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Research Article

Polymorphisms of *MBL* Gene Introns and Their Association with *MBL* Serum Levels in Hu Sheep

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Abstract

PCR single-strand conformation polymorphism (SSCP) and DNA sequencing techniques were used to analyze the genetic polymorphism of mannose-binding lectin *(MBL)* gene 3 introns in Hu sheep. The results showed that 3 introns of *MBL* gene had polymorphism, 3 genotypes were identified in intron1, which were controlled by 2 alleles, respectively; 3 genotypes were identified in intron2, which were controlled by 2 alleles, respectively; 3 genotypes were identified in intron3, which were controlled by 2 alleles, respectively; 6 new single nucleotide polymorphisms(SNPs) mutation sites were found, g.288T>A in intron1, g. 1091 T>C, g.1096A>C, g.1770G>C in intron 2, g.2297C>T, g.2331G>A in intron3. Use ELISA to detect MBL serum level in Hu sheep, then use One-way ANOVA analyze the relationship of different genotypes and *MBL* serum level. And the result showed that the *MBL* serum level of AA genotype was higher than BB genotype ($P<0.05$) in intron1, CC genotype was higher than DD, GG genotype was higher than HH ($P<0.05$) in intron2. So, AA, CC and GG genotypes may be related to disease resistance; BB, DD and HH genotypes be related to disease susceptibility. The point mutation in intron3 couldn't lead to the change of MBL serum level.

Keywords: *Sheep, MBL, Mycoplasma pneumonia, PCR-SSCP, ELISA, Introns*

Hu Koyunlarında MBL Gen İntronlarının Polimorfizmi ve MBL Serum Seviyeleri ile İlişkisi

Öz

PCR single-strand conformation polymorphism (SSCP) ve DNA sekanslama teknikleri, Hu koyunlarında mannoz bağlayan lektin *(MBL)* gen 3 intronunda genetik polimorfizmi analiz etmede kullanılmıştır. Sonuçlar *MBL* geninin 3 intronunda polimorfizmin olduğunu gösterdi, ve intron 1'de sırayla 2 allel tarafından kontrol edilen 3 genotip, intron 2'de sırayla 2 allel tarafından kontrol edilen 3 genotip, intron 3'de de sırayla 2 allel tarafından kontrol edilen 3 genotip tespit edildi. 6 yeni tek nükleotid polimorfizm (SNPs) mutasyon alanı (g.288T>A intron 1'de, g. 1091 T>C, g.1096A>C, g.1770G>C intron 2'de, g.2297C>T, g.2331G>A intron 3'de) bulundu. Hu koyunlarında *MBL* serum seviyelerini belirlemek amacıyla ELİSA uygulandı, sonrasında Tek yönlü ANOVA kullanılarak farklı genotipler ile *MBL* serum seviyeleri arasındaki ilişki analiz edildi. Sonuçlar intron 1'de AA genotipinin *MBL* serum seviyesinin BB genotipinden daha fazla olduğunu ($P<0.05$), intron 2'de CC genotipinin DD genotipinden, GG genotipinin HH genotipinden daha fazla olduğunu ($P<0.05$) gösterdi. Bu nedenle AA, CC ve GG genotipleri hastalık dirençliliği ile ilişkili olabilirken BB, DD ve HH genotipleri hastalık duyarlılığı ile ilişkili olabilir. İntron 3'de nokta mutasyon MBL serum seviyesinde değişime neden olamaz.

Anahtar sözcükler: *Koyun, MBL, Mycoplasma pneumonia, PCR-SSCP, ELISA, İntronlar*

INTRODUCTION

Mannose-binding lectin *(MBL)* is a serum protein mainly produced by the liver and belongs to the C-type calcium ion-dependent lectin, which plays an important role in innate immunity. *MBL* function involves the formation of a complex-activated complement system, which binding to a serine protease associated with MBL in the lectin pathway [1]. It plays an important role in adaptive immune responses, and inflammatory responses by affecting

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cytokine release [2]. Sheep *MBL* gene has 4 exons and 3 introns with a full length 4462 base pair (bp) [3]. The study found that there are 5 exons in the *MBL* gene, and 6 single nucleotide polymorphisms (SNPs) associated with *MBL* expression levels [4]. The above polymorphism and their haplotype have the greatest influence on serum *MBL2* content, at the same time, it's essential for its anti-infective effect to maintain a certain level of *MBL* serum concentration, so *MBL* polymorphism has become a hot topic in current research. The study has been shown that the level of *MBL* in the blood is mainly determined by the structure of the *MBL* gene, which is affected by the variation of the gene structure region and the regulation of the activity of the gene promoter region [5]. For example, three SNPs in the exon I of the human *MBL* gene, known as the D-allele (Arg52Cys), B-allele (Gly54Asp) and C-allele (Gly57Glu), interfere with the formation of high *MBL* oligomers, It is speculated that it can affect the level of serum *MBL* by initiating different levels of gene expression [6-8].

Promoter is a cis-actin element of eukaryotic gene expression regulation, contains important information of gene expression regulation network, determines the degree of gene expression and its specificity [9,10]. On the other hand, the mutation of the intron area may influence the transcription, which results in incomplete translation in the functional areas. Therefore, this makes *MBL* protein to change in structure, and hinders the realization of *MBL* biological function. Resulting in a significant decrease in *MBL* serum levels of the body, and ultimately the disease resistance is reduced due to weakening of the body's immunity.

We use PCR-SSCP and DNA sequencing techniques to analyze the genetic polymorphism of 3 introns in the Hu sheep's *MBL* gene, and conduct the statistical analysis on the association between different genotypes in the *MBL* gene and *MBL* serum levels in Hu sheep. In order to lay the foundation for comprehensive study, the study on the correlation between the polymorphisms of the sheep *MBL* gene and diseases.

MATERIAL and METHODS

Collection of Sheep Blood Samples

Whole blood was collected from 105 healthy individuals of the Hu sheep that aged 4 months and weighted 10-12 kg were from different sheep farms in the ninth agricultural unit of the Xinjiang Production and Construction Corps, Tacheng, China. Fresh blood samples were mixed immediately with EDTA buffer. Sera were separated by centrifugation at 3000×g for 10 min, and were then transferred to 1.5 mL Eppendorf tubes and stored at -80°C.

DNA Extraction, Primer Design and PCR Amplification

Genomic DNA was extracted from EDTA anticoagulated blood samples using phenol/chloroform method [11]. Primer sequences of sheep *MBL* introns-1,2,3 and annealing temperatures of the PCR *(Table 1)* were designed with Primer 5.0 from the sequences of the MBL gene of sheep available in GenBank (accession numbers FJ977629). Primers were synthesized at Sangon Biological Engineering Technology Company (SBETC, Shanghai, China) and were used in a 25 µL PCR reaction to amplify a some sections of the intron-1,2,3 region of the *MBL* gene *(Table 1)*. A 25 µL PCR reaction contains 1 µL (50 ng) of genomic DNA extracted from an individual Hu sheep, 2.5 µL 10× PCR buffer, 1 µL (5mM) of each primer, 2.5 µL dNTPs (2.5 mM), 1.5 µL $MgCl_2$ (15 mM), 0.6 µL (1.5 units) Taq DNA polymerase, and 14.9 µL MilliQ H_2O. The PCR reagents were supplied by the SBETC. The conditions for PCR reactions are 94°C for 5min, followed by 30 cycles of 30 s at 94°C, 45 s at annealing temp *(Table 1)*, 30 s at 72°C, and a final extension at 72°C for 10 min. PCR products were electrophoresed on 1.5% agarose-gel using 0.5×TBE buffer; the agarose gel was stained with ethidium bromide.

PCR Single-Strand Conformation Polymorphism Analysis

PCR products were analysed by SSCP, following protocols described [12]. Aliquots of 2 µL PCR products were mixed with 8 µL denaturing solution (98% formamide, 25 mM EDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), were incubated at 98°C for 10 min and were then chilled on ice for 10 min. Denatured PCR products were electrophoresed on 12% PAGE gel (80 mm×73 mm×0.75 mm) in 0.5×TBE buffer at 140V and 12°C for 20 h. The gel was stained with 0.1% silver nitrate solution.

Cloning of PCR Products and DNA Sequencing

PCR products representative of different SSCP patterns in the Hu sheep were cloned using pGEM-T Easy Vector System (Promega) and competent *Escherichia coli* cells following the manufacturer's instruction. 6 to 12 colonies were selected for each SSCP pattern and cultured overnight in Terrific Broth medium that contained 50 mg/mL ampicillin. To isolate plasmids, a 50-mL aliquot of the overnight culture was centrifuged at 13.000 rpm for 2 min; the supernatant was discarded. The pellet was mixed with 30 mL (10×) TE buffer, was boiled for 10 min, and was then centrifuged at 13.000 rpm for 2 min. One µL of the supernatant was used in a PCR with primers MBLF and MBLR (see above for primer sequences). The PCR products from isolated plasmids were electrophoresed on 12% PAGE gels under the same conditions described above for the PCR products from the genomic DNA. The PCR products with *MBL*F and *MBL*R from both isolated plasmids and genomic DNA were sequenced at BGI (Beijing, China; http://www.genomics.cn).

Measurement of MBL Protein Levels in Serum

Serum samples from the Hu sheep were stored at −80°C. *MBL* levels in serum samples were measured using the *MBL*

Table 1. *Primer sequences Information of MBL Gene*

Serial Number	Loci	Sequence	Location	Length/bp	Annealing Temperature/°C
1	Intron1	F: GTGATGGTGCCAAGGGAGAA R: GGGATGCCAGAATCAGAGCC	1145-1329	185	58
2		F: ATCATTTGAAACAGAGGCACG R: TCCCAGGGGAAAGGAGACAC	1289-1494	206	56
3	Intron2	F: GTTTACTTTAGCAAGGTCCAG R: CAGGCATCTCACAAGGGTTT	1696-1917	222	59
4		F: AGCCAAACCCTTGTGAGATG R: ACAATAGCCAGCGTGTAAGT	1894-2111	218	58
5		F: GTCTCACTTACACGCTGGCTAT R: AATACAACGTGGTGGAAGCA	2087-2290	204	59
6		F: TGCTTCCACCACGTTGTATT R: TCCCTGAGTTTGTCCTGTTAA	2271-2478	208	59
7		F: TAACAGGACAAACTCAGGGA R: TGCCAAGCTACTCACTAATT	2458-2650	193	60
8		F:AGTAGCTTGGCATGTGGAGA R:GGGGTAGGGTACCTTTTGAA	2639-2914	276	60
9	Intron3	F: CTGAAGTTTGGTAAAGTGAA R: CTCATTAGTTCTATGCGTTT	3062-3231	170	60
10		F: GCATAGAACTAATGAGTAGCA R: TCACTTGGGTCAGTCGTGTC	3215-3488	270	59
11		F: CGACTGACCCAAGTGAGCAT R: GTCTCAGGGCAAGCAACAGG	3473-3653	181	60
12		F: CACCTCTTTCCCTTTGTTATG R GGTTAAATCTAGCAGCCCTAA	3583-3800	218	57
13		F:TGTTCAGATTAGGGCTGCTAGA R:GCCGCATAAAATATGGTATGTCC	3771-3978	208	59

Oligomer ELISA Kit (ADL, America), which contains a 96-well test plate, standards of known *MBL* concentrations, wash buffers, a *MBL* antigen and a biotinylated monoclonal antibody specific to *MBL*, an enzyme (streptavidin-peroxidase) and a substrate solution. Serum samples from the Hu sheep and standards of known *MBL* concentrations were loaded into the wells on the test plate: 50 µL of each serum sample or standard per well. The *MBL* antigen and the biotinylated monoclonal antibody specific to *MBL* were added to each well and were incubated at 37°C for 60 min. The wells were washed and the enzyme, streptavidin-peroxidase, was added. After incubation at 37°C for 30 min, the wells were washed to remove unbound enzymes; the substrate solution, which reacted with the bound enzyme to induce a colour, was added. The intensity of the colour was proportional to the concentration of *MBL* protein present in the serum samples. It was measured with an ELISA reader at 450 nm and was then converted into *MBL* concentration (µg/L) in serum, using an established human antigenic *MBL* level of 1670 µg/L as a reference.

Statistical Analysis of the Association Between Polymorphisms in MBL Gene and MBL Protein Levels in Serum

Differences in haplotype frequencies were analysed using a chi-square test (χ^2 test). The association between polymorphisms in *MBL* gene and *MBL* protein levels in serum were evaluated using One-way ANOVA test. All statistical analyses were performed with SPSS for Windows (version 17.0).

RESULTS

PCR products for *MBL* gene had 13 specific fragments containing 3 introns. They were subject to a test by 1.5% agarose gel electrophoresis and then found to be consistent with the results of the target fragment without specific bands so could be subject to SSCP analysis.

PCR-SSCP analysis results showed that 1, 4, 6, 8,10 and 13 primers had polymorphisms. Primers 1 is intron 1 had 3 genotypes, respectively defined as AA, BB, AB, which were controlled by A and B alleles. primers 3 is intron 2-1 had 2 genotypes, respectively defined as PP and OP, which were controlled by O and P alleles. primers 4 is intron 2-2 had 3 genotypes, respectively defined as of CC, CD, and DD, which were controlled by C and D alleles. primers 6 is intron 2-4 had 3 genotypes, respectively defined as EE, EF and FF, which were controlled by E and F alleles. primer 8 is intron 2-5 had 3 genotypes, respectively defined as GG, GH and HH, which were controlled by G and H alleles. primers 10 is intron 3-2 had 3 genotypes, respectively defined as II, IJ and JJ, which were controlled by I and J alleles. primers 13 is intron 3-5 had 3 genotypes, respectively defined as KK, KM and MM,

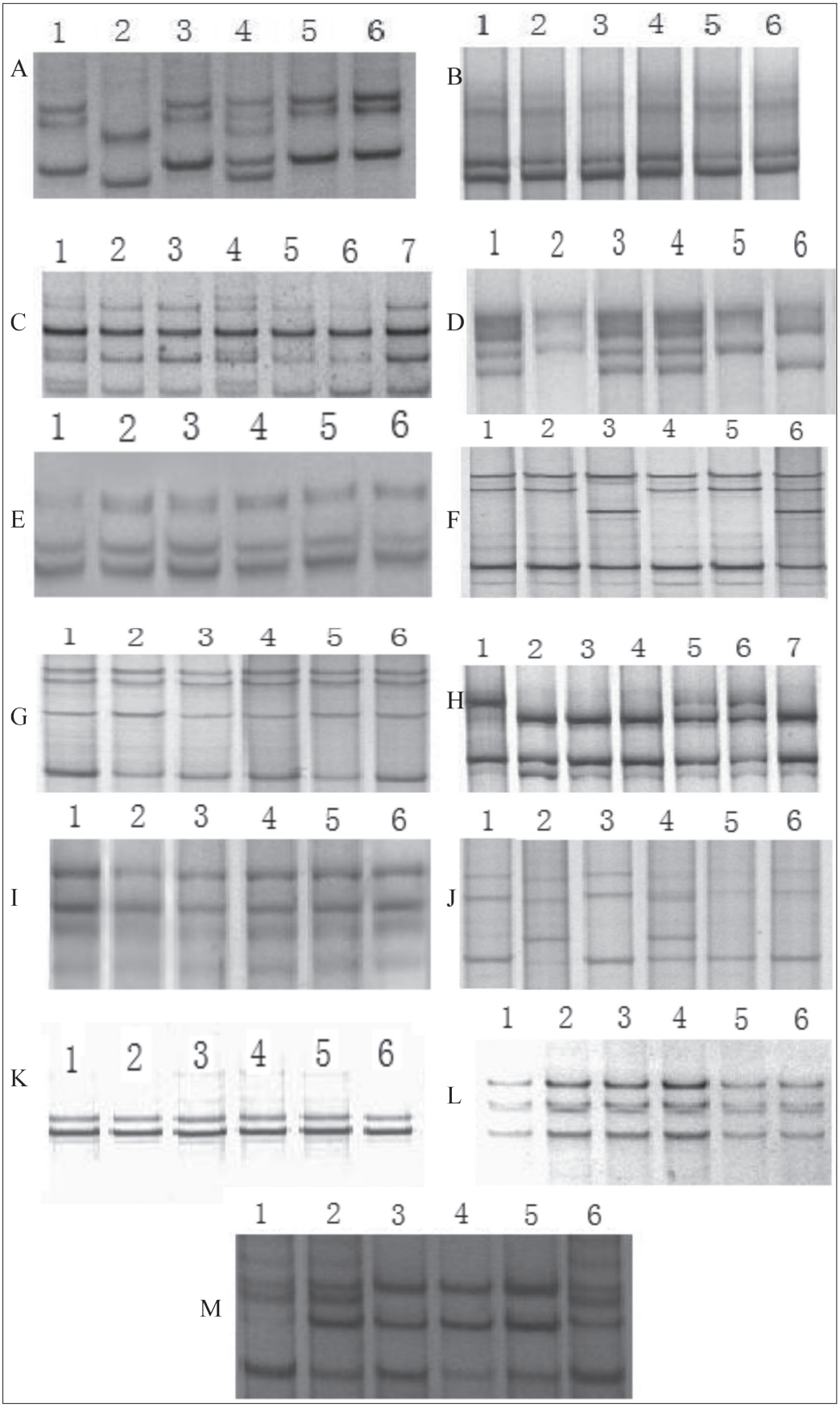

Fig 1. SSCP analysis of PCR products amplified with primers. Each product is represented by one of these letters A-M. These letters correspond to the primer 1-13, respectively

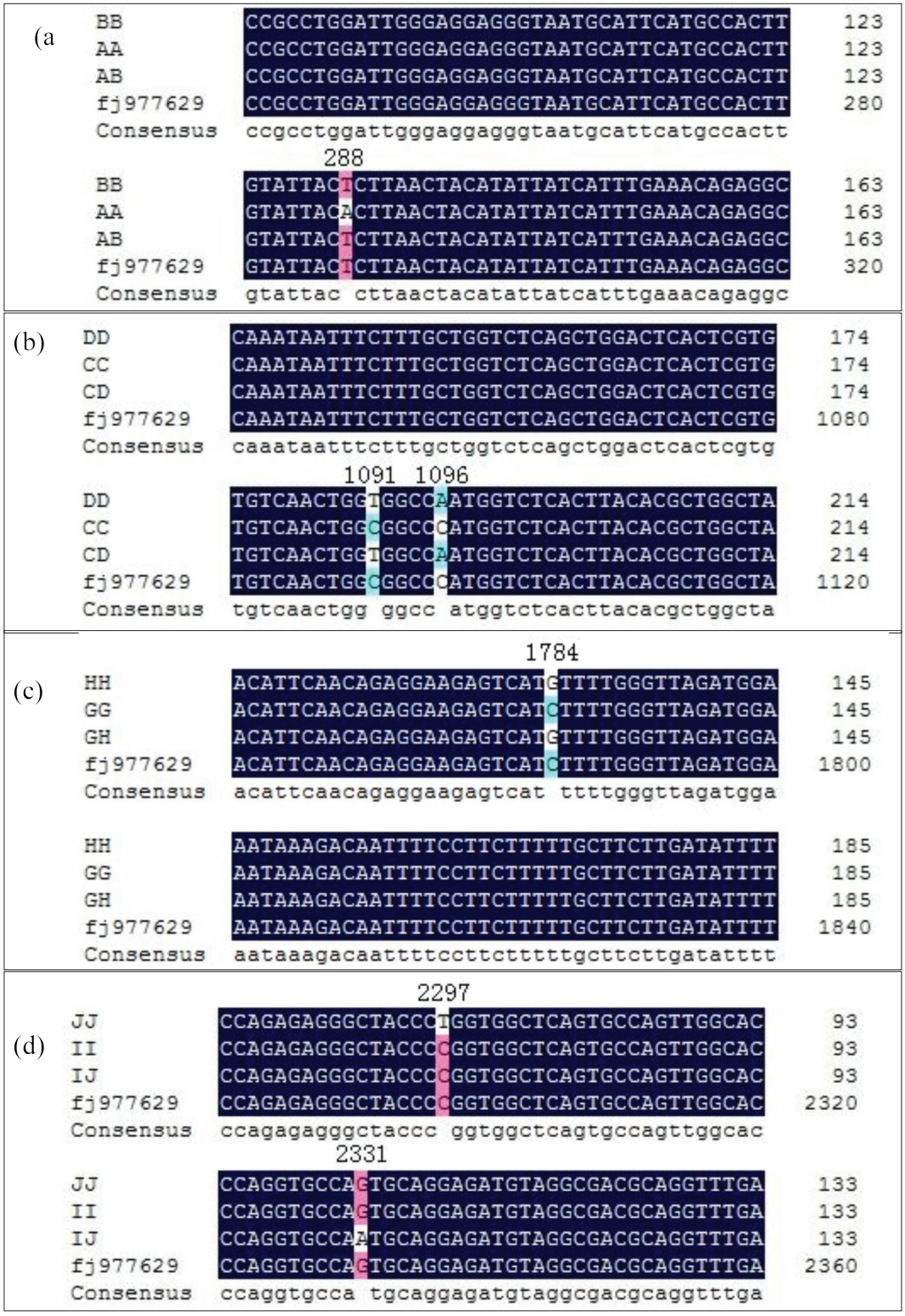

Fig 2. Comparison of sequences of different genotypes of MBL intron primer in Hu sheep (a- primer1; b- primer4; c- primer8; d- primer10)

which were controlled by K and M alleles. Primer 2, 5, 7, 9 were not found to have genetic polymorphisms *(Fig. 1)*.

Let the PCR fragments of different genotypes for all paired primers be cloned and sequenced. The comparison of sequencing results indicated that Hu sheep *MBL* gene intron had 6 single nucleotide mutation points, respectively being intron 1's g.288T> A *(Fig. 2-a)*; intron 2's g. 1091 T> C, g.1096A> C *(Fig. 2-b)*, and g.1784G> C *(Fig. 2-c)*; intron 3's g.2297C> T and g.2331G> A *(Fig. 2-d)*.

Table 2. *Statistics of frequency of genotype and allele at the MBL gene intron in Hu sheep*

Primer Number	Genotype	Genotype Frequency	Allele	Allele Frequency	χ^2
1	AA	0.467 (49)	A	0.619	13.147**
	AB	0.305 (32)	B	0.381	
	BB	0.229 (24)			
4	CC	0.343 (36)	C	0.552	2.445**
	CD	0.419 (44)	D	0.448	
	DD	0.238 (25)			
6	EE	0.238 (25)	E	0.571	13.692**
	EF	0.333 (70)	F	0.429	
	FF	0.095 (10)			
8	GG	0.181 (19)	G	0.519	13.184**
	GH	0.676 (71)	H	0.481	
	HH	0.143 (15)			
10	II	0.553 (58)	I	0.710	5.925**
	IJ	0.314 (33)	J	0.290	
	JJ	0.133 (14)			
13	KK	0.114 (12)	K	0.443	11.551**
	KM	0.657 (69)	M	0.557	
	MM	0.229 (24)			

*$\chi^2_{0.01(100)} = 119.56$; ** $P<0.01$*

According to the phenotype indicated in the electrophoretogram, then statisticed genotype frequencies. Carried out χ^2 test on different genotypes of 6 pairs of primers distributed in Hu sheep, and results showed all pair of primers had a genotype frequency distributed in Hu sheep which was characterized by an extremely significant difference ($P<0.01$), respectively *(Table 2)*.

According to the introns and sheep *MBL* ELISA test kit results, it was indicated that in this study with 105 Hu sheep, *MBL* gene intron had 3 genotypes, i.e. AA, BB and AB genotypes whose number was respectively 49, 24, and 32. According to the different genotype of intron 1, the single factor ANOVA analysis was made for *MBL* serum levels; the results showed that there were extremely significant differences between AA and AB ($P<0.01$), AB and BB ($P<0.01$), AA and BB ($P<0.01$) *(Fig. 3)*.

According to the introns and sheep *MBL* ELISA test kit results, it was indicated that in this study with 105 sheep, *MBL* gene intron 2-2 had 3 genotypes i.e. CC, CD and DD. The number of CC, CD, and DD was respectively 36, 44 and 25. According to the different genotype of intron 1, the single factor ANOVA analysis was made for the *MBL* serum level; the results showed that there were extremely significant differences between CC and CD ($P<0.01$), CD and DD ($P<0.01$), CC and DD ($P<0.01$) *(Fig. 3)*.

Mannose-binding lectin gene intron 2-6 had 3 genotypes i.e. GG, GH and HH whose number was respectively 19, 71 and 15. According to the different genotype of intron 2-6, the single factor ANOVA analysis was made for the *MBL* serum level; the results showed that there were extremely significant differences between GG and GH ($P<0.01$), GH and HH ($P<0.01$), GG and HH ($P<0.01$) *(Fig. 3)*.

Mannose-binding lectin gene intron 3 had 3 genotypes i.e. II, IJ, and JJ whose number was respectively 58, 33, and 14. According to the different genotype of intron 3, the One-way ANOVA analysis was made for the *MBL* serum level; the results didn't show significant differences between II and IJ ($P>0.05$), ($P>0.05$), II and JJ ($P>0.05$) *(Fig. 3)*.

DISCUSSION

Mannose-binding lectin is the most important natural anti-infective immune molecule in humans and animals [13]. It is secreted by the liver and secreted into the blood. It induces and activates the body's immune response before the antigen-antibody reacts specifically. *MBL's* protection features are closely related to *MBL* levels. In other words, a certain concentration of circulating *MBL* is maintained. Level is the basis of its physiological function. The lower the *MBL* level, the higher the susceptibility to pathogenic microorganisms. The expression level of MBL in serum is closely related to the *MBL* gene polymorphism, that is, *MBL* serum level is mainly removed by *MBL* gene. Affected by structural gene mutations and promoter region activity, mutations in the *MBL* gene can result in decreased *MBL* levels in serum, affecting the complement activation system [5,14].

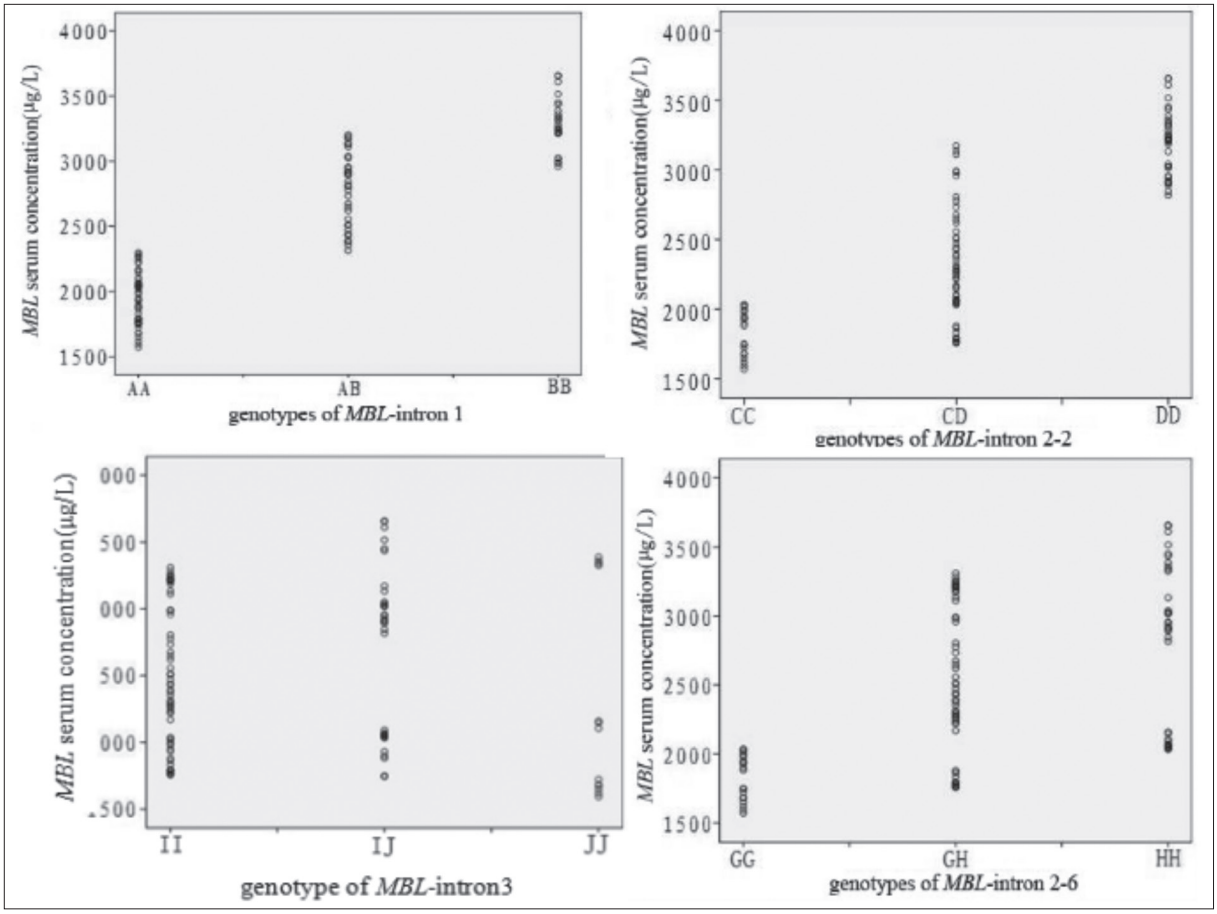

Fig 3. Comparison of MBL serum concentration in different genotypes of MBL

An intron, corresponds to an exon and is a special type of non-coding DNA sequence. The exons are alternately arranged to form an intervening gene [15]. It does not occur in mature mRNA sequences as it is cleaved during transcriptional translation of the precursor RNA. Very few connates have been found in prokaryotic genome sequences, and almost all introns are included in eukaryotic genome sequences, except that inferior eukaryotes have relatively few intron sequences. Therefore, in this experiment, 3 introns of the *MBL* gene were not involved in protein synthesis, but their mutations affected the exon coordination and the encoded protein. There also may be a position effect, it also may affect the correct shearing in the *MBL* gene expression. Thereby affecting the translation of the protein, leading to low *MBL* serum levels, ultimately making the body susceptible to the disease [16]. However, the specific function needs a further research.

This paper carried out the polymorphism analysis for 3 intron areas of the *MBL* gene in Hu sheep, detected and compared all the *MBL* serums which corresponded with genotypes. The results showed that in the Hu sheep *MBL* gene, intron 1's AA-type corresponds to a low level of *MBL* serum concentration, BB-type and AB-type correspond to a high level, so it can be predicted that BB is resistant and AA susceptible. Similarly, intron 2-2's CC-type is susceptible and DD resistant; intron 2-6's GG-type is resistant and HH susceptible; in intron 3, the difference between II and JJ is not significant, so the mutations affect the *MBL* serum level. From the above, it is indicated that in the *MBL* gene, AA of intron 1 and CC and CG of intron 2 are resistant, while BB, DD and HH are susceptible. Intron 1 and 2 genotype differences in *MBL* serum levels are significant, which indicates that *MBL* gene can be used as candidate gene for disease resistance.it found that +328 site of the pig *MBL*1 intron has a C/T mutation and that different genotypes have a significant difference in serum C3c concentration and complement hemolytic activity [17], further confirming that the pig *MBL*1 gene can be used as a function and a positional candidate gene for complement hemolytic activity.

As conclusion, the Hu sheep's *MBL* gene intron has a wealth of genetic polymorphisms, and lays the genetic basis for relevant genetic markers in screening, generation, disease resistance or susceptibility. According to the analysis for the correction of Hu sheep intron polymorphisms with *MBL* serum levels, this experiment screens the resistant and susceptible alleles to lay a solid foundation for the further validation of whether resistant alleles can be used as genetic markers for resistance to mycoplasma pneumoniae in sheep.

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