SNPs of CD14 change the mastitis morbidity of Chinese Holstein

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Abstract. Gram-negative (GN) bacterial infection is a main cause of bovine mastitis. The cluster of differentiation (CD) 14 gene serves an essential role in GN bacterium-induced innate immune response. CD14 works as a bacterial lipopolysaccharide (LPS) receptor, combines with LPS-liposaccharide binding protein complex, and causes cellular activation. However, the effects of CD14 single nucleotide polymorphisms (SNPs) on morbidity of clinical mastitis remain unclear. In the present study, To investigate the polymorphisms of CD14 gene and its effects on cows' susceptibility to mastitis, polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) assay was used to detect SNPs of CD14 gene in 134 Chinese Holsteins. SNPs were identified in PCR products amplified with 3 sets of primers in CD14 exon 2. A total of three SNPs were located in that exon: g.528 A→C (147Ser→Arg) in allele B; g.612 A→G (175Asn→Asp) in allele D; and g.1022 A→G in allele F (synonymous mutation). The SNPs in alleles B and D affected the secondary structure of CD14. A 3-dimensional (3D) structural analysis predicted three potential protein forms with a similar structure and indicated that the changes of the above-mentioned alleles were on the concave surface of the protein. In more detail, 147 Ser→Arg induced a protein kinase C phosphorylation site to move forward, as assessed by the motif analysis. The morbidity rate of AB (mixed type g.528 A/C) and CD (mixed type g.612 A/G) was the highest among all genotypes presented in the current study, and via of tumor necrosis factor-α and interleukin-6 mRNA levels were upregulated in animals of this genotype compared with others. Taken together, the CD14 SNPs identified in the present study, may be closely associated with the morbidity of mastitis.

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Introduction

Bovine mastitis can directly result in the reduction of milk quality and production leading to a large economic loss (1). Gram-negative (GN) bacterial infection is the main reason for bovine mastitis, which leads to acute mastitis with clinical symptoms (2-4). Lipopolysaccharide (LPS) is a key component of the outer membrane of all GN bacteria, which can be secreted by them under different physiological conditions (5,6). LPS can stimulate secretion of cytokines-mediated inflammation pathways (7). In addition, mammals, through a cluster of differentiation (CD)14-dependent pathway can detect LPS concentration, which activates host cells to start an inflammatory response for bacteria resistance (6).

CD14 serves as a receptor for the complex of LPS-liposaccharide binding protein (LPS-LBP) complex in animals (8,9). When LPS is released from bacterial outer membrane and intrudes into the host organism, CD14 binds to LPS-LBP to form the LPS-LBP-CD14 complex (10). Subsequently, macrophages can secret inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukins (IL)-1β, -6 and -8 (11) activating the toll-like receptor-4 (TLR-4) signaling pathway. As a result, this binding induces an innate defense response against GN bacteria (12). Investigation of CD14 involved in the activation of the TLR signaling pathway may contribute to blocking the inflammatory cascade prior to inflammatory cytokines release (13).

In addition, it is well known that there are two forms of CD14, a membrane-bound CD14 (mCD14) and a soluble one (sCD14), which serves inessential role in the recognition of GN bacteria. mCD14 is expressed on the cell surface of monocytes, macrophages and polymorphonuclear neutrophils (PMNs) (9-11). Under low LPS concentrations, mCD14 activate phagocytes in the presence of LBP. Notably, sCD14 is only present in body fluid such as the serum or breast

milk (7,8). Either mCD14 or sCD14 can directly strengthen LPS response and associated signaling pathways in epithelial and endothelial cells, even in the absence of mCD14 (14-17). sCD14 binding to LPS can stimulate innate response to GN bacterial infection under low concentrations (6,17), alleviate stress induced by mastitis and weakens LPS toxicity (8). Previous studies revealed the difference in susceptibility to GN bacterial infection between dairy cows (18) and the polymorphisms in the coding and promoter region of CD14 gene-associated with the risk of several diseases in humans and animals (11,19-22). Therefore, it seems that genetic variations in cow CD14 may affect the risk for GN bacterial infections (23). However, little is known about the correlation between CD14 polymorphisms and mastitis. For this reason, the single-strand conformation polymorphism (SSCP) method was used to analyze CD14 polymorphisms and their correlation with clinical mastitis in Chinese Holstein, which will be important for breeding anti-mastitis dairy cows.

Materials and methods

Animal selection, ethical approval and genomic DNA extraction. In the present study, 134 dairy cows (Chinese Holstein, Wuhu Dairy Co., Ltd., Wuhu, China) had complete records of Dairy Herd Improvement. They shared the same season (Spring, February) of the last deliveries and physical conditions (2 years old, 2 fetus, same lactation period). They were raised under the same housing and management conditions, in which 66 cows were regarded as clinical mastitis cases defined by the red and swollen udder and somatic cell count (SCC) of milk (SCC >1,000,000/ml). These data were used to evaluate animal status (24). The other 68 cows without clinical symptoms were regarded as non-mastitis cows. Animals and their care protocols followed a previous study (5) and were approved by the Animal Care and Use Committee of Nanjing Agricultural University and Wuhu Dairy Ltd (approval no. 20150930). Blood samples were harvested from caudal veins of cattle and stored at -81˚C. DNA extraction from the blood samples was followed by the conventional phenol-chloroform method. DNA was dissolved in TE buffer (made in our laboratory) and stored at -20˚C until further use.

Primer design and polymerase chain reaction (PCR). Primers were designed to amplify coding sequence of *CD14* gene (NCBI Reference Sequence: AC_000164.1). PCR reaction contained: 2.5 μl of 10XPCR buffer (Takara Biotechnology Co., Ltd., Dalian, China) 1.5 μl Mg^{2+} (25 mM), 0.5 μl dNTP (10 mM), 0.2 μl Taq DNA polymerase (0.5 U/0.5 μl; Takara Biotechnology Co., Ltd.), 1 μl each primer (10 μM/μl), and 2 μl template DNA (50 ng/μl) diluted with ddH_2O to a final volume of 25 μl. PCR amplification reaction program: Pre-denaturation for 4 min at 94˚C; then 35 cycles (denaturation for 40 sec at 94˚C, renaturation for 30 sec at annealing temperature, elongation for 1 min at 72˚C); finally elongation for 7 min at 72˚C. The primer sequences, sequence site, predicted size of segment and annealing temperatures are listed in Table I.

PCR-SSCP assay. PCR products (5 μl) were denatured with the denaturation buffer solution (Takara Biotechnology Co., Ltd.) at 97˚C for 7 min and put on ice for 20 min, separated with 30% polyacrylamide gel at 200 V/12-15 h and stained with silver nitrate (Tiangen Co., Ltd., Beijing) for 15-20 min at room temperature.

DNA sequencing and analysis of different genotypes. PCR products were purified using a kit from Tiangen Biotech Co., Ltd. (Beijing, China) and sent for sequencing and analysis. The differences and polymorphism of different genotypes were analyzed with MegAlign software version 2.0 (DNASTAR, Inc., Madison, WI, USA).

Prediction of protein and functional site of CD14 protein. Protean software version 2.0 (DNASTAR, Inc.) was applied to analyze the hydrophilicity and secondary structure. The 3-dimensional (3D) structure was also built through the Hopp-Woods and Homology-based modeling method (25-28).

The modeling construction can be divided into two steps: In the first, protein crystal structure data was selected from the ExPDB database; in the second, protein sequences were placed to the SWISS-MODEL website (www.expasy.org/swissmod/SWISS-MODEL.html). Subsequently, the results of the analysis of homology modeling, modification and optimization of the protein were obtained. The quality (QMEAN-score) of 3D structure was evaluated in QMEAN website (swissmodel.expasy.org/qmean) (29,30). Coding sequences and functional sites were also predicted on the ExPASy Molecular Biology Server (www.expasy.org/tools/). The website (http://prosite.expasy.org/) of PROSITE database was used to assay protein kinase C (PKC) phosphorylation site. A Z-score of the model was also analyzed with QMEAN including four regular statistical aspects (C beta interaction, all-atom pair wise, salvation and torsion angle energy). Finally, 3D structures were presented with Cn3d 4.1 software (NIH, Bethesda, MD, USA).

Reverse transcription-quantitative PCR (RT-qPCR). Blood samples of mastitis cows were collected and anti-coagulated with sodium heparin. Ficoll reagent (Shanghai Shenggong Co., Ltd., Shanghai, China) was used to dissociate lymphocytes from peripheral blood. Total RNA was extracted from lymphocytes with TRIzol following manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and then SuperScript First-Strand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc.) was applied to perform the reverse transcription following manufacturer's protocol. The 2XiTaq Universal SYBR-Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to amplify the target genes. PCR reactions (40 cycles for 95˚C for 15 sec and 60˚C for 1 min) were run on ABI StepOne machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mRNA level of target genes was calculated with StepOne software version 2.1, using 18s rRNA (18s rRNA kit, Ambion; Thermo Fisher Scientific, Inc.) as internal control. The following primers of TNF-α and IL-6 were used: Bovine TNF-α forward, 5'-CAGGGCTCCAGAAGTTGCTTG-3' and reverse, 5'-GAACCAGAGGGCTGTTGATGG-3'; Bovine IL-6 forward, 5'-GTGATGACTTCTGCTTTCCCTACC-3' and reverse, 5'-TCTGCCAGTGTCTCCTTGCTG-3'.

Table I. Primer pairs of CD14 gene designed for its amplification.

Primer	Forward (5'-3')	Reverse (5'-3')	Annealing temperature (˚C)	Expected segment size (bp)	Amplified site size (bp)
1	GTGAGCCACTGTAAAGGAAAGA	TGGAAAGACAGCGGAGGT	57.8	172	-80-91
2	GGCTCTGAGAATCTACCGACTA	ACTGCTTCGGGTTGGTGT	54.4	352	-21-330
3	GCCGTTCAGTGTATGGTTGCCGTCG	GGCACCTCCTGTTGTCCACGATACG	54.4	330	234-563
4	AGGAACTGACGCTTGAGGA	CGCCGAGACTGGGATTGT	57.2	270	442-711
5	CAGGGTGCTGAACATTGCC	GCGAGTTGTGGCTGAGGT	57.2	266	602-867
6	GACCTCAGCCACAACTCGC	GGCTCCAGGGTCCAGAAA	55.4	243	849-1,091
7	GCTGCCCGAGGTAAATGA	TAGAAGGCTGGTTGGTT GAG	58	272	1,034-1,305

CD14, cluster of differentiation 14; bp, base pairs.

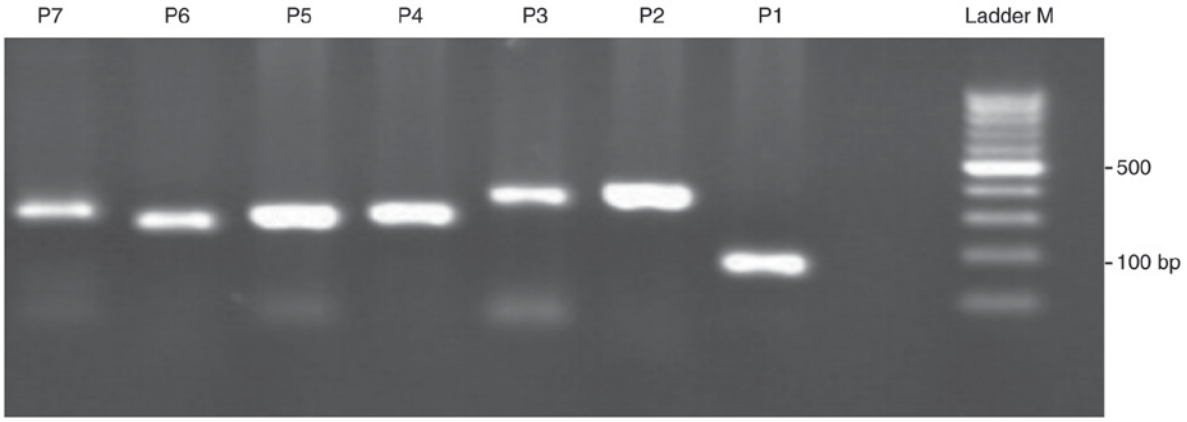

Figure 1. PCR products amplified by seven pairs of primers. M, marker; P1-P7, PCR products amplified by primer pairs presented in Table I. PCR, polymerase chain reaction.

Statistical analysis. Pop Gene software version 3.1 (https://sites.ualberta.ca/~fyeh/popgene.html) (5) was used to assess if frequencies of the alleles, diploid data of the single-population and the significance of differences of single locus [including allele number, frequency, loci and polymorphism information content (PIC)] had a P-value <0.05. Data are expressed as the mean ± standard deviation. Association between single nucleotide polymorphisms (SNPs) in the *CD14* gene and the morbidity of clinical mastitis was analyzed with crosstabs methods (including Pearson χ^2, Continuity correction and Fisher' exact test) in SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Gene amplification. Seven pairs of primers were tested to amplify *CD14* and product sizes were as expected (Table I; Fig. 1). Therefore, *CD14* genotypes were analyzed further by SSCP.

Analysis of SSCP and sequences. The SSCP analysis revealed the polymorphisms in the *CD14* gene all of which were found in exon 2 (Fig. 2). Three genotypes (AA, AB and BB) were found in PCR products amplified with primer pair 3 (P3)

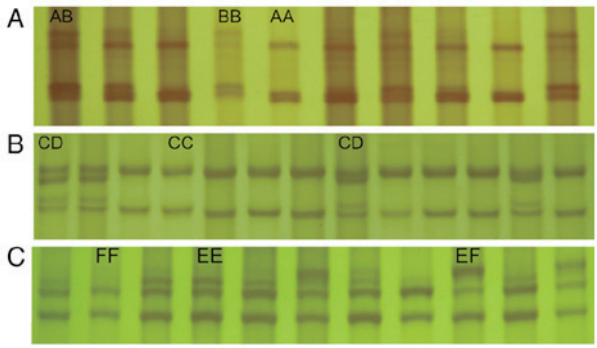

Figure 2. The SSCP pattern amplified by (A) primer pair 3 with three genotypes identified (AA, AB and BB), by (B) primer pair 4 with two genotypes identified (CC and CD); and by (C) primer pair 6. with three genotypes identified (EE, EF, FF). SSCP, single-strand conformation polymorphism.

including one SNP: g.528 A→C in allele B (Fig. 2A). Two genotypes (CC, CD) were found in PCR products amplified with primer pair 4 (P4) including one SNP: g.612 A→G in allele D (Fig. 2B); three genotypes (EE, EF and FF) were found in PCR products amplified with primer pair 6 (P6) including one SNP: g.1022 A→G in allele F (Fig. 2C). All genotypes are demonstrated in Fig. 2 and SNPs in each genotype of exon 2

Table II. Three SNPs identified in *CD14* gene.

Primer pair	Allele	SNP
3	B	g.528 A→C
4	D	g.612 A→G
6	F	g.1022 A→G

SNP, single nucleotide polymorphism; CD14, cluster of differentiation 14.

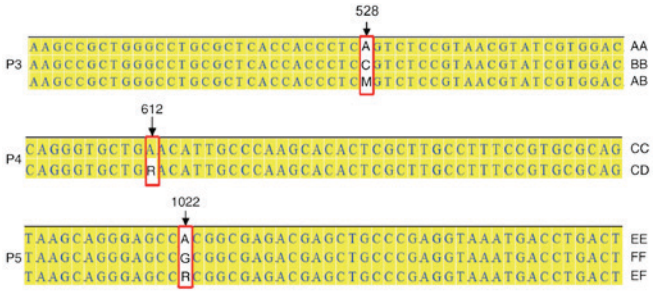

Figure 3. DNA sequence alignments of P3, P4 and P6 primer pairs, as assessed by DNASTAR 7.0 MegAlign software. Nucleic acid substitutions (M, pyrimidine; R, purine) in the different CD14 genotypes are demonstrated. CD14, cluster of differentiation 14.

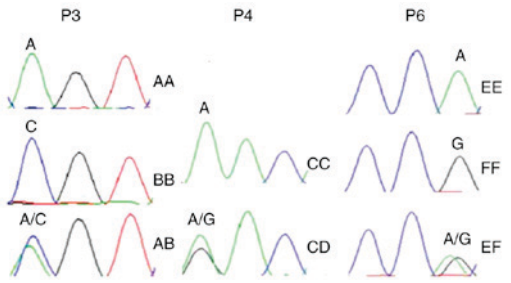

Figure 4. Representative oscillograms of the different genotypes, illustrating the gene mutations found by P3, P4 and P6 primer pairs.

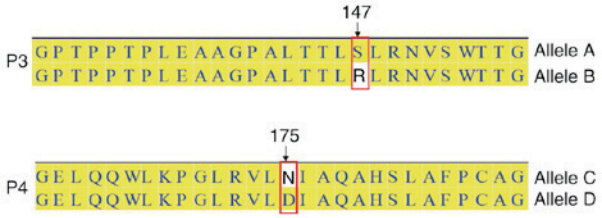

Figure 5. SNP alignments identified by P3, P4 and P6 primer pairs.

were compared and illustrated in Fig. 3. DNA sequence oscillograms of different genotypes are illustrated in Fig. 4 and the 3 different SNPs are presented in Table II. Except for the SNP in allele F, these SNPs cause the following amino acid changes: g.528 A→C (147 Ser→Arg) in allele B; g.612 A→G (175 Asn→Asp) in allele D (Fig. 5).

Prediction of CD14 molecular structure and motif analysis of exon 2. Via the haplotype analysis, only three protein variants encoded by CD14 were predicted in animals. Protein information, types, combinations and their associations with the morbidity of mastitis are listed in Table III, and the different sequences of these three proteins are presented in Fig. 6.

Secondary structure analysis confirmed that bovine CD14 was a mixed-type protein with extensive hydrophilic and hydrophobic regions consisting of 36.4% α-helix, 38.7% β-pleated sheet, 0.7% turns and 4.2% coiled coils domains. The two amino acid mutations lead to changes of secondary structure: 147 Ser→Arg in allele B made a β-pleated sheet a little shorter; 175 Asn→Asp in allele D made β-pleated sheet fall into α-helix (Fig. 7). 147 Ser→Arg in allele B made the isoelectric point of protein 2 higher than protein 1; 175 Asn→Asp in allele D caused the isoelectric point of protein 3 lower than protein 1 (Tables IV-VI).

3D structures of these three proteins were also predicted (Fig. 8A). The QMEAN score was 0.457 (mean Z score: -4.94; Fig. 8B), which demonstrates the reliability of the model (29). It was demonstrated that 147 Ser→Arg induced a protein kinase C (PKC) phosphorylation site moving forward in protein 3 by using the PROSITE database Motif assay (Fig. 6).

Genetic analysis of the genotypes and SNPs. The genetic information of the CD14 gene is listed in Table VII. In the fragment amplified by P3, three genotypes were found (AA, AB and BB), and allele A accounts for 70.90% and in a moderate polymorphism (0.2<PIC<0.5); Two genotypes were found

Table III. Protein types encoded by *CD14*, protein combinations and the association with bovine mastitis.

Protein type and protein combination	Alleles	Frequency of each protein type or protein group (%)	Number of clinical individuals	Number of non-clinical individuals	Morbidity (%)
1	AA, CC	49.2	20	46	30.3[a]
2	BB, CC	19.4	12	14	46.2[b]
3	AA, DD	0	0	0	0
1 and 2	A/B, CC	19.4	21	5	80.8[b]
1 and 3	AA, C/D	12	13	3	81.3[b]

[a]P>0.05; [b]P<0.01, χ^2 test. CD14, cluster of differentiation 14.

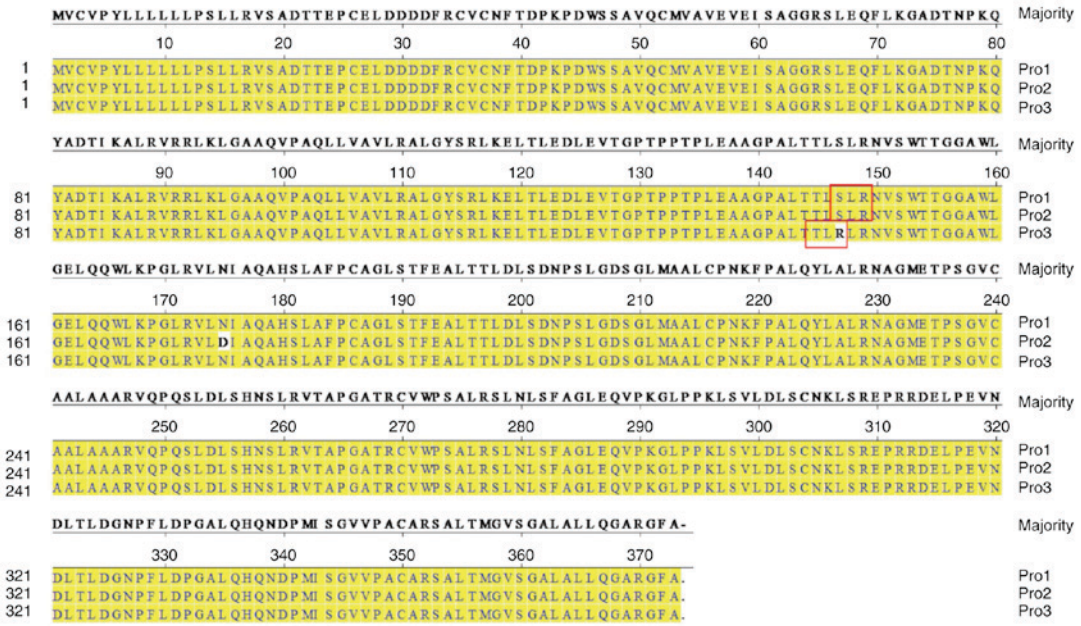

Figure 6. Sequence alignments with the amino acid sequence and the sequence motif of the three predicted proteins. Red box is the PKC phosphorylation site. It was predicted that the mutation 147 Ser→Arg causes a PKC phosphorylation site to move forward. PKC, protein kinase C; CD14, cluster of differentiation 14.

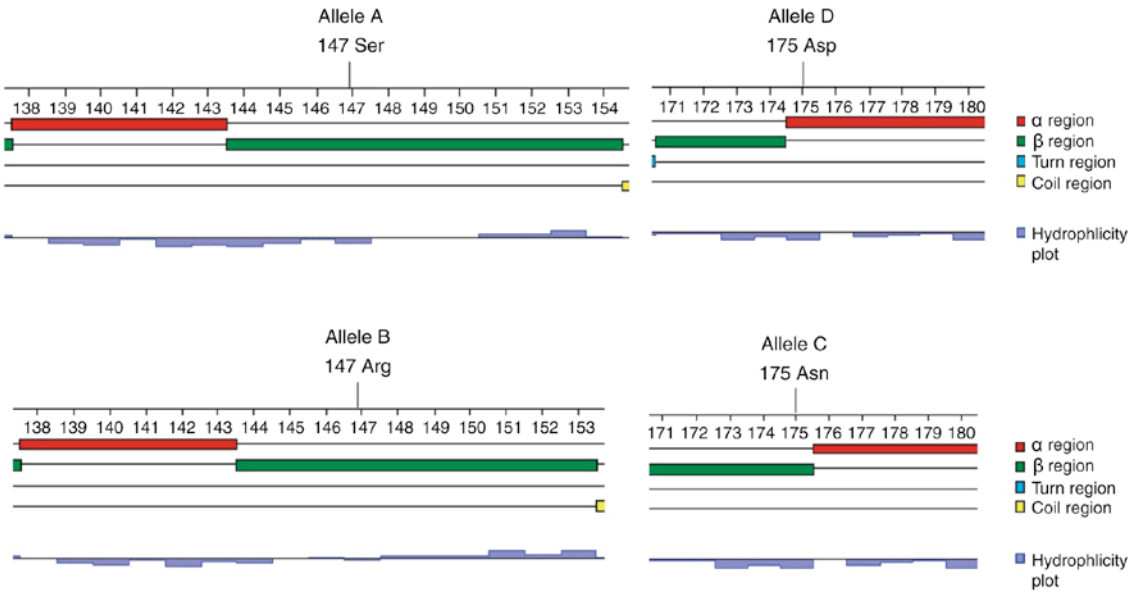

Figure 7. Secondary structure and hydrophilicity of CD14. It was demonstrated that 147 Ser→Arg shortened the β-pleated sheet in Allele B. In addition, 175 Asn→Asp in Allele D made β-pleated sheets into α-helices. CD14, cluster of differentiation 14.

Table IV. Isoelectric point of each protein.

Predicted protein	Isoelectric point
1	5.32
2	5.54
3	5.17

(CC and CD) amplified by P4, allele C is prevalent accounting for 94.03% and in low polymorphism (PIC<0.25); A total of three genotypes were found (EE, EF and FF) amplified by P6, allele E accounts for 70.15% and in a moderate polymorphism (0.2<PIC<0.5). Genetic linkage analysis indicated that alleles B and F were in high linkage disequilibrium (P<0.01; Table V). χ^2 test revealed that genotyping by P4 and P6 was in Hardy-Weinberg equilibrium (P>0.05) but P3 was not (P<0.01; Table VII).

Association studies. The association between SNPs and mastitis morbidity are presented in Table VIII, and AB has a higher morbidity rate than AA (χ^2=12.97, P<0.01) and BB (χ^2=6.72, P<0.01) in the fragments amplified by P3, while susceptibility to mastitis of CD individuals is higher than for CC (χ^2=7.44, P<0.01) in the fragments amplified by P4 (P<0.01; Table VIII).

Table V. Linkage disequilibrium between A, B, E and F alleles.

P3 allele	P6 allele	Burrows	Correlation	χ^2	P-value
A	E	0.0810	0.03258	14.12	0.0002
B	F	0.0810	0.03258	14.12	0.0002

Table VI. Key SNPs in exon 2 of *CD14* gene, and their influence on the protein structure and motif profiles.

Allele	SNP	Primary structure	Secondary structure	Motif profiles	Location in the 3D structure
B	g.528 A→C	147 Ser→Arg	Shorter β-pleated sheets	Protein kinase C phosphorylation site moved forward	Concave surface of CD14 protein
D	g.612 A→G	175 Asn→Asp	β-pleated sheets into α-helices	No motif profile	Concave surface of CD14 protein

CD14, cluster of differentiation; SNP, single nucleotide polymorphism; 3D, three-dimensional.

Table VII. Distribution of genotypes and alleles of *CD14* gene.

Genotype	Number of cattle	Genotype frequency (%)	Allele	Allele frequency (%)	χ^2 test for Hardy Weinberg equilibrium	Polymorphism information content
Primer pair 3					P<0.01	0.31
AA	82	61.20	A	70.9		
BB	26	19.40	B	29.1		
AB	26	19.40				
Primer pair 4					P>0.05	0.12
CC	118	88.06	C	94.03		
CD	16	11.94	D	5.97		
Primer pair 6					P>0.05	0.33
EE	64	47.76	E	70.15		
FF	10	7.46	F	29.85		
EF	60	44.78				

CD14, cluster of differentiation.

However, SNPs in the fragments amplified by P6 had no difference among different genotypes (P>0.05). Protein types (including combinations of them) and their risk of disease are illustrated in Table III, and prevalence rates of protein 1/protein 2 and protein 1/protein 3 combinations were higher than that of protein 1 (P<0.01) and protein 2 (P<0.05). QRT-qPCR analysis of the clinical samples, revealed that mRNA expression levels of TNF-α and IL-6 of AB and CD genotypes were higher than that of the other genotypes (P<0.01; Fig. 9).

Discussion

The DD genotype may be susceptible to pathogens, and eventually lead to morbidity in embryonic or adult animals, but real evidences of lethality remains to be identified. Previous studies have suggested that SNPs in the immune response-associated genes contribute to susceptibility to diseases among individuals (30-32). LPS is the first molecule that GN bacteria recognize and induce infection (5,9). Generally, sCD14 in the milk or blood blocks mCD14 function of macrophages or PMN (8). When GN bacteria invade the host body, mCD14 on monocytes, macrophages and PMNs will form a complex with LPS; Notably, sCD14 also binds to LPS, mediating activation of cells without mCD14 under low concentration of LPS (33,34). Subsequently, the sCD14-LPS complex induces the release and translocation of nuclear factor-κB (NF-κB), which upregulates the expression of pro-inflammatory cytokines (33). It has been demonstrated that recombinant sCD14 can relieve inflammation activation in the mammary gland induced by *E. coli* (17).

Table VIII. Statistical analysis of association between genotypes and susceptibility of clinical mastitis in Chinese Holstein.

Genotype	Number of clinical individuals	Number of non-clinical individuals	Morbidity (%)
Primer pair 3			
AA	33	49	40.2
BB	12	14	46.2
AB	21	5	80.8[a]
Primer pair 4			
CC	53	65	44.9
CD	13	3	81.3[a]
Primer pair 6			
EE	30	34	46.9
FF	4	6	40
EF	28	32	46.7

[a]P<0.01, χ^2 test.

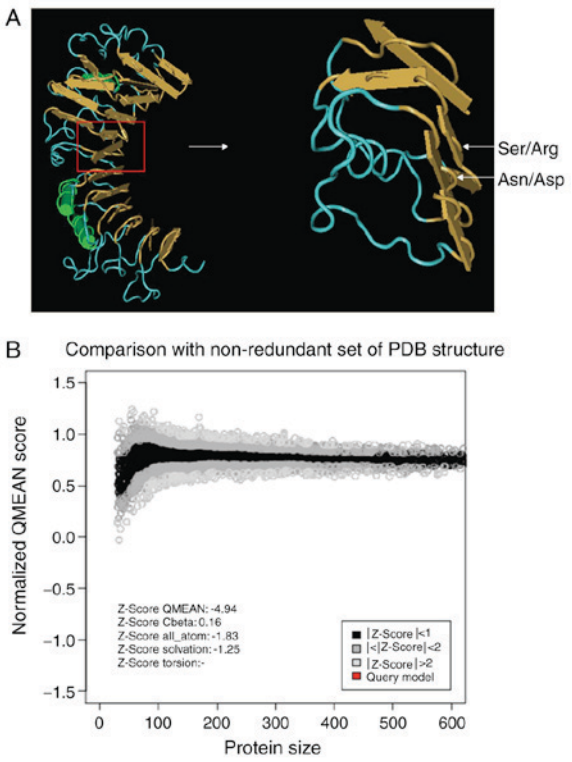

Figure 8. Protein analysis of the 3D structure of CD14. (A) Structural representation of the potential CD14 protein and the location of two mutations leading to an amino acid change. 147 Ser→Arg and 175 Asn→Asp are on the concave surface of the protein. (B) QMEAN analysis results. Total QMEAN-score of each protein is 0.457 (mean Z score: -4.94; estimated model reliability 0-1).

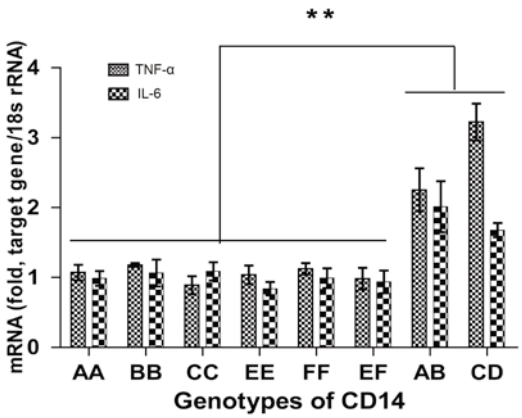

Figure 9. mRNA expression levels of TNF-α and IL-6 in different CD 14 genotypes in clinical samples with mastitis. Data are expressed as the mean ± standard deviation. **P<0.01. CD, cluster of differentiation; TNF, tumor necrosis factor; IL, interleukin.

Two SNPs in the coding sequence (CDS) of CD14 result in primary and secondary structure changes to the protein, shifting of a PKC phosphorylation site and changes in the mRNA expression level of TNF-α and IL-6. CD14 serves an essential role in host immunity, which is the cellular receptor for LPS (8,35). In the present study, three SNPs located in the coding sequence, two of which lead to amino acids changes, in which g.528 A→C (147 Ser→Arg) occurs at a functional site, causing a PKC phosphorylation site to move forward. PKC functions in the LPS-induced immune responses through activation of mitogen-activated protein kinase/NF-κB and subsequent transcriptional activation of the target genes. In addition, previous studies revealed that TNF-α and IL-6 can be translationally regulated by PKC activation in LPS response (35,36). Phosphorylation, a post-translational modification (37) exerts significant effects on many physiological processes, such as signaling transduction, gene regulation and cell differentiation. The 528 site SNP found in the present study may affect PKC phosphorylation. In addition, g.528 A→C (147 Ser→Arg) and g.612 A→G (175Asn→Asp) can change the isoelectric points of protein 2 and protein 3. As a result, it may change the secondary structure leading to a functional change of CD14. AA genotype crossed with BB and the offspring of cross, AB, perhaps induced mutations of its linked genes, and eventually elevated TNF-α and IL-6.

SNPs in CD14 protein may change morbidity by altering CD14 expression on the surface of neutrophils. Researchers

indicated that the CDS mutations occurring in CD14 may change its expression on neutrophils (38). Neutrophils can protect animals from GN bacterial infection via building a defense line, and CD14 confers neutrophils protection against LPS sensitivity (39), which is indispensable for innate immunity. LBP strengthens the formation of toll-like receptor 4-CD14-myeloid differentiation protein 2 complexes, participate in inflammation signaling pathway transduction, promoting cytokine release (40-42). These cytokines mediate the antibiosis and change the abundance of molecules on surface of neutrophils (33). The present study will shed light on controlling the inflammatory response and will contribute to the development of therapeutics for bovine mastitis.

In conclusion, a total of three SNPs were identified in CDS region of bovine CD14 gene in Chinese Holstein. SNPs g.528A→C (147 Ser→Arg) and g.612 A→G (175 Asn→Asp) that may be associated with the expression of CD14 on the surface of neutrophils. The morbidity ratio of AB and CD was higher than that of other genotypes, suggesting CD14 genotyping could serve as a molecular marker for breeding anti-mastitis dairy cows.

Acknowledgements

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