https://doi.org/10.56093/ijans.v90i9.109447

Indian Journal of Animal Sciences **90** (9): 1245–1249, September 2020/Article

Correlation of SNPs in Myeloid differentiation-2 (MD-2) gene with the susceptibility to clinical mastitis in Chinese Holstein dairy cows

JINGCHENG XU[1], SHANSHAN GUO[2], XINSHENG TANG[1] and YAFEI CAI[2]✉

College of Life and Environment Sciences, Huangshan University, Huangshan city, Anhui province, 245041 China

Received: 27 October 209; Accepted: 21 January 2020

ABSTRACT

Myeloid differentiation-2 (MD-2), as an essential component of the CD14-TLR4/MD-2 receptor complex, is critical in identifying bacterial Lipopolysaccharide (LPS) and activating innate immune responses. To evaluate the relationship between MD-2 polymorphisms (including 5′ end and exon regions) and clinical mastitis, population genetic analysis was performed via PCR single strand conformation polymorphism (PCR-SSCP) and direct sequencing in Chinese Holstein dairy cows. Eleven pairs of primer PCR products for SSCP analysis: six pairs of primers (P1-P6) for the 5′-end, four (P7–P10) for the exon regions, and one (P11) for 3′-untranslational region. There were six SSCP bands (named: EE, EF, FF, EQ, EM and EN genotype) in the PCR amplification products of primer P1, two bands in P4 (CD and DD) and three bands in P5 (AA, AB, and BB). Total of five (g.-2173 C/G, g.-2148 C/T, g.-2089 G/T, g.-555 G/A and g.-121 C/A) single nucleotides polymorphism sites (SNPs) were identified in 5′-end of the MD-2 gene. Data showed that SNPs g.-555 (G/A) had significant differences ($P<0.01$). However, only Gram-negative bacteria (e.g. *E. coli*) were screened in the milk of the clinical mastitis cows, indicating that this SNPs g.-555 (G/A) in MD-2 gene may play an important role in susceptibility to clinical mastitis infected with Gram-negative bacteria in Chinese Holstein dairy cows.

Keywords: Chinese Holstein, Clinical mastitis, MD-2, PCR-SSCP, SNPs

Bovine mastitis is a common and costly disease, including reduced milk production and quality, treatment costs and premature elimination of dairy cows, causing huge economic losses to dairy farmers and industry (Heikkila *et al.* 2018). Intramammary pathogens infection (Gram-negative, *Escherichia coli*; Gram-positive, *Streptococcus uberis* and *Staphlyococcus aureus*) are main cause of mastitis, including clinical mastitis and sub-clinical mastitis (Cheng *et al.* 2019). It is well known that gram-negative bacteria are one of the main causes of clinical mastitis; clinical mastitis affects the yield and quality of milk, and also reduces the breeding efficiency of dairy cow. Therefore, better control of gram-negative bacterial mastitis is urgent.

MD-2, is also known as lymphocyte antigen 96 (LY96) in *Bos taurus*, a cysteine-rich glycoprotein that forms a complex with Lipopolysaccharide (LPS) and Toll-like receptor 4 (TLR4), plays a crucial role in the innate immune response (Steimle *et al.* 2019). LPS binds to the TLR4/MD-2 heterodimer and assembles into an active tetramer complex, which constitutes the cell membrane signal center, and then activates NF-κB and interferon regulatory factors in the package to promote pro-inflammatory factors (IL-1β, TNF-α) and interferon regulatory factors (IRF3) (Pizzuto *et al.* 2019). Although the specific molecular mechanisms still need further research support, the role of TLR4/MD-2 in inflammatory diseases has been well-documented (Chen *et al.* 2018). LPS stimulates bovine mammary epithelial cells activates TLR4/NF-κB pathway, which, in turn, induces ER stress, apoptosis, autophagy and oxidative stress in mammary epithelial cells, thereby reducing inflammation and cell damage (Li *et al.* 2019). However, in Holstein cows, the association between MD-2 gene (especially genetic polymorphisms) and clinical mastitis also remains unclear.

In this study, in order to evaluate the association between MD-2 gene polymorphisms and clinical mastitis caused by gram-negative bacteria, the 5′-end and exon regions of the MD-2 gene were identified by PCR-SSCP and direct sequencing in Chinese Holstein dairy cows, and attempted to determine the key SNPs associated with clinical mastitis traits.

MATERIALS AND METHODS

Sample collection and genomic DNA extraction: Total of 221 Chinese Holstein dairy cows were provided by Wuhu Weigang Dairy Co., Ltd., Anhui Province, including 117 clinical mastitis cows infected with gram-negative bacteria and 104 normal healthy cows. All cows have the same feeding standards and environment, similar production

Present address: [1]College of Life and Environment Sciences, Huangshan University, Huangshan City, Anhui Province, 245041 China; [2]College of Animal Science and Technology, Nanjing Agricultural University, Nanjing City, Jiangsu Province 210095 China. ✉Corresponding author e-mail: ycai @njau.edu.cn

levels, the same parity and lactation period (3rd parity). Milk samples of the clinical mastitis cows were collected via sterile centrifuge tubes (50 mL) and brought back to laboratory for bacterial isolation and identification. Blood samples were collected aseptically and transferred into 10 mL Eppendorf tubes (blood samples/anticoagulants (EDTA-K2) = 6/1) and stored at –70°C. Genomic DNA was exacted from whole blood using phenol/chloroform method, then dissolved in TE buffer and kept at –20°C.

Isolation and identification of pathogenic bacteria: Four selective medium (*Salmonella-Shigella* Agar (SS), Triple Sugar Iron Agar (TSI), MacConkey Agar (MAC), and Blood Agar (BA) plate medium) were used for the isolation and screening of pathogenic bacteria in milk. The milk mixed samples were inoculated in four kinds of selective medium via scribing method, and cultured at 37°C for 18 to 24 h. Then the gram-negative oxidase and Gram staining tests were used initially to identify pathogenic bacteria.

Primer design and PCR amplification: Eleven pairs of primers for PCR were designed according to MD-2 gene (NCBI Reference Sequence: AC_000171.1) which has been deposited in GenBank to amplify the 5'-end region, exon regions and 3'-untranslational region (UTR) of the MD-2 gene (Table 1). PCR reaction system: 10×PCR buffer 2.5 μL, Mg^{2+} (25 mM) 1.5 μL, dNTP (10 mM) 0.5 μL, forward and reward primers (10 μM/μL) 1 μL each, Taq DNA polymerase (0.5 U/μL) 0.2 μL and template DNA (50 ng/μL) 2.0 μL diluted with H_2O to 25 μL. The PCR reactions were run at 94°C for 5 min followed by 33 cycles of denaturation for 45s at 9°C, renaturation for 30s at annealing temperature, elongation for 1 min at 72°C; finally elongation for 10 min at 72°C.

PCR-SSCP assay: The PCR products (5 μL) were electrophoresed in 1% agarose gels containing ethidium bromide (30 min), and then staining was captured by UV gel imaging system. The PCR products with a single and bright stand in UV gel imaging system were analyzed by SSCP as followed: 5 μL PCR products were mixed with the same volume of the denaturation buffer solution at 97°C for 7 min, and immediately placed on the ice at –20°C for 20 min, then run on 30% polyacrylamide gels (1×TBE buffer, 180 V, 12 to 15 h) and stained with $AgNO_3$ (15 to 20 min).

DNA sequencing and genotyping of SNPs: PCR amplification products containing different SSCP bands were directly subjected to DNA bidirectional sequencing. Different SSCP bands represent different genotypes. The identity and diversities of sequences with different genotypes were analyzed with the software of DNAstar 7.0 MegAlign.

Statistical analysis: The significance of differences in single locus (Genotype frequency, Allele number, Allele frequency, Polymorphic loci, Polymorphic information content) and multi-locus were evaluated by means of Pop Gene 32. Results were considered to be significant if $P<0.05$. The frequencies of genotypes and alleles were

Table 1. Primers of MD-2 gene designed for PCR-SSCP analysis

Primer	Sequence (5′-3′)	Annealing temperature Tm/°C	Expected segment Size/bp	Amplified site Size/bp
P1	F: GGTATGTTGACTCCTTGCTTTTAG R: CAACAGAAACCCAGCACAAATCCC	60	496	–2207 to –1711
P2	F: GGATTTGTGCTGGGTTTCTGTTGCT R: GTCTTTGTTCCTTTCAGTGTGTTCT	51	522	–1733 to –1111
P3	F: AACACACTGAAAGGAACAAAGACT R: ACTTTATGTAAGGTGTGGGTTTTAC	58	398	–1134 to –737
P4	F: GTAGTTGGCTAAAGTGAGGGCATTC R: CTCTTTATCCCTCTGTGTTGCTTGC	60	368	–702 to –335
P5	F: TTGGGAGAATAGCAAGCAACACAGA R: AACTAATCTTCAGACTCTTCCCCTT	59	330	–370 to –41
P6	F: GGGAAGAGTCTGAAGATTAGT R: TGTGAAAACACCCTTGGAATGG	54.4	229	–62 to 167
P7	F: AAAGGTAGTTTTGAGGAGGGGGC R: GCCAGACTACCGACACACAAAG	58	374	15409 to 15782
P8	F: TAATGTAGGAAAAGCACAAGGC R: GCTAAAATAAAAACATTGGGTGG	58	379	21166 to 21544
P9	F: AACCTGTGTCTCTTACATCTCCTG R: GGTTTCCTTTGGTGTAAGTTTTC	54.4	358	31579 to 31936
P10	F: TAATAATGATGGGCAATGTTAGATA R: TCAGGGTAATGTATGATGGTAAAGT	50.2	367	32393 to 32758
P11	F: GCTCTTTTGCTTGAACTTTACTATC R: GGAACCCAATCTGCTGACAAT	59.7	232	32720 to 32951

MD-2, Myeloid differentiation-2; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism.

calculated (Kurz *et al.* 2019), and the significant differences were tested by χ^2 text. The results of calculation were used to analyze Hardy-Weinberg balance of mutation sites. The linkage disequilibrium of the SNPs and alleles was determined in this study using Two-locus LD method according to the diploid data of the single-population.

Crosstabs method (Pearson Chi-square test, Continuity correction test, Fisher' exact text) in SPSS 13.0 was adopted in order to measure the association between SNPs in MD-2 gene and the relative risk of clinical mastitis in Chinese Holstein dairy cows.

RESULTS AND DISCUSSION

Gram-negative bacteria can cause clinical mastitis in Chinese Holstein dairy cows: There were no bacterial single colonies in medium SS, indicating that *Salmonella* and *Shigella* were not isolated from the milk sample. There were also no single colonies in the BA medium, suggesting no positive pathogens such as *Streptococci* and *Staphylococcus aureus* in the milk. Gram-negative bacilli oxidase test was negative, and some single bacteria produced acid in TSI and MAC medium contained red colony, suggesting that bovine milk sample contained the bovine mammary gland gram-negative pathogenic bacteria, such as *E. Coli*. Finally, gram staining test also confirmed that gram negative bacteria caused clinical mastitis in Chinese Holstein dairy cows.

Genetic polymorphisms in the 5'-end of MD-2 gene in Chinese Holstein dairy cows: Eleven pairs of dairy cows MD-2 gene primers were successfully cloned. Agarose gel electrophoresis showed that they were all single bands and can be used for SSCP typing analysis. SSCP results observed that the PCR products of the primers P1, P4, and P5 have 6, 2, and 3 types of bands, respectively. It's suggested that these regions of the MD-2 gene have single nucleotide polymorphisms. The SSCP bands of primer P1 PCR products were named EE, EF, FF, EQ, EM, and EN genotype and contained five alleles (Fig.1A), the P4 PCR products had two genotypes (DD and CD) (Fig.1B), while three genotypes (AA, AB, and BB) were identified in P5 PCR products (Fig. 1C). In addition, χ^2 test revealed that genotypes in the fragment amplified by P5 were in Hardy-Weinberg equilibrium (P>0.05), while the genotypes in the P1 and P4 PCR products were not in Hardy-Weinberg Equilibrium (P<0.01). Data indicated that there are SNPs at the 5'-end of the MD-2 gene in Chinese Holstein dairy cows.

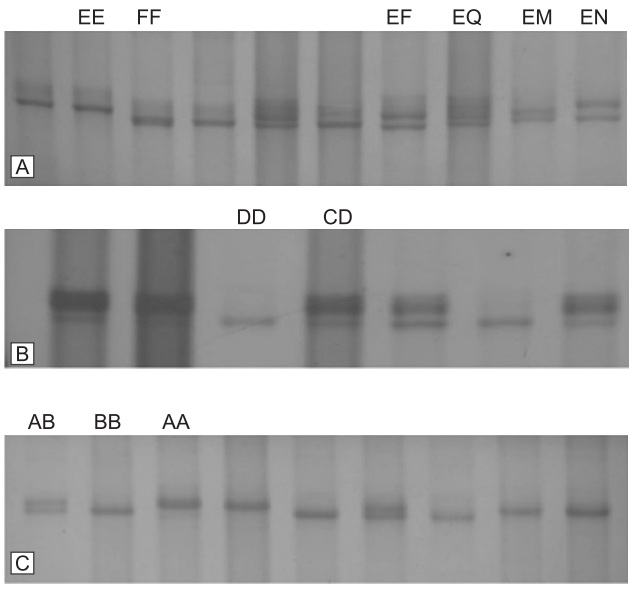

Fig.1. SSCP analysis for the PCR products of primer 1 (A), primer 4 (B) and primer 5 (C). Three genotypes were detected in panel A (EE, FF, EF, EQ, EM, EN), two genotypes were detected in panel B (DD, CD) and three genotypes were detected in panel C (AA, AB, BB).

Novel SNPs in 5'*-end region of the MD-2 gene was identified in Chinese Holstein dairy cows:* Directing sequencing results of all primers showed that the PCR products were bovine MD-2 gene fragments in NCBI database, suggesting that our PCR-SSCP analysis results were correct. Total of novel five SNPs were detected in P1, P4 and P5 primer PCR products at the 5′-end region of the MD-2 gene in Chinese Holstein dairy cows via direct sequencing (Fig. 2). Interestingly, these polymorphisms only appeared in the 2.3 kb sequence near the translation starting site of the MD-2 gene promoter region, among which the SNPs g.-121, g.-2173, and g.-2089 had three types (homozygote and heterozygote), while SNPs g.-555 and g.-2148 had two types, respectively. It is well known that the single nucleotide polymorphism at the 5' end of the gene is significantly associated with gene function (Yang *et al.* 2019). Therefore, the single nucleotide polymorphisms at the 5' end of MD-2 gene are bound to affect its function.

Novel SNPs (*g.-555* (*A/G*)) *at the 5' end of MD-2 gene was detected to be associated with clinical mastitis in Chinese Holstein dairy cows:* Lipopolysaccharide (LPs) can induce immune response and inflammatory response in the bovine mammary gland (Sun *et al.* 2019). However, MD-2 was co-expressed with TLR4 and the solubility was significantly increased, and participates in the cascade response caused by LPS stimulation and promotes cytokine release (Tian *et al.* 2019). Genomics studies have shown that key gene SNP are associated with mastitis in dairy goats (Oget *et al.* 2019), TLR4 SNP as well. Therefore, as a pivotal upstream factor of TLR4 signaling pathway, the MD-2 gene polymorphism is potentially associated with susceptibility of clinical mastitis with gram-negative infection in Chinese Holstein dairy cows. In this study, SSCP analysis showed that morbidity rate of DD is higher than CD (P<0.01) in the fragment amplified with P4 (Table 2). However, SNPs in the fragmentary amplified with P1 and P5 did not change the morbidity ratio significantly (P>0.05). Statistical results indicated that the SNP (g.-555A/G) in DD genotype might affect the secretion of MD-2 protein and regulate the binding ability of MD-2 protein to TLR4. Therefore, this polymorphism may be an important risk factor for the susceptibility to clinical mastitis with gram-negative infection in Chinese Holstein dairy cows.

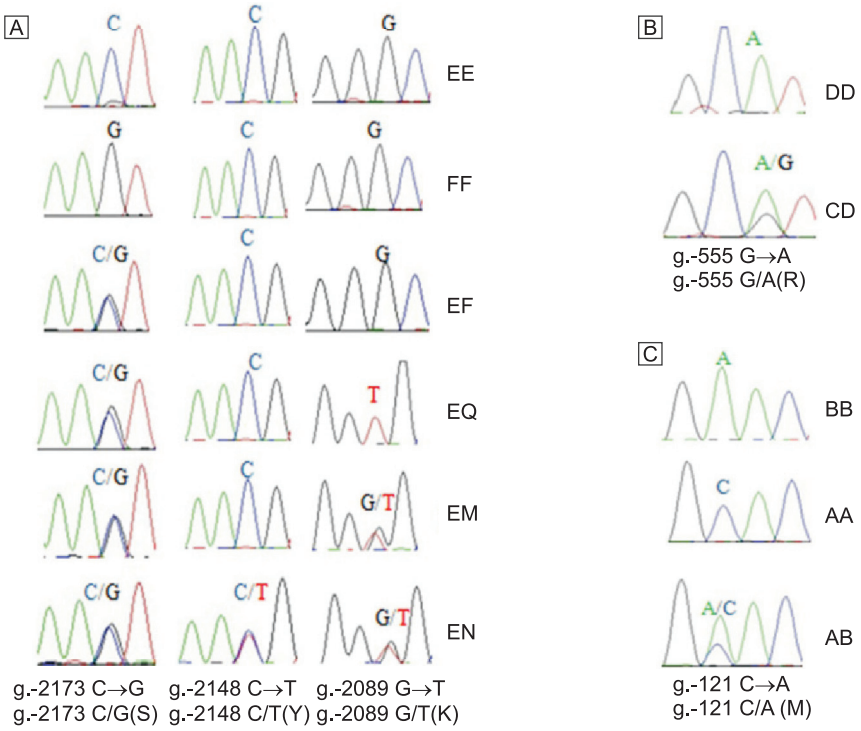

Fig. 2. Oscillogram of each genotype showing the mutations in primer 1, primer 4 and primer 5.

Table 2. Statistical analysis of the association between genotypes and susceptibility of clinical mastitis in Chinese Holstein

Location	Genotype	Number of clinical individuals	Number of non-clinical individuals	Morbidity (%)
P1	EE	6	11	35.29
	FF	43	48	47.25
	EF	5	8	38.46
	EQ	12	17	41.38
	EM	21	28	42.86
	EN	9	13	40.91
P4	CD	94	110	46.08
	DD	16	1	94.12**
P5	AB	37	29	56.06
	BB	3	0	100.00
	AA	71	81	46.70

**P value of Crosstabs test smaller than 0.01.

MD-2, a key component of the TLR4/CD14 combination, is associated with LPS-induced inflammatory response in mammary epithelial cells. In this study, five novel SNPs were identified at the 5′ end of the MD-2 gene by PCR-SSCP and direct sequencing. Trait correlation analysis showed that the SNPs (g.-555A/G) may be associated with susceptibility to mastitis in Holstein dairy cows.

ACKNOWLEDGEMENTS

This study was supported by Agricultural Innovation fund of Jiangsu Province [grant no. CX (18)3014]; National Key R&D Program of China [grant no. 2018YFC1200201]; Major Program of Natural Science Foundation of Anhui Province Education Department (KJ2015ZD38 and KJ2019A0612); Start-up grant from Nanjing Agricultural University [grant no. 804090].

REFERENCES

Chen L, Fu W, Zheng L, Wang Y and Liang G. 2018. Recent progress in the discovery of myeloid differentiation 2 (MD2) modulators for inflammatory diseases. *Drug Discovery Today* **23**: 1187–1202.

Cheng J, Qu W, Barkema H W, Nobrega DB, Gao J, Liu G, De Buck J, Kastelic J P, Sun H and Han B. 2019. Antimicrobial resistance profiles of 5 common bovine mastitis pathogens in large Chinese dairy herds. *Journal of Dairy Science* **102**: 2416–26.

Heikkila AM, Liski E, Pyorala S and Taponen S. 2018. Pathogen-specific production losses in bovine mastitis. *Journal of Dairy Science* **101**: 9493–504.

Kurz J P, Yang Z, Weiss R B, Wilson D J, Rood K A, Liu G E and Wang Z. 2019. A genome-wide association study for mastitis resistance in phenotypically well-characterized Holstein dairy cattle using a selective genotyping approach. *Immunogenetics* **71**: 35–47.

Li C, Li L, Chen K, Wang Y, Yang F and Wang G. 2019. UFL1 Alleviates Lipopolysaccharide-Induced Cell Damage and Inflammation via Regulation of the TLR4/NF-kappaB Pathway in Bovine Mammary Epithelial Cells. *Oxidative Medicine and Cellular Longevity* **2019**: 6505373.

Oget C, Teissier M, Astruc J M, Tosser-Klopp G and Rupp R. 2019. Alternative methods improve the accuracy of genomic prediction using information from a causal point mutation in a dairy sheep model. *BMC Genomics* **20**: 719. 10.1186/s12864-019-6068-4

Pizzuto M, Lonez C, Baroja-Mazo A, Martinez-Banaclocha H, Tourlomousis P, Gangloff M, Pelegrin P, Ruysschaert J M, Gay N J and Bryant C E. 2019. Saturation of acyl chains converts cardiolipin from an antagonist to an activator of Toll-like receptor-4. *Cellular and Molecular Life Sciences* **10**.1007/s00018-019-03113-5

Steimle A, Michaelis L, Di Lorenzo F, Kliem T, Munzner T, Maerz J K, Schafer A, Lange A, Parusel R, Gronbach K, Fuchs K, Silipo A, Oz H H, Pichler B J, Autenrieth I B, Molinaro A, and Frick J S. 2019. Weak agonistic LPS restores intestinal immune homeostasis. *Molecular Therapy* **10**.1016/j.ymthe.2019.07.007

Sun L, Chen L, Wang F, Zheng X, Yuan C, Niu Q, Li Z, Deng L, Zheng B, Li C and Zhou X. 2019. Exogenous hydrogen sulfide prevents lipopolysaccharide-induced inflammation by blocking the TLR4/NF-kappaB pathway in MAC-T cells. *Gene* **710**: 114–21.

Tian R, Seim I, Zhang Z, Yang Y, Ren W, Xu S and Yang G. 2019. Distinct evolution of toll-like receptor signaling pathway genes in cetaceans. *Genes and Genomics* **10**.

Yang J, Wang J, Liang X, Zhao H, Lu J, Ma Q and Tian F. 2019. Relationship Between Genetic Polymorphisms of the TNF Gene and Hallux Valgus Susceptibility. *Genetic Testing and Molecular Biomarkers* **23**: 380–86.