the sequences of the first exon of the MBL2 gene in the sheep, a g.1030G>A mutation was identified at the nucleotide 44 of the leader peptide coding sequence and found to be responsible for the p.Val15Met aa change. Such a mutation could give rise to a potential second AUG starting codon, thus originating new forms of products, an alternative to the canonic ones.

It is known that the alternative translational initiation is one mechanism that increases the complexity level of an organism by the alternative gene expression pathways. In general, in eukaryotes, it is the first AUG starting codon from the 5' end of the mRNA (first-AUG rule) that is used for initiation. Unlike prokaryotes, the ribosome does not assemble directly on the starting codon, but the minor subunit links to the cap at the 5' end and flows along the mRNA (scanning mechanism) till it stops at the level of the first AUG, where the other components of the translation machinery assemble (Kozak 2005).

The presence of the alternative translation initiation codons in a single mRNA might, therefore, contribute to the generation of protein diversity. The genes produce two or more versions of the encoded proteins, and the shorter version, initiated from a downstream in-frame start codon, lacks the N-terminal amino acids fragment of the full-length isoform version. Since the first discovery of the alternative translation initiation, a small but growing number of mRNAs have been reported to

initiate translation from alternative start codons. This phenomenon involves nearly 15% of human mRNA (Kozak 2005).

In the present study, a high level of polymorphism has also been detected at the promoter level. Particularly, six point mutations were observed: three transitions, in positions -171 (g.740G>A), -619 (g.292G>A), and -725 (g.186A>G); two transversions, in positions -477 (g.434A>C) and -664 (g.247A>C); and a single nucleotide deletion (g.113delT).

Detection of mutations at the promoter level of MBL2 is of particular interest. Several studies have shown that the presence of genetic polymorphism in the human promoter region of MBL2, especially at the -550 and -221 positions, affects serum protein levels (Madsen et al. 1995; Steffensen et al. 2000; Lee et al. 2005) and, consequently, increases susceptibility to infectious diseases (Madsen et al. 1995; Prohaszka et al. 1997). Analogously, analysis of the regulatory region in sheep MBL2 gave evidence for six point mutations. Analysis of the transcription factor binding site using the online program AliBaba version 2.1 (http://www.generegulation.com/pub/programs/alibaba2/index.html) shows that both mutations g.740G>A and g.247A>C change the consensus sequences of the Sp1 factor.

Similarly, the g.434A>C mutation would seem to alter the consensus sequence of the HNF1 (hepatocyte nuclear factor) gene, whereas the g.113delT mutation would determine the disappearance of a consensus site for the factor C/EBPalpha (CCAAT-enhancer-binding proteins), a basic region/leucine zipper (bZIP) transcription factor. Both of these are liver-enriched transcription factors C, which are implicated in the regulation of numerous liver-specific genes (Miura et al. 1993; Lee et al. 1997). Finally, the g.292G>A mutation would create a new consensus sequence for glucocorticoid-responsive elements. The increased release of glucocorticoid hormones by the adrenals is an important part of the response to stress and results in elevated serum concentrations, mainly of cortisol. Thus, the glucocorticoid-responsive elements in the promoter region of the MBP gene may also serve to increase MBL expression during the acute phase response (Taylor et al. 1989).

Polymorphism Detection in Goats

With a template of genomic DNA obtained from three goats of the Nicastrese breed reared in the province of Catanzaro, the MBL2 gene was sequenced from the first nucleotide of the first exon to nt 417 of the fourth exon, for a total of 3554 bases plus 961 bp of the 5' UT region (EMBL acc. no. AM933377). Analysis and comparison of the sequences showed a total of 12 polymorphic sites. Two of these were localized at the level of the first intron (g.1213C>T and g.1325T>C), three in the second intron (g.1853T>G, g.1929C>T, and g.2900T>G), one in the third intron (g.3369A>G), and five at the promoter level (g.20G>A, g.68T>C, g.160G>A, g.515C>T, g.900C>A). Only one mutation was found at the level of the second exon, at nucleotide 116 (g.1662G>A), being of the missense type, responsible for the p.Met102Ile aa change. Since the presence of guanine (g.1662G>A) also characterizes the MBL2 gene in sheep and cattle, it is likely that its presence might be indicative of an ancestral condition.

In contrast to the sheep locus, the only exonic mutation shown at the goat MBL2 locus does not seem to influence the expression of the gene directly. In addition, none of the observed mutations at the intronic level seems to be responsible for differences in the expression of the MBL2 gene, mainly because none of them, at least apparently, alters the canonic sites of splicing. Moreover, we failed to find any significant difference in the transcription factor binding when wild alleles were replaced by mutant ones. Thus, the role of the promoter SNPs on the transcription activity of the goat MBL2 gene and, in turn, its role in disease progression cannot be predicted.

Further research is needed to verify if the observed mutations in sheep and goats might compromise the gene expression or might be responsible for alternative products of the MBL2 gene and, in such a case, to elucidate the biological significance that these forms might have in the network of inborn immunity in response to disease severity.

Genotyping of Sheep MBL2 Alleles

Of the seven exonic mutations observed, we genotyped the transition g.1030G>A, which is known to be responsible for the p.Val15Met aa change that could represent an alternative starting codon of translation. Such a mutation is also responsible for the creation of an HphI endonuclease restriction site (GGTGANNNNNNNN!) and, therefore, would allow identification of G-carriers.

For that purpose, the DNA spanning the 50 region and the first exon (1174 bp) was amplified. The HphI digestion of amplicons in subjects homozygous for the presence of adenine produced two monomorphic restriction fragments (34 and 956 bp) and a polymorphic band of nearly 184 bp; the same digestion in subjects homozygous for guanine produced the same monomorphic fragments (34 and 956 bp) plus two sub-bands of 51 and 133 bp, deriving from the 184 bp band.

Heterozygous individuals produced a pattern characterized by all five restriction fragments (Fig. 1).

The presence of guanine at the first exon of the sheep MBL2 gene would represent the ancestral condition of the gene, since it is also present in other species. Therefore, the allele containing guanine has been named MBLA; and the allele containing adenine has been named MBLB. Using this method, we typed 102 ewes belonging to the Sarda breed.

The population survey at the sheep locus showed a genotype distribution of 78 A/A, 20 A/B, and 4 B/B, with a frequency of 0.86 for the A allele and 0.14 for the B.

For this marker, the population was determined to be in Hardy–Weinberg equilibrium (1 g/l, χ^2 = 3.02, $P \leq 0.05$).

Genotyping of Goat MBL2 Alleles

Genotyping concerned only five of the 12 markers: g.20G>A, g.160G>A, g.900C>A, g.1662G>A, and g.1853T>G. To identify carriers of the g.1662G>A exonic transition quickly and easily, we established a method based on the amplification-created restriction site (ACRS) PCR, with a modification of the forward primer to create a polymorphic restriction site of the Hsp92II endonuclease. The amplified tract had a length of 117 bp, including part of the second exon and second intron.

In subjects homozygous for the presence of adenine, the Hsp92II digestion produced two monomorphic restriction fragments of 9 and 21 bp (not visible electrophoretically) and a polymorphic band of 87 bp; in subjects homozygous for guanine, the 87 bp band produced two fragments of 67 and 20 bp (the latter not visible electrophoretically). The heterozygous individuals produced a pattern characterized by all five restriction fragments: 87, 67, 21, 20, and 9 bp (Fig. 2).

The method developed to genotype the carriers of the intronic transversion g.1853T>G was based on PCR-RFLP. For this purpose, the DNA tract from nt 236 of the first intron to nt 344 of the second intron was amplified, for a total of 615 bp. The g.1853T>G transversion was responsible for the disappearance of one of the three restriction sites of the HinfI (G!ANTC) enzyme within the amplified tract. In subjects homozygous for the presence of guanine, digestion produced two monomorphic restriction fragments of 127 and 34 bp (the latter not visible electrophoretically) and a polymorphic band of 454 bp. In subjects homozygous for the presence of thymine, the 454 bp band produced two fragments, of 157 and 297 bp. The heterozygous individuals produced a pattern characterized by all five restriction fragments: 454, 297, 157, 127, and 34 bp (Fig. 3).

To investigate the three mutations in the promoter region (g.20G>A, g.160G>A, g.900C>A), 218 samples of caprine DNA were genotyped at the KBiosciences laboratory (Herts, UK, http://www.kbioscience.co.uk).

The population survey for the five chosen markers included 218 individual samples of DNA obtained from goats of the Nicastrese breed reared in the province of Catanzaro. Four of the five mutations (g.20G>A, g.1853T>G, g.900C>A, and g.1662G>A) were found to be strictly associated (cis phase) and therefore can be considered a single biallelic locus. Frequencies of the alleles were 0.74 (A) and 0.26 (B), with the following genotype distribution: 119 A/A, 83 A/B, 16 B/B. The population under investigation was shown to be in Hardy–Weinberg equilibrium (1 g/l, $\chi^2 = 0.08$, $P \leq 0.05$).

The genotype distribution of the g.160G?A mutation at the MBL2 locus was 168 G/G, 47 G/A, and 3 A/A, with an allele frequency of 0.88 (G allele) and 0.12 (A allele). The population under

investigation was shown to be in Hardy–Weinberg equilibrium (1 g/l, $\chi^2 = 0.019$, $P \leq 0.05$) at this marker.

Natural genetic resistance can be considered an alternative route to vaccination for the control of infectious diseases in domestic animals. Innate immunity plays an important role during infections, since it decreases the initial number of bacteria and may influence the development of a protective adaptive immunity. The high genetic variability found in the Nicastrese goat and Sardinia sheep breeds at the MBL2 candidate gene may be important in the study of innate resistance to infectious diseases such as brucellosis and may serve as a useful tool for large-scale screening of goat and sheep populations, so that a marker-assisted selection program can be developed for the genetic improvement of these species.

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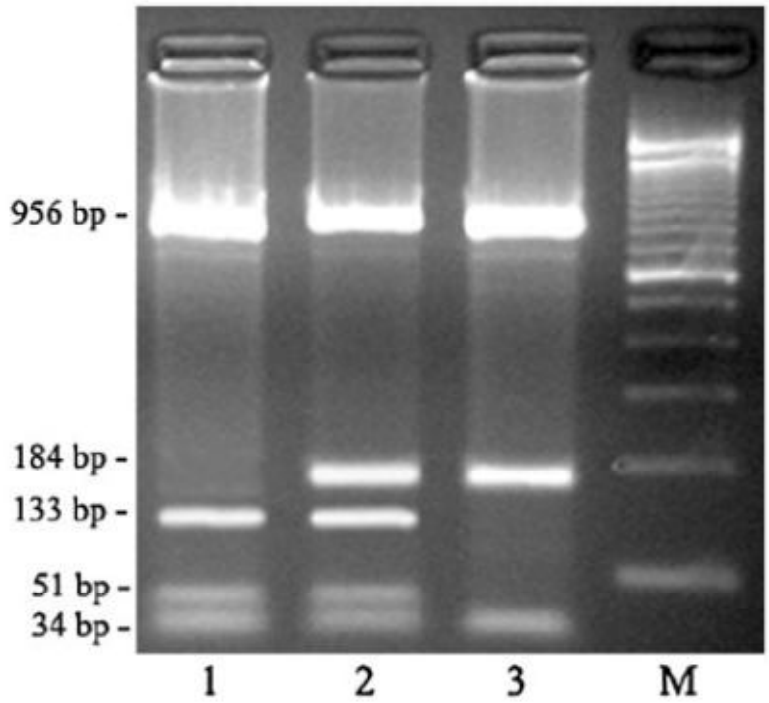

Fig. 1 Sheep MBL2 genotypes revealed by HphI digestion. DNA electrophoretic patterns obtained after digestion with HphI endonuclease of the DNA region spanning the 5' region and the first exon of the sheep MBL2 gene. Lane 1 A/A (subjects homozygous for guanine), lane 2 A/B (heterozygous subjects), lane 3 B/B (subjects homozygous for adenine). Lane M 100 bp marker

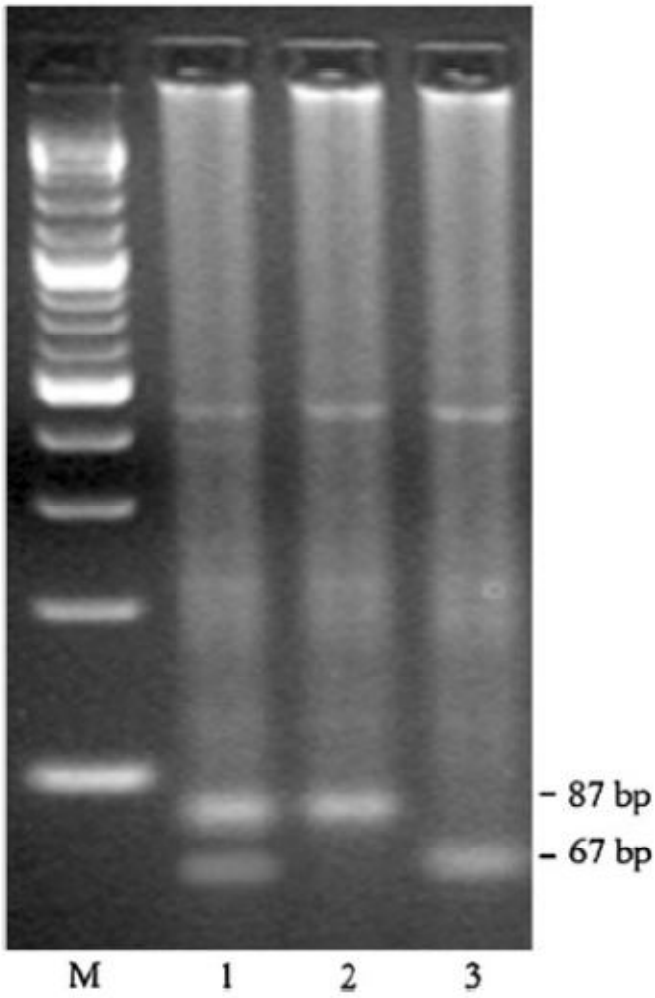

Fig. 2 Goat MBL2 genotypes revealed by Hsp92II digestion. DNA electrophoretic patterns of ACRS-PCR obtained after digestion with Hsp92II endonuclease of the DNA region spanning part of the second exon and second intron of the goat MBL2 gene. Lane 1 A/G (heterozygous), lane 2 A/A (homozygous for adenine), lane 3 G/G (homozygous for guanine). Lane M 100 bp marker

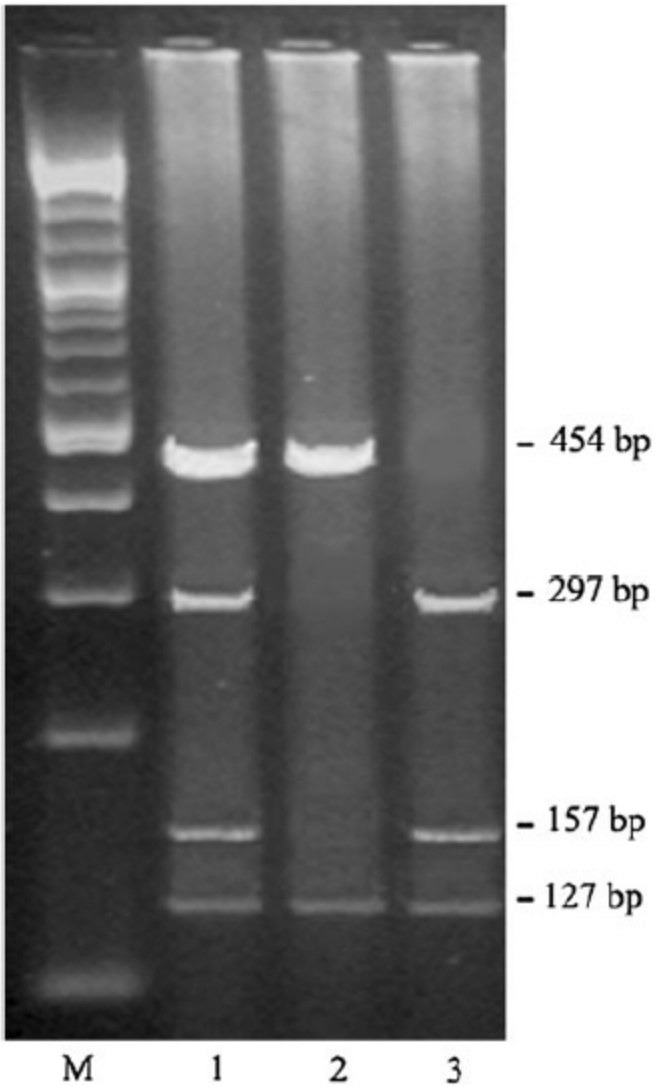

Fig. 3 Goat MBL2 genotypes revealed by HinfI digestion. DNA electrophoretic patterns obtained after digestion with HinfI endonuclease of the DNA region spanning nucleotide 236 of the first intron to nt 344 of the second intron of the goat MBL2 gene. Lane 1 T/G, lane 2 G/G, lane 3 T/T. Lane M 100 bp marker

Table 1 Oligonucleotide primers, positions and restriction enzymes for PCR-RFLPs and ACRS-PCR assays

mutation	Position nt		Primers sequence (5'-3')	Genotyping
g.1853T>G	1393-1412 [(1)]	Forward	GCTTTTCTTCTATGGTCCTT	HinfI PCR-RFLP
	Complementary to: 1988-2007 [(1)]	Reverse	ATATACCATGTATCTACCGC	
g.1662G>A	1643-1661[(1)]	Forward	GAGACCCTGGAGAAAA***C***AT	Hsp92II ACRS-PCR
	Complementary to: 1742-1759 [(1)]	Reverse	CCCAGGAAGCAGCAGAAG	
g.1030G>A	1-18 [(2)]	Forward	GGCTGGACTGATCTGGA	HphI PCR-RFLP
	Complementary to: 1154-1174 [(2)]	Reverse	GTTCTCCCTTTTCTCCCTTG	

[(1) (2)] Numbering of primers agrees with the nucleotide sequence of the goat (EMBL Acc. No. AM933377) and sheep (EMBL Acc. No. AM933378) MBL2 gene, respectively.

Table 2 Thermal amplification programs for goat a) HinfI PCR-RFLP and b) ACRS-PCR and for sheep c) HphI PCR-RFLP

a)

Cycle	Denaturation	Annealing	Extension
1	97 ºC – 2 min		
29	94 ºC – 45 sec	58 ºC – 45 sec	72 ºC – 1.30 min
1			72 ºC – 10 min

b)

Cycle	Denaturation	Annealing	Extension
1	95 ºC – 4 min		
29	95 ºC – 1 min	64.5 ºC – 45 sec	72 ºC – 1 min
1			72 ºC – 10 min

c)

Cycle	Denaturation	Annealing	Extension
1	97 ºC – 2 min		
29	94 ºC – 45 sec	62 ºC – 45 sec	72 ºC – 2 min
1			72 ºC – 10 min