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Sherly Ignetious
M.V.Sc. Scholar, Department of Animal Genetics & Breeding, College of Veterinary Science and Animal Husbandry, Mhow, Indore, Madhya Pradesh, India

Shrikant Joshi
Professor, Department of Animal Genetics & Breeding, College of Veterinary Science and Animal Husbandry, Mhow, Indore, Madhya Pradesh, India

Ranjit Aich
Assistant Professor, Department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry, Mhow, Indore, Madhya Pradesh, India

Shefali Macwan
Research Associate, Department of Animal Genetics and Breeding, Anand Agricultural University, College of Veterinary Science and Animal Husbandry, Anand, Gujarat, India

Corresponding Author:
Ranjit Aich
Assistant Professor, Department of Veterinary Biochemistry, College of Veterinary Science and Animal Husbandry, Mhow, Indore, Madhya Pradesh, India

Genetic studies on bovine leukocyte adhesion deficiency in Holstein Friesian crossbred cattle

Sherly Ignetious, Shrikant Joshi, Ranjit Aich and Shefali Macwan

Abstract
Bovine Leukocyte Adhesion Deficiency (BLAD) is a lethal autosomal recessive disorder and known to affect Holstein cattle breed throughout the world. In order to determine the area of mutation in CD 18 gene for BLAD, the PCR products of 367 bp were digested with *Taq I* endonuclease enzyme. After digestion of the PCR products, the normal BLAD allele in unaffected cattle produced two fragments of 313 bp and 54 bp. BLAD carriers exhibited three fragments of 367 bp, 313 bp and 54 bp; and the DNA of control affected cattle obtained from Department of Animal Genetics and Breeding, College of Veterinary Science and A.H., Anand Agricultural University exhibited only one fragment of 367 bp. The present study revealed two out of fifty Holstein cattle crosses were BLAD carriers. The DNA of the carriers of BLAD was sequenced by using ABI PRISM 310 Genetic Analyzer. Sequence obtained was deposited in NCBI (Accession number PBM17.sqn PBM17 KT032069). The genotype frequency of BLAD carriers was found to be 0.04. The gene frequency of dominant allele was estimated to 0.98 and that of recessive allele to 0.02. Out of fifty animals screened, 4% of animals were found to be heterozygous for BLAD.

Keywords: BLAD, CD18 gene, mutation, inherited disorder, Holstein Friesian

Introduction
Holstein Friesian (HF) breed of cattle is being widely used for genetic improvement since decades as the crossbred technology in the Indian dairy industry is augmented with the viability by increase in milk production per animal. Bovine leukocyte adhesion deficiency (BLAD) is a lethal autosomal recessive disorder and known to affect Holstein cattle breed throughout the world (Shuster *et al*., 1992) [22]. BLAD was first identified in Holstein-Friesian cattle at the beginning of the eighties and no study has reported the occurrence and etiology of the disease in other breeds (Kumar and Chakarvarty, 2015) [8]. The disease is result of single point mutation i.e. Adenine to Guanine (A→G) of nucleotide 383 in integrin β2 (CD18) gene located on the first chromosome of bovine, forming defective β chain of integrin receptors involved in interaction with other cell and extracellular matrix. Defective glycoprotein receptors lead to less or lack of migration of leukocytes at the infection site and it is associated with persistent marked neutrophilia in the affected homozygote calves. Due to immature immune mechanism, newly born calves are the most affected. Affected homozygous cattle die at an early age due to immature immunity and recurrent bacterial infection (Debnath *et al.*, 2016) [4]. There were identification records for BLAD (Meydan *et al.*, 2010; Adamov *et al.*, 2014; Khade *et al*., 2014; Kaya *et al.*, 2016) [11, 2, 7, 6] as inherited bovine disorder and non incidence of BLAD carrier (Abraham *et al*., 2019) in Holstein Friesian in different regions of the world but there was insufficient information of BLAD determination and its incidence in Madhya Pradesh, India. Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) is the most reliable and inexpensive method for identification of BLAD. The investigation was therefore, undertaken with the objectives to standardize PCR-RFLP technique for the detection of BLAD in Holstein Friesian crossbred cattle, to find out the incidence of BLAD and to determine gene and genotype frequency.

Materials and Methods
The experiment was designed to screen 25 males and 25 females Holstein Friesian crossbred cattle from Bull Mother Farm, Bhadbhada, Bhopal; Livestock unit, College of Veterinary Science and A.H., Mhow and nearby villages. Blood samples were collected from jugular veins in 2 ml capacity vaccutainers (K_2 EDTA).

The collected blood samples were brought to the laboratory on ice with proper care and stored at 4 ºC until further procedure. DNA was extracted from collected blood samples by John's method (John *et al.*, 1991) [5] with some modifications and whole blood extraction kit (Merck Genei, India; Catalogue number: 612102300011730). The required solutions were prepared in the laboratory for DNA extraction from blood samples using molecular (Sigma Aldrich, USA) grade chemicals. For isolation of DNA 500 µl and 200 µl of blood was used for manual and kit method respectively. DNA was eluted in 100 µl of 0.3 X TE buffer and 100µl of elution buffer for manual method and kit respectively. The quality of DNA was checked and quantization was done by double beam Spectrophotometer and thermo scientific NanoDrop 1000 and 2% agarose gel electrophoresis. The DNA was stored at -20 ºC.

The PCR was used to amplify one region of CD18 gene for BLAD for each sample as described by Meydan *et al.*, 2010 [11].

Table 1: The sequence of oligonucleotide primers employed in PCR assay

Gene	Genetic disorder	Primers (5' to 3')	RE	Reference
CD18	BLAD	F: 5′-GAATAGGCATCCTGCATCATATCCACCA-3′ R: 5′-CTTGGGGTTTCAGGGGAAGATGGAGTAG-3′	TaqI	Meydan *et al.*, 2010 [11]

Amplification reactions of the DNA samples extracted from the blood were prepared in a final volume of 25 µl in PCR tube. The PCR components was used were: 12.5 µl of 2X PCR Master Mix with final concentration of 1X (Merck Genei cat no. 6106022 00031730), 7.5 µl of de-ionized Water, 1.0 µl of Forward Primer (10 pmoles), 1.0 µl of Reverse Primer (10 pmoles) and 3.0 µl of Genomic DNA (30 ng/µl). A round of PCR in a final volume of 25 µl was carried out in a PCR thermal cycler (Applied Biosystems, USA). The cycling conditions were as: initial denaturation at 94 ºC for 3 minutes; 35 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds and extension at 72 ºC for 30 seconds; and final extension at 72 ºC for 5 minutes. The PCR amplification was determined by 2% agarose gel electrophoresis. The gel was visualized under gel documentation system (Bio-Rad, USA) for gene amplification. The restriction digestion of 367 bp with restriction enzymes *Taq I* (ILS, Gurgaon; Cat#R0149S) was used for BLAD. PCR products of the amplified gene (5 µl) were digested separately with restriction enzymes (1 µl) with the manufacturer's 10 X restriction buffer (3 µl) with water (21 µl) in the final reaction volume of 30 µl. The reaction mixture was centrifuged for few seconds for uniform mixing and then incubated at 65 ºC for 1 hr temperature specific for the given restriction enzyme. The polymorphism was checked by 3% agarose gel electrophoresis. The DNA sequencing of carrier was performed using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). Sequence obtained was verified by the sequence present in NCBI, USA. The gene frequencies, genotype frequencies and incidence were calculated by standard formulas.

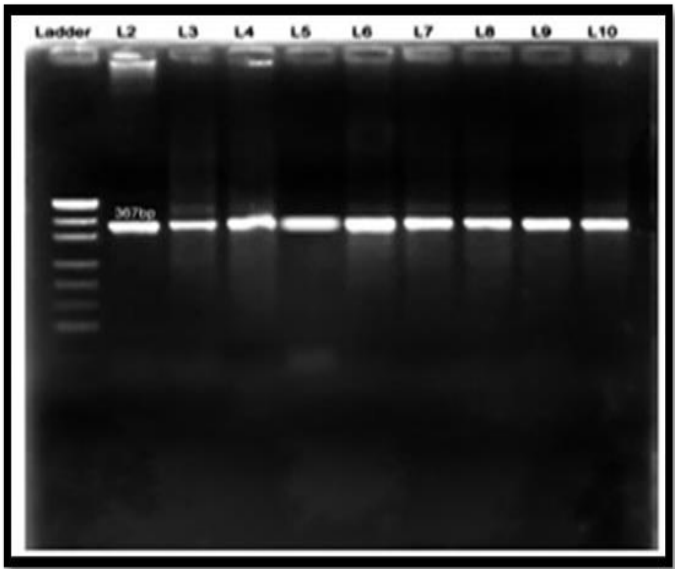

Fig 1: Amplified DNA fragment of 367 bp for BLAD

Results and Discussion

The DNA was isolated from the blood samples by manual method (John *et al.*, 1991) [5] and by using whole blood extraction kit. All the samples were found to be within acceptable purity ratio of 1.6-1.9. Previous studies have obtained satisfactory results with standard phenol-chloroform method (Patel *et al.*, 2006; Roy *et al.*, 2012) [18, 21]; Guanidium -Thiucianate Silica gel method (Rezaee *et al.*, 2008) [19]; salting-out method (Meydan *et al.*, 2010) [11]; using extraction kits (Oner *et al.*, 2010; Mahdipour *et al.*, 2010; Adamov *et al.*, 2014) [15, 10, 2]. The primers used in the present study successfully amplified the DNA fragments of 367 bp for BLAD in all the 50 animals. The size of amplification product was same for all the animals (Fig. 1).

The annealing temperature and time optimized in this study were 55 ºC for 30 seconds for 35 cycles and primer used was F: 5'GAATAGGCATCCTGCATCATATCCACCA 3' and R: 5' CTTGGGGTTTCAGGGGAAGATGGAGTAG 3'. This is accordance with the work of Meydan *et al.* (2010) [11]. PCR amplification of 367 bp fragments by the same set of primers as used in the present study was also reported by Padeeri *et al.* (1999) [16], Adamov *et al.* (2014) [2] and Khade *et al.* (2014) [7]. However, Patel *et al.* (2006) [18] amplified DNA fragment by adding a forward primer (5' AGG TCA GGC AGT TGC CTT CAA 3') and a reverse primer (5' GGG GAG CAC CGT CTT GTC CAC 3'). PCR-RFLP technique was applied to detect genetic variation in the amplified region of CD 18 gene. The restriction endonuclease *Taq I* was used to digest the PCR product in the present study as earlier used by Patel *et al.* (2006) [18], Meydan *et al.* (2010) [11], Khade *et al.* (2014) [7] and Abraham *et al.*, 2019 [1]. On the contrary, Adamov *et al.* (2014) [2] used two restriction endonucleases Taq I and Hae III. The amplified PCR product of 367 bp for the CD18 locus upon digestion with the *Taq I* restriction enzyme yielded two bands. Normal animals were found to have two bands of 313 bp and 54 bp whereas two carrier animals showed three bands each of 367 bp, 313 bp and 54 bp. None of the animals found homozygous for mutant CD18 gene (Fig. 2, Fig. 3).

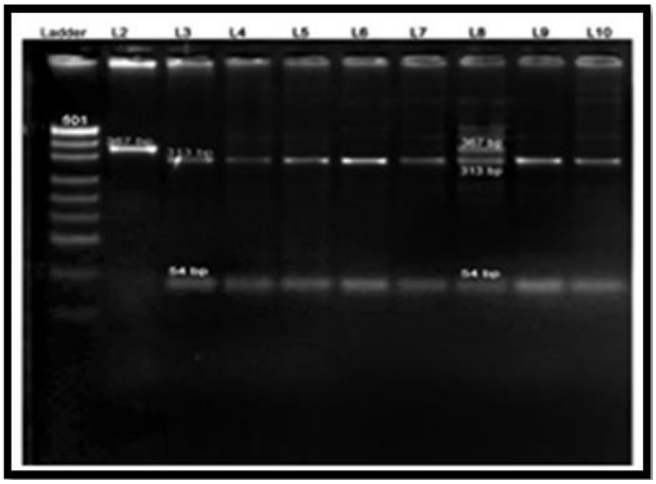

Fig 2: Agarose gel electrophoresis of *Taq I* digested products

Lane1: Molecular ladder (pUC19/ mspI: 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 34, 26.); Lane 2: Control; Lane 3, 4, 5, 6, 7, 9 and 10 shows two bands of 313 bp and 54 bp of homozygous normal animals; Lane 8 shows three band of 367 bp, 313 bp and 54 bp of heterozygote carrier animal

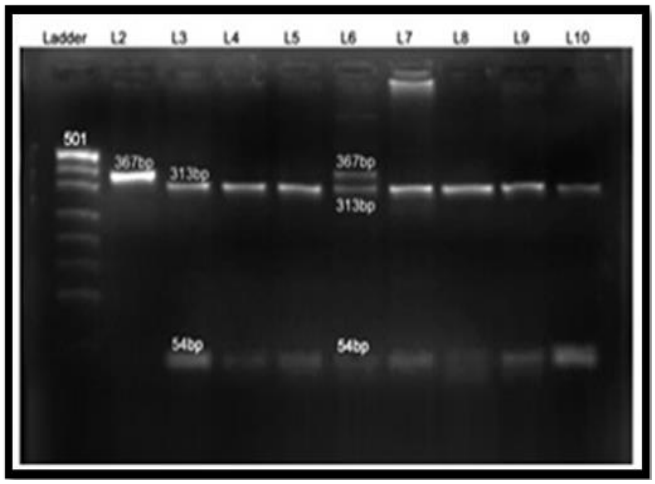

Fig 3: Agarose gel electrophoresis of *Taq I* digested product

Lane 1: Molecular ladder (pUC19/ mspI), Lane 2: correspond to control; Lane 6: BLAD carrier animal; and remaining lanes correspond to DNA from BLAD –free animals.

This is in agreement to the findings of Patel *et al.* (2006) [18] and Meydan *et al.* (2010) [11]. The genotype frequency of BLAD carrier animal, normal allele and defective recessive allele was estimated to 0.04, 0.98 and 0.02, respectively. Previously carriers of BLAD have been identified in India in Holstein cattle (Padeeri *et al.*, 1999; Patel *et al.*, 2007) [16, 17]. As far as the Indian animal population is concerned, it has become important for a routine screening of all HF and other crossbred animals to prevent spread of this disease.

BLAD has been reported to a frequency of 0.2% in USA (Shuster *et al*., 1992) [22]; 13.5% in Germany (Biochard *et al.*, 1995) [3]; 0.32% in Japan (Nagahata *et al.*, 1997) [13]; 5.7% in Brazil (Riberio *et al.*, 2000) [20]; 3.2% in Iran (Nassiry *et al.*, 2005) [14] and 1.33% (Muraleedharan *et al*., 1999) [12]; 3.23% (Patel *et al*., 2007) [17] and 21.82% (Kumar, 2009) [9] in India. The reason for this discrepancy is probably sampling of different populations i.e. cattle in different regions, although it may also be influences by the use of carrier sires over time.

The sequence of DNA of the carriers of BLAD obtained in the present study was registered in NCBI (PBM17.sqn PBM17 KT032069). The findings are in agreement with those reported by Meydan *et al*. (2010) [11].

Conclusion

The study demonstrates the prevalence of BLAD carriers among Holstein crosses in Madhya Pradesh, although at a low frequency. The carrier animals are suggested to be culled to avoid spread of this inherited disorder in the population. PCR-RFLP analysis was found to be a strong and reliable method for identification of BLAD. The study demonstrates a need for further examination of more Holstein cattle in Madhya Pradesh, preferably by testing the breeding sires to avoid unrecognized spread of this genetic disorder.

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