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

INTRODUCTION

Mannan-binding lectin (MBL) is a member of C-type lectin superfamily that recognizes 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.
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 \[2,3\]. Similarly, some other studies also reported that A-type MBL can has antibacterial function against Porcine Actinobacillus, Actinobacillus pleuropneumoniae, Haemophilus parasuis \[4,5\]. 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 \[6,7\]. 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 -20\(^\circ\)C.

**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 37\(^\circ\)C 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 4\(^\circ\)C 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 -20\(^\circ\)C.

**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 MBLF (5-CTGCCTGTATACACTCTTCCT-3) and MBLR (5-CAGTGTACCTGGTTCTCCCT-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\(_2\)O. The PCR reagents were supplied by the SBETC. The procedure for PCR reactions are 94\(^\circ\)C denaturation for 30 s, annealing for 45 s, 72\(^\circ\)C extension for 30 s, 35 cycles, final extension at 72\(^\circ\)C 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 mM MEDTA, 0.025% xylene-cyanole and 0.025% bromophenol blue), were incubated at 98\(^\circ\)C 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 xTBE buffer at 140 V and 12°C 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 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, 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 χ2-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...
all individuals were negative before artificial infection. However, after 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 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...
**Figure 4.** Comparison of serum MBL concentration in different genotypes of MBL-exon1 after infection

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

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<tr>
<th>Sheep No. Genotypes</th>
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<td>DD (susceptible group)</td>
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Numbers 1-9 just represent the number of sheep in each group. + represents incidence and - no incidence

Decay 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 oligomerization 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

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 produced 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 converges 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 phagocytic 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 be a positive correlation between the susceptibility individuals and exon-1 mutations in MBL. Interestingly, in our study, there were four 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.86G > C loci DD genotype in China Merino sheep that was significantly correlated with 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.

Acknowledgements

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References


