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Investigation of Bovine Leukocyte Adhesion Deficiency (BLAD) and Complex Vertebral Malformation (CVM) in a Population of Iranian Holstein Cows

Research Article

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ABSTRACT

In the present research, molecular detection of bovine leukocyte adhesion deficiency (BLAD) and complex vertebral malformation (CVM) in a population of Iranian Holstein cows has been carried outusing milk somatic cells by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The BLAD and CVM are monogenic and autosomal recessive heredity lethal syndrome in Holstein-Friesian cattle. BLAD characterized by affecting the haematopoietic system via reduced expression of the adhesion molecules on neutrophils. CVM characterized by intra-uterine mortality with disorders such as short neck, curved legs, abnormality of ribs and some certain heart abnormalities. In the first step of our research program, tank milk samples from 50 herds were collected. PCR-RFLP was performed to detect a point mutation of both CVM and BLAD genes. After DNA extraction, PCR was amplified using specific primers for 136 bp DNA (CD18 gene) and 233 bp DNA (SLC35A3 gene). *TaqI* and *EcoT22I* enzymes were used to identify both BLAD and CVM alleles of both genes by digestion of PCR products. In these herds, we did not find any affected herd with the mutant allele of BLAD comparing with a positive evidence but the mutation of SLC35A3 gene found in 17 different herds. In the next step of our study a herd with 120 cows was randomly selected for individual test using blood samples. We showed two cows out of 120 were identified as carriers of this gene. In this herd, the total number of dominant homozygote (AA), heterozygote (Aa) and recessive homozygote (aa) genotypes for CVM were 118, 2 and 0, respectively and the frequency of A and a alleles were 0.992 and 0.008, respectively. The other affected herds will be tested in the next step of our research program.

KEY WORDS genetic defect, PCR-RFLP, single nucleotide polymorphism.

INTRODUCTION

Livestock species can be affected by different types of genetic defects. Recently, we are faced with genetic defects in dairy cattle such as bovine leukocyte adhesion deficiency (BLAD) and complex vertebral malformation (CVM) both are autosomal recessive defects leads to an inherited lethal disease in Holstein breed. BLAD is caused by a deficiency in leukocyte surface glycoproteins known as integrin which is a sticky protein generally found on the surface of leukocytes and it is reduced in animals carrying recessive alleles. These proteins are responsible for the cell-cell interactions necessary for neutrophils to adhere to vascular endothelium and enter the tissue for destroying pathogens. It can be found on all T-cells, B-cells, macrophages and neutrophils. Two point mutations have been identified in the gene that

encodes bovine CD18 in Holstein cattle affected by BLAD (Vatasescu-Balcan *et al.* 2007). One mutation replaces adenine with guanine at nucleotide 383 and the other replaces cytosine with thymine at nucleotide 775. The latter is a silent mutation and the other mutation replaces glycine by aspartic acid at nucleotide 383 changing at amino acid 128. One more silent mutation has recently been detected in CD18 (Gen-Bank accession No: KF840683) gene which replaces T > C at 348 position in exon 4, which does not change amino acid asparagine, AAT > AAC (Vatasescu-Balcan *et al.* 2007). The mutation at 383 bp positions eliminates a *TaqI* restriction site and creates a *HaeIII* site, which allows the identification of normal, carrier and affected animals (Adamov *et al.* 2013). The defective leukocyte adherence leads to inadequate mucosal immunity. BLAD-affected cattle have severe and recurrent mucosal infections such as pneumonia, ulcerative gingivitis, periodontitis, papillomatosis and loss of teeth, dermatophytosis and stunted growth (Nagahata *et al.* 1987). CVM characterized by intra-uterine mortality with some disorders such as short neck, curved legs, abnormality of ribs and some certain heart abnormalities (Nagahata *et al.* 1987). The molecular cause of CVM is a point mutation with substitution of guanine by thymine at the nucleotide positions 559 of exon 4 gene SLC35A3 (Gen-Bank accession No: AY160683) chromosome 3, encoding a uridin 5-diphosphate-N-acetyl-glucosamine transporter which results in the substitution of valine amino acid by phenylalanine at position 180 (Thomsen *et al.* 2006). Disease symptoms have not been observed in both CVM and BLAD carriers but clinical characterization of CVM affected calves have a composite phenotype with multiple malformations of the cervical thoracic and lumber regions of the vertebral column, abnormality of ribs and legs, low body weight and lateral rotation of the fetlock joints, most affected fetuses are aborted at gestation before day 260 and others are usually stillborn or prematurely born with a neck shorter than normal, some with heart malformation in which the main joining blood veins are incorrect, injuries were observed in almost all bones (Patel, 2012; Rezaei *et al.* 2009).

The most economic impact of this gene is loss of fetus and also longer calving interval. Therefore use of molecular techniques for monitoring of genetic defects may help farmers to reduce calving interval by preventing the mating between heterozygote sires and cows.

MATERIALS AND METHODS

Milk samples were collected from 50 randomly selected dairy cattle herds in Tehran and Alborz provinces of Iran. All cows in selected herds had pedigree and recorded data. Thirty mL of milk sample from milk tank of each herd were collected and then samples were coded and transferred to the laboratory.

Genomic DNA was extracted from milk somatic cells using an extraction kit of Qiagene Company (Dnasy tissue). Extracted genomic DNA was electrophoresed on 1% agarose gel.

Nonodrop spectrophotometer was used to determine quality and quantity of the extracted DNA. After DNA extraction, special primers were used for amplification of exon 4, chromosome 3 of SLC35A3 gene and exon 5, chromosome 1 of CD 18 gene containing our related mutations, thermo-cycler amplified 233 bp part of SLC35A3 gene (as shown in Figure 1)and 136 bp part of CD18 gene, then they were loaded on 1% agarose gel. PCR products of SLC35A3 gene were loaded on 1% agarose gel after digestion by *EcoT22I* enzyme for showing affected herds with CVM carriers or non-affected herds and PCR products of CD 18 gene were loaded on 1% agarose gel after digestion by *Taq I* enzyme for showing affected herds with BLAD carriers and non-affected herds. PCR reaction was performed for amplification of polymorphic regions of both SLC35A3 and CD 18 genes using primer sequences shown at Table 1.

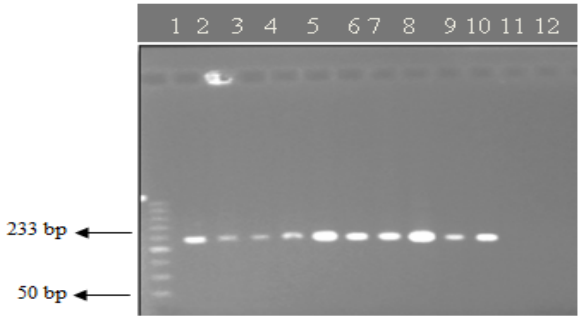

Figure1 PCR products of SLC35A3 in Iranian Holstein cows using milk somatic cells
Lane 1: ladder 500 bp; lanes 2-10 PCR products; lane 12: negative control

The PCR reaction was done by QIAGENE company PCR kit (Hot StarTaq PCR). The temperature programs of polymerase chain reaction forSLC35A3 and CD18 was as below:

PCR temperature program and cycling for SLC35A3

1: Initial denaturation at 94 ˚C for 3 min to ensure the complete separation of the DNA strands.
2: Amplification after 30 cycles of strand denaturation at 94 ˚C for 45 s, primer annealing at 55 ˚C for 20 s and primer extension at 72 ˚C for 40 s.
3: Final extension at 72 ˚C for 10 min.

Table1 Primers for PCR amplification of the CVM and BLAD loci

Forward	5' CACAATTTGTAGGTCTCACTGCA 3'	Primer sequences designed for SLC35A3 Kepenek (2007)
Reverse	5' CGATGAAAAAGGAACCAAAAGGG 3'	
Forward	5´ CCT TCC GGA GGG CCA AGG GCT 3´	Primer sequences designed for CD18 Vatasescu-Balcan *et al.* (2007)
Reverse	5´ CCT GGT GAT GCC ATT GAG GGC 3´	

PCR temperature program and cycling for CD18

1: Initial denaturation at 95 ˚C for 3 min to ensure the complete separation of the DNA strands.

2: Amplification after 40 cycles of strand denaturation at 95 ˚C for 30 s, primer annealing at 57 ˚C for 30 s and primer extension at 72 ˚C for 60 s.

3: Final extension at 72 ˚C for 10 min.

Then PCR products were electrophoresed on 1% agarose gel. In order to confirm our results, 8 samples of our PCR products were transferred to Kavoosh-Kosar Company and sequencing results were compared with data available in NCBI (national center for biotechnology information) Genebank.

We used *EcoT22I* (Ferments) enzyme for detection of single nucleotide mutation on *EcoT22I* site in the amplified products of SLC35A3 gene and *HaeIII* (Ferments) enzyme for detection of single nucleotide mutation on *HaeIII* site in the amplified products of CD18 gene. The PCR products of each herd were digested by *EcoT22I* and *HaeIII* enzymes-for 12 hours at 37 ˚C. Then, in order to identify the genotypes, digestion products were separated by electrophoresis on 1% agarose gel stained with DNA safe stain and digested products were compared with a positive evidence for recessive genotype (as shown in Figure 2). Then, one of the affected herds having 120 dairy cows was randomly selected and 30 mL milk of each cow was collected, DNA was extracted from each sample and PCR-RFLP (as described) was done for each cow in the herd , 2 carriers out of 120 were detected in this herd.

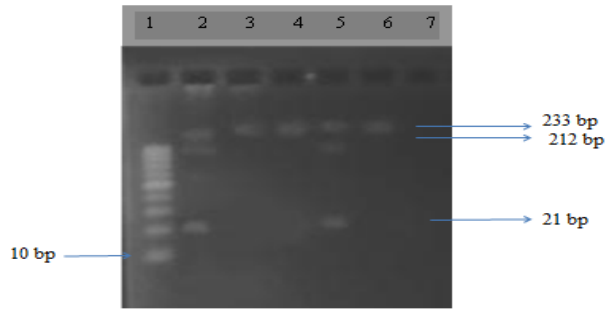

Figure2 PCR products after digestion by *EcoT22I* enzyme
Lane 1: ladder 200 bp; lanes 2 and 5 herds with CVM carriers; lanes 3, 4, 6 non-affected herds with genotype AA; lane 7: negative control

RESULTS AND DISCUSSION

The primers used in this experiment (Table1) were amplified successfully. Out of 50 Holstein herds, 17 herds were diagnosed having CVM carrier cows with 3 fragments of 233 bp, 212 bp and 21 bp (Figure 2). We didn't find any CVM carrier or CVM affected (with a single 233 bp fragment) in the 33 herds and any BLAD carrier in all 50 herds. In the next step of our study, one of the CVM carrier herds having 120 dairy cows was randomly selected. In order to detect carrier cows in the herd, 30 mL milk of each cow was collected and PCR-RFLP (as described) was done for each cow in the herd and 2 carriers were detected. After studying the pedigree of these two carriers, we found, they are full sib sisters. After blood sampling from their father and their mother, our results showed that they received this recessive allele from their heterozygote father. Many CVM carrier fetuses are aborted at gestation day 159 while other CVM affected calves are prematurely born or usually stillborn. The major morphological changes in CVM-affected calves with recessive genotype delivered after gestation day 260. Therefore it was normal that we didn't find any CVM-affected calf in the cattle.

The total number of aa, AA and Aa genotypes for CVM were obtained 0, 118 and 2 respectively and the frequency of A and a alleles were 0.992 and 0.008 respectively. CVM carriers had no clinical reproductive problems. Rezaei *et al.* (2009) reported that there are no CVM carriers in Iranian Holstein bulls in both Abbas Abad breeding center and Ferdowsi University of Mashhad's dairy farm. Agerholm *et al.* (2001), Agerholm *et al.* (2004), Nagahata *et al.* (2002), Chu *et al.* (2008), Betka *et al.* (2008), Kotikalapudi *et al.* (2013) and Ghanem *et al.* (2008) determined different frequencies of CVM carriers in their cattle.

CONCLUSION

In this study we found that 33 herds are not infected by CVM defect and if we assume the average of 100 dairy cows for each non-infected herd. We have done 33 testes instead of 3300. DNA extraction from milk somatic cells doesn't have any stress for cows. Moreover, we are not often allowed to collect blood samples from pregnant cows. One of the aims of our work was to optimize PCR-RFLP as a diagnostic molecular test for genetic defects such as BLAD and CVM carrier cows using milk somatic cells instead of blood. In addition, the DNA testing for CVM was developed in 2001 (Agerholm *et al.* 2001). This means that semen from some CVM-carrier bulls have been used-before 2001 without being identified (Boujenane *et al.* 2009).

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