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Research Article

Association Study Between Mannose-Binding Lectin *(MBL)* Polymorphisms and Serum *MBL* Protein Levels After *Mycoplasma ovipneumoniae* Infection in Sheep

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Abstract

Mannose Binding Lectin (*MBL*) is a liver-derived, circulating plasma protein that plays a pivotal role in innate immunity, however, *MBL* polymorphisms in sheep were associated with the incidence of *MO* infection. The objective of this study was to determine the relationships between the different genotypes of *MBL* and the resistance against *MO* infection disease, including three hundred and thirty-six individuals of the China Merino sheep. Single-Strand Conformation Polymorphism (SSCP) analyses of PCR amplicons of the exon-1 region of the *MBL* gene revealed four patterns: BB, BC, CC and DD. However, BB, BC and DD genotype have 3 SNPs, 2 SNPs, and 4 SNPs respectively, and CC genotype has only an identical sequence to the reference, among which there are 3 synonymous SNPs and 6 non-synonymous SNPs. Our statistic analysis showed that the DD genotype *MBLs* in China Merino sheep were associated with the decrease of *MBL* protein level in serum. The sheep carrying this kind of genotype were more susceptible to *MO* infection, however, an opposite result was found in sheep having CC genotype, which will provide a reference for molecular breeding of sheep breeds resistant to *MO* infection.

Keywords: *Polymorphisms, China Merino sheep, Mannose-binding lectin, PCR-SSCP, Mycoplasma ovipneumoniae infection*

Koyunlarda Mannoz Bağlayan Lektin *(MBL)* Polimorfizmi ve *Mycoplasma ovipneumoniae* Enfeksiyonu Sonrasında Serum *MBL* Protein Seviyeleri İle İlişkisi

Özet

Mannoz Bağlayan Lektin *(MBL)* karaciğer kaynaklı, dolaşımda yer alan plazma protein olup doğal bağışıklıkta önemli bir rol oynar. Koyunlarda *MBL* polimorfizmi *Mycoplasma ovipneumoniae (MO)* enfeksiyonu ile ilgilidir. Bu çalışmanın amacı 136 Çin Merinos koyununda farklı MBL genotipleri ile *MO* enfeksiyonuna karşı direnç arasındaki ilişkiyi araştırmaktır. *MBL* geninin ekzon-1 bölgesinin PCR amplikonlarının Tek Zincir Konformasyon Polimorfizm (SSCP) analizi BB, BC, CC ve DD olmak üzere dört şekil bulunduğunu tespit etti. BB, BC ve DD genotipleri sırasıyla 3 SNP, 2 SNP ve 4 SNP'ye sahipti. CC genotipi referans ile aynı bir sekansa sahip olup aralarında 3 sinonim SNP ve 6 sinonim olmayan SNP vardı. İstatistiksel analizler, Çin Merinos koyunlarında DD genotip MBL'nin serumda azalmış *MBL* protein seviyesi ile ilişkili olduğunu gösterdi. Bu tip bir genotipe sahip olan koyunlar *MO* enfeksiyonuna daha duyarlıyken CC genotipe sahip olanlarda tam tersine bir görüntü elde edildi. Bu durum *MO* enfeksiyonuna dirençli koyunların elde edilmesi için bir referans oluşturmaktadır.

Anahtar sözcükler: *Polimorfizm, Çin Merinos koyunu, Mannoz bağlayan lektin, PCR-SSCP, Mycoplasma ovipneumoniae enfeksiyonu*

INTRODUCTION

Mannan-binding lectin *(MBL)* is a member of C-type lectin superfamily lectins collagen family, which can selectively identify Mannan, N an acetyl Glucosamine and mannose, N an acetyl mannosamine, etc. *MBL* can bind to a variety of bacteria, viruses, fungi, *Mycoplasma*, parasites, etc. It is an important part of anti-inflammatory immune response

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and constitutes the first defense line against infection by activating complement and opsonophagocytosis [1].

Serum *MBL* level and *MBL* gene mutation are closely associated with many kinds of diseases. *The MBL - defect* could result in many kinds of acute or chronic pathogen infections. Abnormal serum *MBL* levels might also cause certain autoimmune diseases, the mutations in exon-1 of the human *MBL* gene can interfere the formation of a stable *MBL* functional polymer which decreased serum *MBL* levels and lowered the body's physiological function significantly, such as *Systemic lupus erythematosus*, *Clinical repeated infections*, *Hepatitis B virus*, and *other viral infections* in humans [2-5]; Similarly, some other studies also reported that A-type *MBL* can has antibacterial function against *Porcine Actinobacillus*, *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis* [6,7]. It showed that *MBL* plays a vital role in the activation of the complement pathway as anti-infective molecules.

Recently, with the increase in breeding stock and changes of the introduction and feeding methods in sheep in Xinjiang, China, the risk of subsequent propagation of *MO* explosive increased and seriously affected the sustainable and healthy development of sheep. Especially, China Merino sheep, as one of the most famous kinds of fine wool sheep in Xinjiang, has been infected seriously which strongly threatened the wool industry. Therefore, in order to expand theoretical and practical knowledge of *MBL* resistance to *MO* and other relevant diseases, it is necessary to study the relationship between *MBL* genotypes and anti-*MO* infection in sheep [8,9]. Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) technology in this study was used to explore the correlations between different *MBL* genotypes and serum *MBL* levels in sheep, which will provide a reference for sheep breeding and genetic research of immune disease in future.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experiments, Animal Science and Technology College, Shihezi University (Number: A2010096). All samples were collected in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

Experimental Animals and Mycoplasma

A total of 336 healthy individuals of the China Merino sheep that aged 3 months and weighted 8-12 kg were from different sheep farms in the ninth agricultural unit of the Xinjiang Production and Construction Corps, China. A strains of *Mycoplasma (Named MO-141)* was isolated from *Hu Sheep* with *MO* infection in shihezi, Xinjiang, which the DNA homology of *MO-141* and standard strain Y-98 reaches 99% by sequence alignment, and then the isolated strain was inoculated with *Mycoplasma* liquid enrichment medium, which were incubated for 7 days under 5% CO_2 at 37ºC condition. After the culture medium turned yellow, it was put in centrifuge that were separated by centrifugation at 20.000 r/min for 20 min at 4ºC condition, when bacterial counts were 10^6 CCU/mL and then used to construct a sheep model with *MO* infection. And then, the experimental groups were injected with *MO* into the trachea 5 mL/each with a syringe, the control group was injected with the same amount of sterile saline trachea.

Collection of Sheep Blood

It selected thirty-six China Merino sheep with four different *MBL* genotypes by using PCR-SSCP technology, which were averaged into four groups, including BB, BC, CC and DD genotypes respectively, and then were marked as No. 1-36, and then the control group was numbered 37-42. Sheep infected with *MO* in the experimental group, which were collected the fresh anticoagulant blood on the day before infection (-1 d), and 1 d, 7 d, 14 d and 21 d after artificial infection respectively. Meanwhile, the fresh anticoagulant blood in the control group were also collected and stored at -20ºC.

Sheep Genomic DNA Extraction, Primer Design and PCR Amplification

Sheep genomic DNA was extracted from whole blood samples of the China Merino sheep using the phenol/chloroform method as described in Sambrook and Russell (2001). Two primers, including *MBL*F (5-CGCTGTTTACAT CACTTCCT-3) and *MBL*R (5-CACTGTACCTGGTTCTCCCT-3), were designed using Primer 5.0 from the sequences of the *MBL* gene of sheep available in GenBank (accession numbers FJ977629). Two Primers were synthesized at Sangon Biological Engineering Technology Company (SBETC, Shanghai, China) and used in 25 μL PCR reaction to amplify a 194 bp section of the exon-1 region of the *MBL* gene. A total volume of 25 μL PCR reaction contains 1 μL (50 ng) of genomic DNA extracted from an individual China Merino sheep, 2.5 μL 10× PCR buffer, 1 μL (10 mM) of each primer, 2.5 μL dNTPs (2.5 mM), 1.5 μL $MgCl_2$ (15 mM), 0.6 μL (2.5 units) Taq DNA polymerase, and 14.9 μL MilliQ H_2O. The PCR reagents were supplied by the SBETC. The procedure for PCR reactions are 94ºC denaturation for 30 s, annealing for 45 s, 72ºC extension for 30 s, 35 cycles, final extension at 72ºC for 10 min. PCR products were detected by 1.5% agarose gel electrophoresis.

Cloning of PCR Products and DNA Sequencing

PCR products were analyzed by SSCP, aliquots of 2 μL PCR products were mixed with 8 μL denaturing solution (98% formamide, 25 mL MEDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), were incubated at 98ºC for 10 min and then chilled on ice for 10 min. Denatured PCR products were run on 12% PAGE gel (80 mm ×73 mm ×

0.75 mm) in 0.5 ×TBE buffer at 140 V and 12ºC for 20 h. The gel was stained with 0.1% silver nitrate solution. According to the result of PCR-SSCP, PCR products containing the single nucleotide polymorphisms (SNPs) site that was recovered by Omega Gel Extraction Kit, connected with the pMD19-T vector, transformed into DH5α competent cells, picked clones, shaked bacteria, and then identified by bacteria PCR. Lastly, the homozygous genotype was send to sequence at BGI (Beijing, China; http://www.genomics.cn).

Measurement of Serum MBL Levels in Sheep

Sheep *MBL* levels in serum were measured using the *MBL* Oligomer ELISA Kit (ADL, America) in the experimental group and the control group before and after artificial infection. Serum samples from the China Merino sheep and standards of known *MBL* concentrations were loaded into 96 wells on the test plate, and then the *MBL* antigen and the biotinylated monoclonal antibody specific to *MBL* were added to each well and were incubated at 37ºC for 60 min. The wells were washed and the enzyme, streptavidin-peroxidase, was added. After incubation at 37ºC for 30 min, the wells were washed to remove unbound enzymes, and the substrate solution was reacted with the bound enzyme to induce a colour. The intensity of the colour was proportional to the concentration of *MBL* protein present in the serum samples, which was measured with an ELISA reader at 450 nm and then converted into *MBL* concentration (μg/L) in serum.

Statistical Analysis

Differences in haplotype frequencies were analyzed using a $\chi 2$-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 19.0).

RESULTS

Polymorphisms in MBL Gene in China Merino Sheep

SSCP analyses of the PCR-amplified fragments in our study from the 194-bp section of the exon-1 of the *MBL* gene showed four distinct banding patterns in China Merino sheep (n = 336), including BB, CC, DD and BC *(Fig. 1)*. We used the sequences of the full-length *MBL* gene of sheep available in GenBank (accession numbers FJ977629) as a reference to compare with the sequences we obtained from China Merino sheep. FJ977629 has identical sequences for the exon-1 region of the *MBL* gene. Our comparisons showed that pattern CC had an identical sequence to the reference, whereas the other three patterns had sequence variation from the reference. Pattern BB and DD had four mutation sites, respectively: one at position 105 that was synonymous, and the other three at position 28, 43 and 86 that were non-synonymous, which resulted in three amino acid changes in the putative *MBL* protein (Leu 10 Phe, Met 15 Val and Thr 29 Ser). Pattern BC had two SNPs: one at position 105 that was synonymous, and the other at position 43 that was non-synonymous, which resulted in amino acid changes in the putative *MBL* protein (Met 15 Val) *(Fig. 2)*.

Association Between Polymorphisms in MBL Gene and MBL Protein Levels in Serum in China Merino Sheep

The serum *MBL* levels of the 42 China Merino sheep were determined before *MO* infection by Sheep *MBL* ELISA kit, and then the results showed that serum *MBL* levels with CC-type was the highest, followed by subsequently BB-, BC- and DD-type, based on which predicted that CC genotype would be as a resistance group and DD genotype was susceptible *(Fig. 3)*. However, the serum *MBL* levels were determined on the 21th day after artificial *MO* infection that the mean values of serum *MBL* levels of 4 genotypes were lower than the control group, among which the rate of decline in CC and BC were bigger than BB and DD *(Fig. 4)*.

Meanwhile, the antibody OD values were detected between the experimental group and the control group before artificially infected with *MO* (OD >2NC + 0.06 was positive), which the results showed that

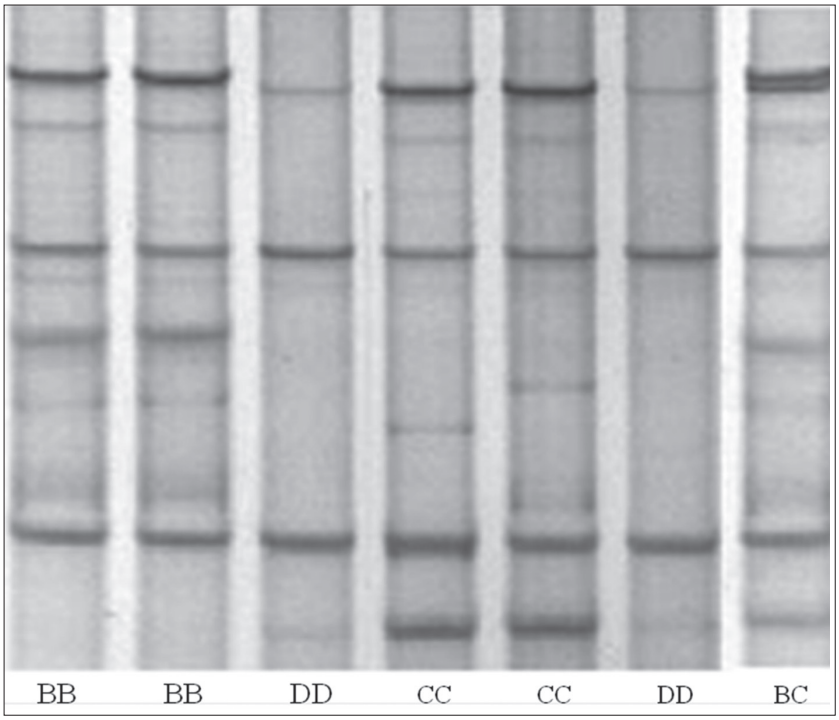

Fig 1. Electrophoretic patterns of PCR-SSCP of *MBL* exon 1 in *Chinese Merino sheep*

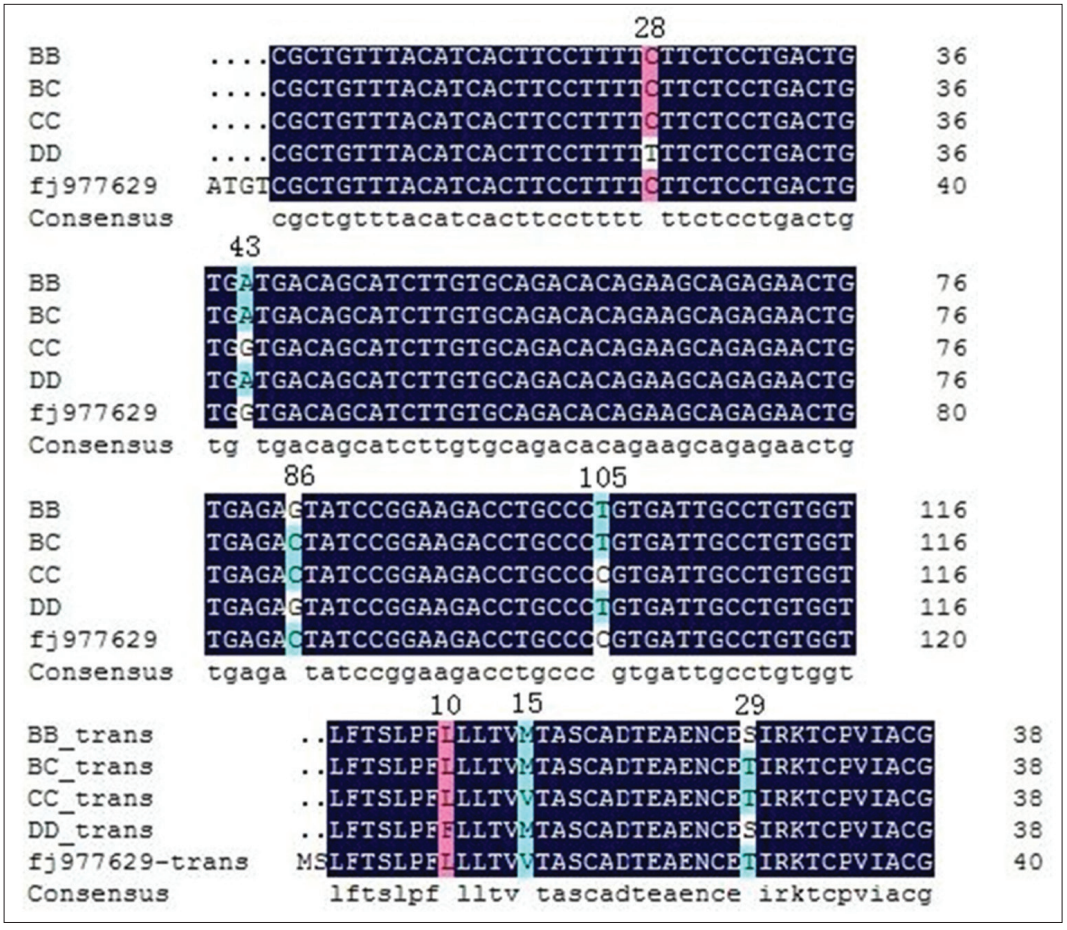

Fig 2. Comparison of the results of *MBL* exon1 polymorphism sequencing and amino acid substitutions in *Chinese Merino Sheep*

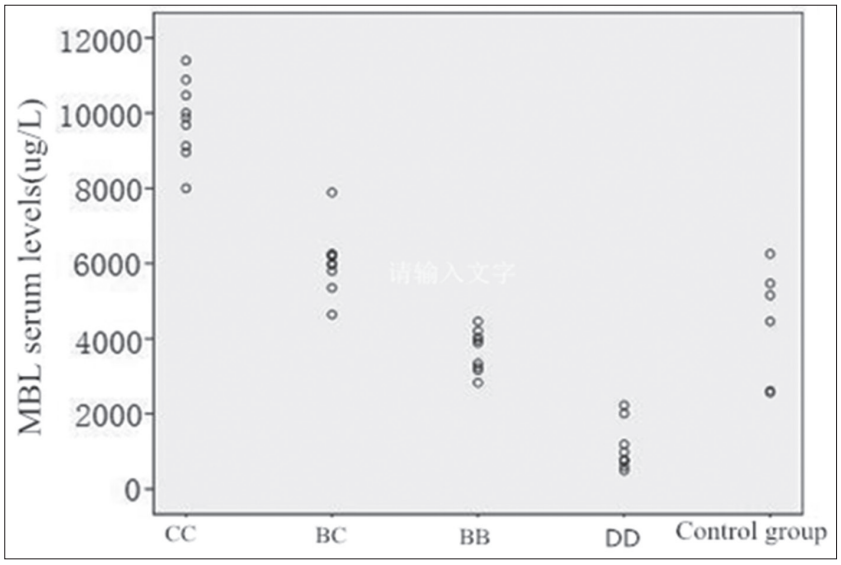

Fig 3. Comparation of *MBL* serum concentration in different genotypes of *MBL*-exon1 before infection

all individuals were negative before artificial infection. However, after *MO* artificial infection, all individuals in the control group were negative, and one half of the individuals in the experimental group were positive. On the 57th day after artificial *MO* infection, there were six sheep died in the experimental group, and then were dissected to carry out pathological findings. Later, all the rest of sheep were determined by ELISA antibody kit, the results showed that there were 2 ill-sheep in DD-type group, 7 ill-sheep in CC-type group, 4 ill-sheep in BC-type group, and 5 ill-sheep in BB-type group, however, all individuals in the control group were not ill *(Table 1)*. Fisher exact statistical analysis showed that the rate of *MO* infection in the DD-type group was significantly lower than the CC-type group ($P<0.05$).

DISCUSSION

Mannan-binding lectin, an important natural anti-infective immune molecule in humans and animals, which is secreted into the blood after synthesis by the liver and induces and activates

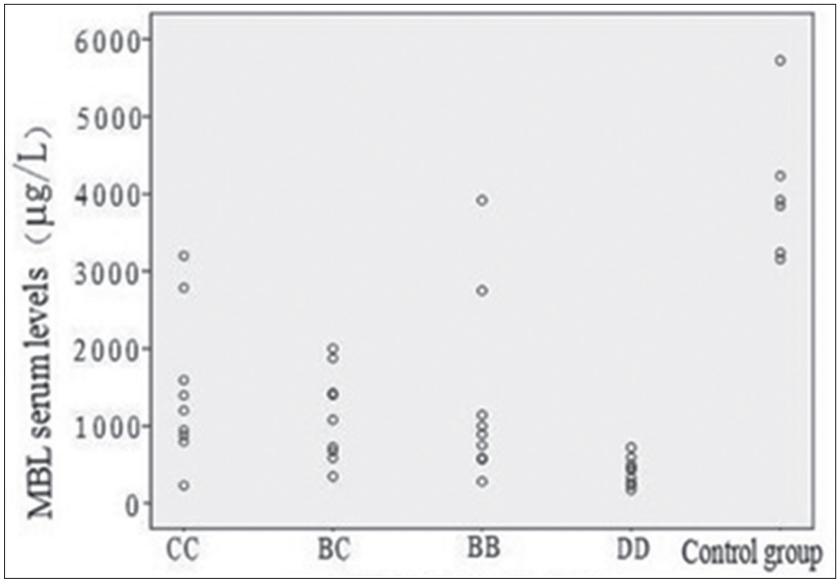

Fig 4. Comparation of serum *MBL* concentration in different genotypes of *MBL*-exon1 after infection

decrease in the serum concentration of *MBL*. Point mutations in exon-1 of the human *MBL* gene are frequently described as being associated with *MBL* plasma concentration, reduced ligand-binding capacity and failure to activate complement [15]. The exon-1 mutations on the protein product are believed to impair oligomer-ization and lead to a functional deficiency. Most mammalian species, including pigs, have 2 forms of *MBL* (A and C) [6,16-18]. Low expression of *MBL-C* was observed in most diseased animals almost all of which had one or both mutations, whereas clinically healthy pigs had a wide range of *MBL-C* expression. As a report that Heterozygotes have reduced serum concentrations of *MBL*, whilst functional multimeric protein is almost absent from the serum of homozygotes

Table 1. *Results of artificial infection with MO in different genotype individuals in Chinese Merino sheep*

Sheep No. Genotypes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
DD (susceptible group)	+	+	+	+	+	+	-	+	-
CC (resistant group)	+	-	-	-	-	-	-	+	-
BC	-	+	+	+	-	-	+	-	-
BB	-	+	+	+	-	-	+	+	-
Control group	-								

Numbers 1-9 just represent the number of sheep in each group. + represents incidence and - no incidence

the body's immune response before the antigen-specific antibody reacts specifically. Functional MBL is a multimeric protein of up to six 96 kDa subunits, which consisted of 3 identical polypeptide chains pro-duced by the liver. There are two pathways by which *MBL* can participate in a host defense response: 1) *MBL* activates the lectin complement pathway via *MBL* associated serine proteases *(MASPs),* that con-verges with the classical complement pathway, at the level of complement C4, and 2) MBL may also act directly as an opsonin, enhancing phagocytosis by binding to cell-surface receptors present on phago-cytic cells [5,6]. Several reports suggest that *MBL* deficiency also predisposes to autoimmune disease, such as systemic lupus erythematosus [10,11], and rheumatoid arthritis [12]. *MBL* deficiency is associated with an increased susceptibility to infection with *Neisseria meningitidis* [13], and severity of atherosclerotic disease [14]. *MBL* binding may facilitate the uptake of *Mycobacterium* by macrophages, thereby promoting infection.

Polymorphisms in the *MBL* promoter have been shown to be associated with the prevalence of infectious diseases. The point mutations, three in exon-1 and two in the promoter region of the *MBL* gene, lead to a dramatic and compound heterozygotes [19]. Similarly, it is also shown in our study these polymorphisms disrupt the assembly of *MBL* peptide trimers or accelerate the *MBL* degradation, and result in profoundly reduced serum levels of functional polymeric *MBL*.

To verify the relationships between the *MBL* genetic polymorphisms and anti-*MO* infection in China Merino sheep that were firstly divided into four types and then different *MBL* genotypes of sheep were artificially infected with *MO*. The results showed that a model of artificial infection of *MO* was successfully established that was used to study the correlation between polymorphisms in *MBL* genotypes and serum *MBL* protein levels in China Merino sheep. Meanwhile, *MO*-infected individuals showed that there were very obviously clinical symptoms, such as a lot of pleural effusion, pulmonary and pleural with cellulose pigmentation, a section of the pneumonia area with status marmoratus and other pathological features. Thus, it revealed that *Mycoplasma* was successfully isolated from the lungs in a sheep model with *MO* infection.

It is worth mentioning that here, Hamvas et al.[20] study showed that there were two thirds of the patients with

Mycoplasma infection accompanied by *MBL* deficiency, whereas there were only one third of the healthy individuals accompanied by *MBL* deficiency, which showed that *MBL* plays an important role in preventing *Mycoplasma* infection and there might a positive correlation between the susceptibility individuals and exon-1 mutations in *MBL*. Interestingly, in our study, there were 4 single-base nucleotide mutations in Exon-1 of *MBL* gene in China Merino sheep, including g.28C > T, g.43A > G, g.86G > C and g.105T > C by using the PCR-SSCP technology to analyze the genetic polymorphism of the exon-1. Of which, g.28C > T, g.43A > G and g.86G > C were non-synonymous respectively, causing the amino acid replacement of Leu10Phe, Met15Val, and Thr29Ser, however, g.105T > C was synonymous. Exon-1 encodes the signal peptide sequence that has cysteine-rich region and eight repeating Gly-X-Y motif. The present study showed that different mutations in exon-1 were located within the first 28 amino acids, which might affect a signal peptide synthesis and hinder the synthesis of the polypeptide chain. At the same time, these mutations in the promoter region of *MBL* gene were thought to reduce *MBL* expression by impairing the binding of transcription factors. The exon-1 mutations in the *MBL* gene were believed to impede the assembly of *MBL* subunits into the basic trimer structure, thereby reducing the amount of functional *MBL* subunits in heterozygous individuals [21,22]. Similarly, a study also showed that polymorphisms in exon 1 of the *MBL*-2 gene were significantly overrepresented in individuals with primary antibody deficiency and culture-proven *Mycoplasma* infections which could result in reducing plasma levels of *MBL* [23]. Therefore, this study further analyzed the relationship between *MBL* exon-1 polymorphisms and serum *MBL* levels in China Merino sheep with *MO* infection. The results in our study showed that serum *MBL* levels of CC-type were significantly higher than DD type in exon-1, which forecasted that CC-type was related to the resistance of sheep with *MO*. In addition, another artificial infection experiment also confirmed that CC-type in China Merino sheep had significant resistance compared with DD-type [24]. Single locus association analysis showed that g.86 G > C loci DD genotype in China Merino sheep that was significantly correlated *MO* and the infection rate was 78%, one explanation which could be that serum *MBL* levels was lower that caused immune dysfunctions to subject to diseases of various kinds, or *MBL* mutant sites might be involved in mediating some cells in *vivo* that were favorable for *Mycoplasma* invasion to make these individuals were more susceptible to *MO*. Thus, g.86G > C loci in exon-1 region of *MBL* can be used as molecular markers for *MO* susceptibility in sheep.

In summary, considering the results obtained here, we can conclude that the exon-1 of *MBL* gene polymorphisms influence *MBL* serum levels in China Merino sheep, which there were four polymorphisms in the exon-1 of the *MBL* gene in China Merino sheep that were closely related to resistance to *MO* of China Merino sheep. More importantly, it could be used as a molecular marker of resistance breeding in sheep that would provide a reference for further studying molecular mechanism of *MBL* gene in livestock breeding for disease resistance.

Competing Interests

There are no potential conflicts of interest.

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