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Comparison of cattle BoLA-DRB3 typing by PCR-RFLP, direct sequencing, and high-resolution DNA melting curve analysis

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Abstract

Major histocompatibility complex (MHC) represents an important genetic marker for manipulation to improve the health and productivity of cattle. It is closely associated with numerous disease susceptibilities and immune responses. Bovine MHC, also called bovine leukocyte antigen (BoLA), is considered as a suitable marker for genetic diversity studies. In cattle, most of the polymorphisms are located in exon 2 of BoLA-DRB3, which encodes the peptide-binding cleft. In this study, the polymorphism of the BoLA-DRB3.2 gene in Holstein's calves was studied using high resolution melting curve analysis (HRM). Observed HRM results were compared to PCR-RFLP and direct sequencing techniques. Eight different HRM and seven different RFLP profiles were identified among the population studied. By comparing to sequencing data, HRM could completely discriminate all genotypes (eight profiles), while the RFLP failed to distinguish between the genotypes *1101/*1001 and *1104/*1501. According to the results, the HRM analysis method gave more accurate results than RFLP by differentiating between the BoLA-DRB3.2 genotypes. Due to the Co-dominant nature of the MHC alleles, HRM technique could be used for investigating the polymorphisms of genotypes and their associations with immune responses.

Introduction

The major histocompatibility complex (MHC) is a group of closely linked genes that forms an important genetic component of the mammalian immune system.[1] The discovery of MHC was done during tissue transplantation studies in mice and was first known for its role in histocompatibility.[2] Consequently, the role of MHC was discovered in immune regulation and several other functions.[3,4] The MHC genes are associated with more human diseases than any other region of the genome. The genetic variation within this region explains a substantial proportion of differential immune respondents between individuals.[5,6] Likewise, genetic variation in the bovine MHC (BoLA) has been associated with numerous disease susceptibilities and immune responses in cattle, and represents an important genomic target for manipulation to improve the health and productivity.[7-10]

The MHC region is known as the most polymorphic loci in vertebrates with polymorphism occurring mainly at sequences that encoding the peptide-binding domains.[11] The BoLA is located on chromosome 23 and spans about 2.50 Mbp.[12,13] Similar to human being and mice, cattle have three MHC gene classes: Class I, II and III.[14,15] MHC Class I molecules are found in almost all cells and exhibit proteins to cytotoxic T cells, while molecules of MHC Class II reside in specific immune cells, particularly on some antigen-presenting cells (APCs) like macrophages and B cells.[16] MHC class I molecules present endogenous peptide antigen to cytotoxic (CD8+) T-cell, whereas class II molecules present exogenous antigen to helper (CD4+) T-cell to generate an immune response.[17] Class II genes are highly polymorphic and this polymorphism is due to a large number of amino acids among alleles at each locus.[18]

MHC polymorphisms arise from point mutations, gene duplication or deletion and intra-locus recombination.[14]

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In cattle, the BoLADRB3 gene is a highly polymorphic locus of class II genes with over one hundred alleles reported. Most of the polymorphisms are located in exon 2, which encodes the peptide binding cleft. Sequence differences play an important role in the variability of immune responsiveness and disease resistance. The study on the polymorphic pattern of the DRB3 gene is also important because it is linked to the immune functions of class II antigen of MHC.[19] However, the high degree of polymorphism in exon 2 leads to difficulty in accurate genotyping, especially heterozygous animals.[20] Several methods have been developed for BoLA typing. These include serology,[21] isoelectric focusings (IEF), direct sequencing,[22,23] heteroduplex analysis,[24] denaturing gradient gel electrophoresis,[25] polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP),[26] and PCR sequence-based typing.[27]

The PCR-based methods have a great value in estimating the number of MHC alleles. Amongst above-mentioned methods, PCR-RFLP is frequently used for BoLADRB3 typing. The protocol included an informative restriction enzyme, which has unique recognition sites present in some alleles but not in others. The enzyme cutting products (RFLP bands) then could be identified by electrophoresis on a vertical polyacrylamide gel. Direct sequencing, as a reference method, is the only technique that detects the nucleotide sequences of an allele. It is widely used for detecting single nucleotide polymorphisms and defining the new alleles.

An alternative method for MHC genotyping is a high resolution melting technique (HRM), a new post-PCR method that depends on the optimal melting condition of the DNA segment. HRM is an automated analytical molecular technique that measures the thermodynamic properties of the PCR products.[28] The HRM takes advantage of a fluorescent dye, which is homogeneously intercalated into the double-stranded DNA and released at melting temperature (Tm). Tm depends on the length, GC content and the complementarity of the target sequence.[28] HRM analysis has been used to discriminate viruses,[29] bacteria,[30-32] nematodes,[33] and fungi.[34] The recent development of better performing saturating DNA dyes and the technical progress that enabled the increased resolution and precision of the instruments have permitted the use of HRM for genotyping.[35]

In this study, the polymorphism of the BoLA-DRB 3.2 gene in Holstein's calves was studied using HRM analysis. Observed HRM results were compared to PCR-RFLP and direct sequencing techniques.

Materials and Methods

Animals and blood sample collection. A total number of 40 blood samples collected from Iranian Holstein calves. The samples were collected from a farm under the ethical statement and instructions of the University of Tehran, faculty t of Veterinary Medicine, 7502015/6/36, 10/05/2016).

DNA Extraction and genotyping of BoLA-DRB3 exon 2 by PCR technique. DNA was extracted from blood samples using a genomic DNA extraction kit (Bioneer, Daejeon, Korea). In the first round of amplification, exon 2 of BoLA-DRB3 gene (284 bp) was amplified by semi-nested polymerase chain reaction (PCR) with HL-030 (5'-ATCCTCTCTCTGCAGCACATTTCC-3') and HL-031 (5'-T TTAATTCGCGCTCACCTCGCCGCT-3') primers. [26]

The first round of PCR amplification was performed with 50.00 ng of DNA in a 25.00 µL reaction mixture (Sinaclon, Tehran, Iran) 10X PCR buffer (2.50 µL), $MgCl_2$ (1.00 mM), dNTPs (0.50 µL), HL-030 and HL-031 primers (1.00 µL each) containing 5.00 pmol μL^{-1}, and Taq DNA polymerase (0.25 µL). The thermal cycling profile for the first round of amplification was initial denaturation of 5 min at 94.00 ˚C, followed by 15 cycles of 1 min at 94.00 ˚C, 2 min at 60.00 ˚C, 1 min at 72.00 ˚C, and final extension of 1 min at 72.00 ˚C.

The second round of PCR amplification was performed with 2.00 µL of first-round PCR product as a DNA template in a separate tube, with the same volume and concentration of contents as described above, using HL-030 and HL-032 primers (Sinaclon, Tehran, Iran). HL-032 primer (5'TCGCCGCTGCACAGTGAAACTCTC-3') is internal to the sequence of the amplified product of the first-round PCR and has eight bases that overlap with HL-031 primer. The thermal cycling profile for the second round was 30 cycles of 1 min at 94.00 ˚C for denaturation and 30 s at 65.00 ˚C for annealing, extension at 72.00 ˚C for 1 min, followed by a final extension of 5 min at 72.00 ˚C. The PCR products were visualized by electrophoresis on 1.50 % agarose gel (Sinaclon, Tehran, Iran) stained with ethidium bromide.

HRM curve analysis. Melting analysis of PCR products (284 bp) in the presence of the fluorescent nucleic acid dye, Eva Green was used for DNA typing of BoLA-DRB3. The PCR amplicons were subjected to the HRM curve analysis, which was performed in a Rotor-Gene™ 6000 thermal cycler (Corbett Life Science Pty Ltd, Hilden, Germany). In order to determine the optimal melting condition for differentiation of the BoLA DRB3 exon2 region, the PCR products were set up at 0.30 ˚C per sec ramping between 70.00 ˚C and 95.00 ˚C. All specimens were tested and their melting profiles analyzed using Rotor-Gene software (version 1.7; Corbett Life Science Pty Ltd., Hilden, Germany) and the HRM algorithm provided. Plots of fluorescence versus temperature were normalized as described by Wittwer.[36] Normalization regions of 85.87 - 86.90 and 90.15 - 91.55 were then used for analysis.

PCR-RFLP analysis. Identification of the enzyme cutting patterns was carried out according to van Eijk *et al*.[26] The RsaI enzyme (Roche, Berlin, Germany) was used to cut the

PCR products and perform the RFLP test. The enzyme cutting products were stained along with an indicator of the MspI-digested pBR322 ladder (Fermentas GmbH, Leon-Rot, Germany) on a 12.00% acrylamide gel for one hour with vertical electrophoresis of 150 volts and then ethidium bromide. Gel images were recorded with a trans-luminant UV device (Bio-Rad, Berkeley, USA).

DNA Sequencing. Samples from each HRM and PCR-RFLP profiles were sent for direct sequencing. Bi-directional sequencing was performed by Auto-Sequencer (ABI 3730 XL; Applied Biosystems, Foster City, USA) and the color tightening method. Sequencing was identified using the BLAST website of the NCBI. The DRB3 sequences obtained by online BioEdit with reference sequences registered on the IPD site were compared.

Results

Based on the direct sequencing results, a total number of 23 variable sites (SNPs) were observed in the sequences considering all the genotypes. HRM, PCR-RFLP and direct sequencing methods were used for identification of the frequency of BoLA-DRB3 alleles and genotypes. The HRM curve analysis showed eight profiles (A-H), (Figs. 1 and 2), while the RFLP results showed seven (a-g), (Fig. 3).

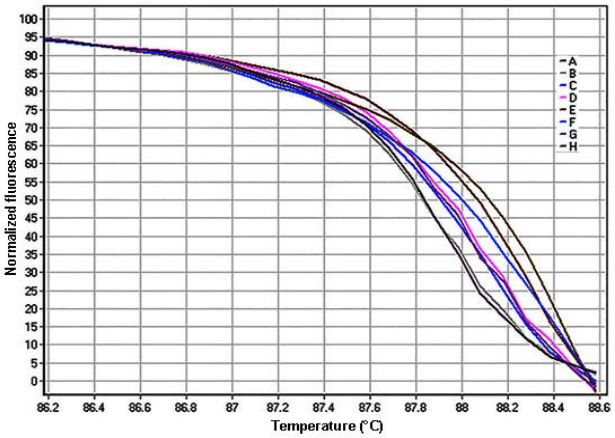

Fig. 1. Eight profiles of BoLA-DRB3.2 identified by HRM analysis.

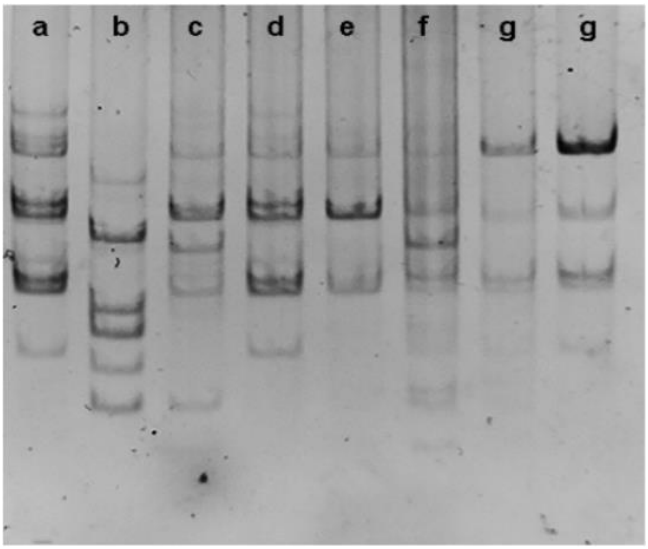

Fig. 3. Seven profiles of BoLA-DRB3.2 identified by PCR-RFLP.

The results are summarized in Table 1. In the case of alleles, the output of both HRM and RFLP analyses were identical showing six alleles named as: *1101, *0101, *2709, *1501, *1104 and, *1001.

Table 1. Frequency of the BoLA-DRB3.2 profiles and genotypes identified by HRM, PCR-RFLP, and sequencing.

HRM profile	samples (no.)	RFLP pattern	Allele sequenced	
A	8	a	*1101	*0101
B	5	b	*0101	*2709
C	7	c	*1101	*1501
D	4	d	*1104	*0101
E	3	e	*1101	*1101
F	4	f	*1001	*1001
G	5	g	*1101	*1001
H	4	g	*1104	*1501

Results indicated that studied HRM profiles contained homozygote and heterozygote genotypes, including two homozygote (E-*1101/*1101 and F-*1001/*1001), and six heterozygote genotypes (A-*1101/*0101, B-*0101/*2709, C-*1101/*1501, D-*1104/*0101, G-*1101/*1001, and, H-*1104/*1501).

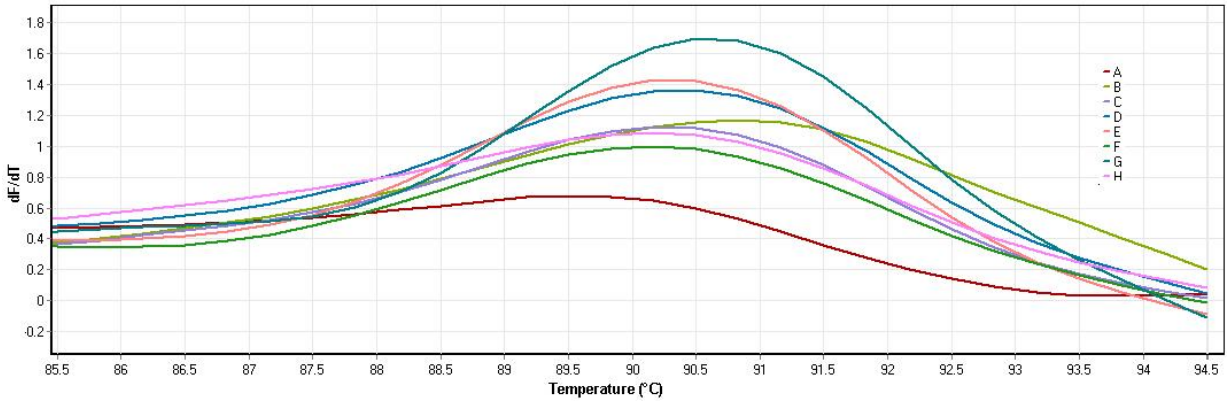

Fig. 2. Eight profiles of BoLA-DRB3.2 identified by normal Tm curve analysis.

In this study, the important difference between the HRM and RFLP analysis was that the HRM could completely discriminate between the genotype G (*1101/*1001) and H (*1104/*1501). Consequently, a total of eight genotype profiles were identified by the HRM method (Figs. 1 and 2). However, the RFLP method was failed to discriminate these two genotypes. Based on the RFLP results, *1101/*1001 and *1104/*1501 were belonged to the "g" profile, and so only seven genotype profiles were observed (Fig. 3).

Discussion

Current methods for MHC typing, such as PCR-RFLP and direct sequencing, had their disadvantages. PCR-RFLP might not detect point mutations and alterations in small bands located close to each other. Also in direct sequencing, resolving the ambiguities due to the heterozygous base-calling fluorescence remains a challenge in chromatogram data analysis. In addition, both methods require expensive equipment and sophisticated laboratory approaches that may not be prepared in all laboratories and for a large number of samples.

In this study, HRM analysis was developed to determine the genetic variations. With regard to the MHC typing, this approach has so far been applied only for human (HLA).[37] Due to the similarity of the structure and diversity of MHC genes among humans and other mammals, HRM typing seems to be appropriate for BoLA-DRB3.2 alleles. This is the first study of the DNA polymorphism of the BoLA-DRB3 gene in cattle by HRM.

Alleles that found in the current study (6 alleles) were similar to those alleles reported for BoLA-DRB3.2 in cattle and no new alleles were found.[26,38,39] In comparison with the previously reported data for Holstein cattle, both HRM and RFLP analysis showed lower homozygosity (17.50%) and higher heterozygosity (82.50%). In a study that the same enzyme was used for RFLP (RsaI), approximately 31.00% of the total crossbred cows were homozygous for the RsaI restriction sites, and 69.00% were found to be heterozygous.[40] Comparing to Iranian indigenous breeds, observed heterozygosity was less than what reported for Sarabi (94.00%) and Najdi (94.00%) cows.[41] Similar to our findings, *1101/*0101 was the most frequent among the 90 genotypes that has been previously reported for Iranian Holstein cow. The similar heterozygosity (88.42%) was also reported for BoLA-DRB3.2 alleles.[10] Differences among the Iranian Holstein populations are not that high and might be largely due to the long term adaptation to geographical and climatic conditions as well as their geographic proximity.

Both normal melting temperature analysis (Tm) and high resolution melting (HRM) were applied to determine the BoLA-DRB3.2 diversity. Although the Tm analysis was consistent with other methods, it did not have the ability to accurately discriminate the genotypes. This is characterized by the very small differences between the peak temperatures of the melting curves for different genotypes. It is worth noting that a temperature difference of just a half-degree Celsius does not have a high degree of specificity and accuracy to distinguish different genotypes. However, this phenomenon is partly related to the structure and sequence of the DRB3 gene of the cattle. The analysis of BoLA-DRB3.2 diversity by the HRM method is very similar to the SSCP method, in which variation is also detectable at a single-nucleotide level. It should be taken into consideration that the differentiation between patterns in the HRM and Tm methods are not ocular and has high repeatability. Nevertheless, like SSCP, it is not possible to detect alleles at the first stage and the results only indicate the diversity of genotype.

Regarding to the most frequent alleles in the population studied, allele *1101 is associated with higher somatic cell in Canadian Holstein,[42] and resistance to mastitis and bovine leukemia virus infection in Polish Holstein Friesian cattle.[43] Allele*0101 were found to be associated with resistance to mastitis [44] and allele *1001 was associated with pregnancy loss[45]. The MHC diversity is important for resistance to infectious diseases, especially those are associated with antigen-specific acquired immunity. As mentioned before, several infectious diseases are associated with BoLA-DRB3 alleles,[46] however, in domesticated and artificially selected populations, polymorphism is low. Therefore, assessing biodiversity using MHC genes as markers is important and can provide precious data for artificial selection to conserve biodiversity.

HRM could be considered as an appropriate method for MHC genotyping in cattle, particularly when combined with DNA sequencing. HRM technique is able to detect homozygote or heterozygote genotypes in a simple, fast and inexpensive method. However, more powerful and accurate approaches such as sequencing might be necessary to confirm the exact number of alleles. Based on the experience presented from the current study, we could first test the large populations by HRM method, to analyze the BoLA-DRB3 gene diversity. It then could be followed by the sequencing or RFLP, which is used for each HRM pattern. As a result, this method would save considerable time and cost. In general, the application of a precise analysis of HRM seems to be of great use in examining the diversity of MHC genes. Although it is not possible to detect precisely the alleles of each of the chromosomes, however, due to the Co-dominant nature of the MHC genes, the HRM results directly (without sequencing) could be used for study the associations between the MHC genes and their immune phenotypes including sensitivity or resistance to the diseases.

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Conflicts of interest

The authors declare no conflict of interest.

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