RESEARCH ARTICLE

Molecular Screening and Prevalence of Inherited Disorders in Indigenous and Crossbred Cattle in India - A Breed-Wise Study

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Abstract

This study was aimed to screen the autosomal recessive genetic disorders like Bovine Leukocyte Adhesion Deficiency (BLAD), Bovine Citrullinemia (BC), Deficiency of Uridine Monophosphate synthase (DUMPS), and Factor XI deficiency (FXI) in cattle that affect the reproductive health of the cattle population. In the present study, 500 animals of different breeds like Holstein Friesian (n=170), Jersey (n=75), Kankrej (n=98), Gir (n=100), and Sahiwal (n=57) were screened. Blood samples were collected from different states/areas of organized farms in India. DNA isolation was carried out by an automated DNA extraction method. The identification of a mutation in the gene was carried out using a set of published primers by conventional PCR. The PCR product was confirmed on 2% agarose gel. Confirmed PCR products were digested by restriction enzymes *Taq1* for BLAD, *AvaII* for BC and *Ava1* for DUMPS. Restriction digestion followed by 3% agarose gel electrophoresis to observe the band length, indicates a normal or carrier animal. The specific PCR method was used for FXI genotype identification. Synthetic genes were synthesized for all four genetic disorders and used as a positive control for the experiment. In our study, out of 500 cattle tested, only three HF crosses were found to be carriers for BLAD, and none of the animals were found to be mutants or carriers for BC, DUMPS, and FXI. The results show the prevalence of BLAD as 0.6% in the population and for BC, DUMPS, and FXI, it was 0%. The prevalence of BLAD carrier animals with heterozygous genotypes was 1.76 % in the HF breed. The genotype frequency of the BLAD carrier was found to be 0.006. BLAD carrier DNA was confirmed by using Sanger sequencing. Identification of genetic disorders in the cattle population and eliminating these animals from the breeding program will help in the selection of healthy animals with desirable traits, which leads to maintaining herd quality, reproductive soundness, and economic viability.

Key words: Cattle, Crossbred, Indigenous, PCR-RFLP, Recessive disorders, Synthetic gene.

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Introduction

Among all the countries in the world, India has the largest cattle population. However, India still has a lot of work to do to maximize milk output (Kumar and Chakravarty, 2015), especially when compared to exotic breeds. Indigenous cattle are well adapted to the local conditions; their milk yield is lower as compared to exotic breeds. To tackle this, various crossbreeding programs were initiated to enhance the qualitative and quantitative traits of cattle. The frozen semen from high-grade exotic breeding bulls was used to perform the cross-breeding program (Debnath *et al*., 2016). Holstein Friesian, Jersey, and *Bos indicus* crosses make up 72% of frozen semen doses used in cattle AI in India. Based on the amount of semen produced in India, Gir, Sahiwal, and Kankrej are the major breeds involved in crossbreeding programs among the *Bos indicus* breeds (Sudhakar *et al*., 2023). There have been reports that some exotic breeds carry genetic disorders. These genetic disorders are simple recessive traits with lethal mutations. The most commonly observed genetic disorders include BLAD, BC, DUMPS, and Factor XI deficiency (Kumar and Chakravarty, 2015: Debnath *et al*., 2016). Lethal mutation causes impaired reproductive performance in affected cattle, including reduced conception rate, an increase in abortion, and fetal death. These recessive lethal mutations were point mutations, which occur due to the addition of nucleotides insertion or removal of nucleotides deletion (Grupe *et al*., 1996; Ignetious *et al*., 2020).

BLAD (Bovine Leukocyte Adhesion Deficiency) is a hereditary disorder in cattle that usually affects Holstein-

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Friesian and crossbred cattle with recent reports in *Bos indicus* breeds, *i.e.,* Sahiwal and Kankrej (Sudhakar *et al*., 2023). This disorder arises due to the single-point mutations in the CD18 gene substitution of A-G leading to defective leukocyte migration and compromised immune response (Nagahata, 2004; Debnath *et al*., 2016). Bovine Citrullinemia (BC) is an autosomal recessive inherited disorder in cattle mainly reported in HF breeds caused by transition C-T in the gene argininosuccinate synthetase (ASS). The mutation leads to defective ASS enzyme which disturbs the urea cycle, resulting in accumulation of ammonia in the bloodstream. Affected calves at the time of birth usually look healthy, but within a few days, they start showing symptoms, which result in death if ignored (Kumar and Chakravarty, 2015).

Deficiency of Uridine Monophosphate Synthase (DUMPS) is another hereditary disorder inherited in an autosomal recessive manner caused by substitution of C-T at exon 5 of the Uridine monophosphate (UMP) synthase gene located on the bovine chromosome one (Meydan *et al.,* 2009). The mutation results in early embryonic lethality in homozygous animals, while heterozygous carriers do not exhibit any symptoms but can transfer the faulty gene to their progeny (Eydivandi and Samadi, 2011). Factor XI deficiency (FXI) is a genetic disorder associated with blood clotting factors (Meydan *et al.,* 2009). FXI is a plasma serine that helps in the formation of stable clots to stop excessive bleeding after injury (Marron *et al.,* 2004). The mutation is characterized by the insertion of a long chain of adenine (A) bases and a stop codon at exon 12 of the factor XI gene on chromosome 27. Affected animal by this deficit experiences improper blood clotting, anaemia, low calving and survival rate, and a higher risk of infectious diseases (Marron *et al.,* 2004; Meydan *et al.,* 2009).

According to the Sudhakar *et al.* (2023), the first time presence of BLAD and Citrullinemia mutant alleles has been identified in *Bos indicus* breeds, specifically Kankrej and Sahiwal. Research findings indicate that a mutation in *Bos indicus* breeds may have occurred due to introgression of mutant alleles of Holstein Friesian genes during past crossbreeding practices involving ancestral animals (Sudhakar *et al*., 2023). Artificial insemination has some advantages and also has some drawbacks as AI animals are carriers of hereditary mutant traits, so the use of such animals in breeding programs may lead to the transfer of genetic disorders to the next generation (Padeeri *et al.,* 1999). The present work was carried out to identify the prevalence of BLAD, BC, DUMPS, and FXI in crossbred and indigenous cattle in India with the use of the PCR_RFLP method.

Materials and Methods

Sample Collection

A total of 500 cattle samples were collected from different organized farms from Maharashtra, Gujarat, and Haryana for different indigenous and crossbred breeds. Samples were collected in EDTA vacutainers and transported to the laboratory by maintaining a cold chain and stored at 4 °C until genomic DNA extraction.

DNA Extraction and PCR Assay

Genomic DNA was isolated from a 200 µL whole blood sample by using Roche Magna Pure Automated DNA extraction system with DNA blood extraction protocol. DNA quantification was done by Nanodrop, and the quality of DNA was checked by agarose gel electrophoresis before proceeding with PCR. Details of the primers sequences used for PCR are provided in Table 1. PCR amplification reactions were performed in a final volume of 30 µL. Each reaction mixture contained 12.5 µL of 1XGoTaq Green PCR master mix (Promega), 1 µL of each primer (10 pmol of forward and reverse primers), 2 µL of genomic DNA (30-100 ng), and nuclease-free water to make up the final reaction volume. Amplification was done following the thermal cycling protocol an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, particular primer annealing temperature for 30 sec, and extension at 72°C for 30 sec. A final extension step was performed at 72°C for 5 min. PCR primers and restriction enzymes were used as per Table 2.

PCR-RFLP was used to identify genotypes based on digestion patterns for disorders BLAD, BC, and DUMPS, and the specific PCR method was used for FXI genotype identification. Amplified PCR product was verified by 2% agarose gel prepared in 1X TAE buffer. The PCR product was

Table 1: Primers used in present study

Genetic disorder	Primer Sequence	References
BLAD	F-GAATAGGCATCCTGCATCATATCCACCA	Meydan *et al.* (2010)
	R-CTTGGGGTTTCAGGGGAAGATGGAGTAG	
BC	F-GGCCAGGGACCGTGTTCATTGAGGACATC	Grupe *et al*. (1996)
	R-TTCCTGGGACCCCGTGAGACACATACTTG	
DUMPS	F-GCAAATGGCTGAAGAACATTCTG	Meydan *et al.* (2010)
	R-GCTTCTAACTGAACTCCTCGAGT	
Factor XI	F-CCCACTGGCTAGGAATCGTT	Marron *et al*. (2004)
	R-CAAGGCAATGTCATATCCAC	

then subjected to digestion with specific restriction enzymes by using a 3% agarose gel stained with ethidium bromide. The resolved PCR fragment pattern was visualized under a UV lamp and photographs were documented with a gel documentation system (Bio-Rad). The Carrier genotype was confirmed with Sanger sequencing, which was outsourced. The sequence data was analyzed by using Chromas software (2.6.6) for mutation analysis. The resulting sequences were edited with the BioEdit sequence editor tool (7.2.5) and compared with reference sequences using NCBI BLASTn. To find the effect of nucleotide change in amino acid sequence, translational analysis was done with ExPASy (Swiss Institute of Bioinformatics).

Synthetic Gene Preparation

Since we don't have existing positive controls, we designed synthetic genes for normal and mutant genes commercially. For this, sequences were retrieved from NCBI (National Centre for Biotechnology Information) with the following accession numbers: Y12672.1 (BLAD), JN039032.1 (DUMPS), FJ853494.1 (BC), and NC037354.1. By using in silico tools (BioEdit, Clustal W), modifications in sequences were done. The difference between nucleotide substitutions is highlighted in Figure 1 to 4. Modified sequences were synthesized and cloned into a pUCminusMCS vector and confirmed through sequencing by a third party (Eurofins). These recombinant constructs were validated in our lab by restriction digestion and used as a positive control in this study.

Table 2: Reaction setup, restriction enzymes and PCR-RFLP band pattern used for identification of mutation for genetic disorder

Genetic disorder	Annealing temp.	Product size(bp)	Restriction enzymes	Digestion Temp & time	Mutant Type	Wild Type	Heterozygous Carrier
BLAD	63°C	357bp	*Taq I*	*65 °C*, 4 h	357 bp	201 bp,156 bp	357 bp,201 bp, 156 bp
BC	56°C	198bp	*AvaII*	37 °C, 4 h	198 bp	109 bp, 89 bp	198bp, 109 bp, 89 bp
DUMPS	58°C	108bp	*AvaI*	37 °C, 4 h	89 bp, 19 bp	53 bp, 36 bp, 19 bp	89 bp, 53 bp, 36 bp, 19 bp
Factor XI	52°C	244bp			320 bp	244 bp	--

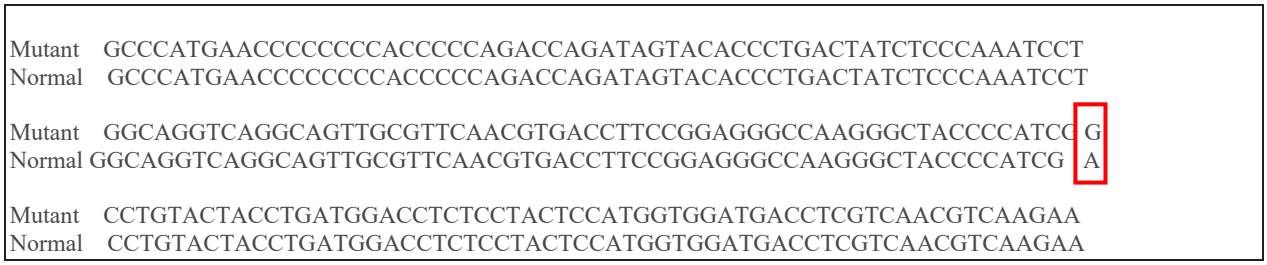

Fig. 1: Alignment of the reference BLAD gene sequence with the designed Mutant sequence. Restriction site for BLAD: *TaqI* (T'CG_A) Cuts at position 201. The modified nucleotides are enclosed in a box to indicate the changes.

Fig. 2: Alignment of the reference BC gene sequence with mutant sequence. Restriction site for BC Gene: *AvaII* (G'GwC_C). Cuts at position 88. The modified nucleotides are enclosed in a box to indicate the changes.

Fig. 3: Alignment of the reference DUMPS gene sequence with Mutant sequence Restriction site for DUMPS Gene: *AvaI* (C'yCGr_G). The modified nucleotides are enclosed in a box to indicate the changes.

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Normal   ACTCCTTAGGGTCAAGTCACCTAATGTGTTGCGTGTCTATAGCGGCATTTTGAATCAATC
Mutant   ACTCCTTAGGGTCAAGTCACCTAATGTGTTGCGTGTCTATAGCGGCATTTTGAATCAATC

Normal   AGAAATAAAAGAGGATACATCTTTCTTTGGGGTTCAAG
Mutant   AGAAATAAAAGAGGATACATCTTTCTTTGGGGTTCAAGAAATAATAATTCAATAAAAAAA

Normal                                                          AAATAA
Mutant   AAAAAAAAAAAAAAAAAAAAATAAAGAAAAAAAAAAAAAAAAAAAAAAAAAAGGAAATAA

Normal   TAATTCATGATCAATATGAAAAGGCAGAAAGTGGATATGACATTGCCTTGTTGAAACTAG
Mutant   TAATTCATGATCAATATGAAAAGGCAGAAAGTGGATATGACATTGCCTTGTTGAAACTAG
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Fig. 4: Alignment of the reference FXI gene normal and mutant sequences. Box indicates the stop codon and further 76 bp insertion fragment

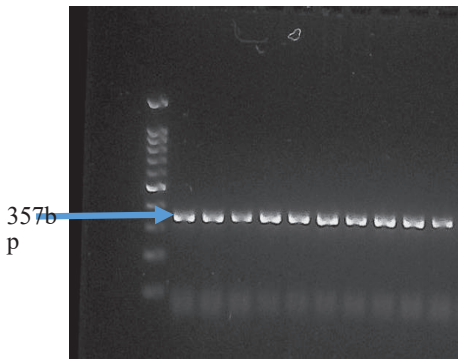

Fig. 5: PCR amplified product of BLAD specific gene on 2% agarose gel electrophoresis M=100 bp ladder; Lanes 1-10=PCR product of BLAD gene (357 bp)

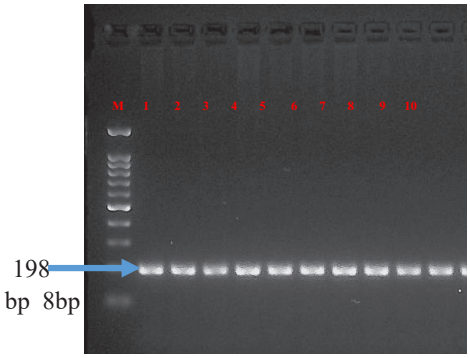

Fig. 6: PCR amplified product of BC specific gene on 2% agarose gel electrophoresis M=100 bp ladder; Lanes 1-10=PCR product of BC gene (198 bp)

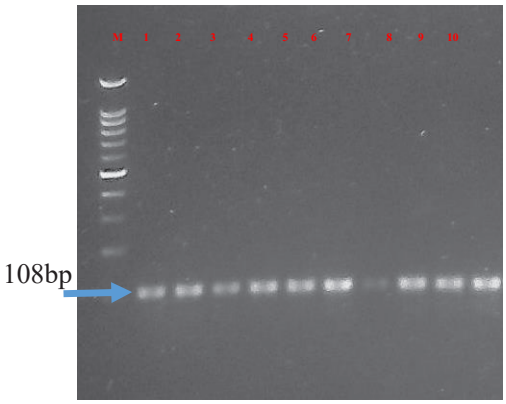

Fig. 7: PCR amplified product of DUMPS specific gene on 2% agarose gel electrophoresis M=100 bp ladder; Lanes 1-10=PCR product of DUMPS gene (108 bp)

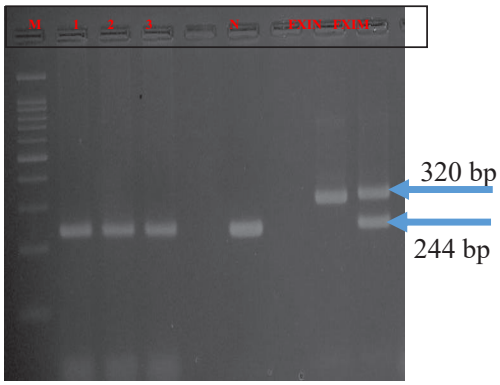

Fig. 8: PCR amplified product of FXI specific gene on 2% agarose gel electrophoresis; M=100 bp ladder; Lanes 1 to 3 = PCR product of FXI gene (244 bp); N=Negative control; FXIN=Normal wild type control; FXIM=Mutant control (320 bp)

Results and Discussion

In the present study, the prevalence of genetic disorders BLAD, BC, DUMPS, and FXI was studied in indigenous and crossbred cattle using gene-specific primers. The amplicon sizes of the PCR products were observed at 357 bp, 198 bp, 108 bp, and 244 bp, as shown in Figure 5 to 8 for BLAD, BC, DUMPS, and FXI, respectively.

The PCR product for the BLAD gene was digested with the *Taqa1* enzyme. After digestion, two band patterns were observed at 201 bp and 156 bp for the wild type, and three bands were observed at 357 bp, 201 bp, and 156 bp for heterozygous carrier animals. A single band was observed at 357 bp for the homozygous mutant type on 3% agarose gel electrophoresis (Fig. 9). Among the animals tested, three HF cattle were identified as heterozygous carriers for the BLAD disorder, and the remaining animals were homozygous normal (Fig. 9). The genotype for carrier animals was confirmed with Sanger sequencing. The sequencing analysis of the chromatogram showed distinct overlapping peaks at the mutation site confirming heterozygosity (Fig. 12-14). The prevalence of BLAD carrier

animals with heterozygous genotypes was 1.76 % only in the HF breed. The PCR product for the BC gene was digested using the restriction enzyme *AvaII,* it showed 109 bp and 89 bp fragments for the wild genotype in all the samples and no banding pattern was observed corresponding to 198 bp,109 bp, and 89 bp for carrier animals (Fig. 10). DUMPS-specific PCR product digested with *AvaI* enzyme exhibited normal three bands in all the samples at 53 bp, 36 bp, and 19 bp, no affected animals were identified in the population which would have exhibited only two bands at 89 bp, and 19 bp (Fig. 11). The PCR amplified for the FXI gene produced a single 244 bp fragment in all tested animals, indicating the presence of the wild-type allele. A mutant allele associated with 320 bp was not observed in any of the samples studied (Fig. 8).

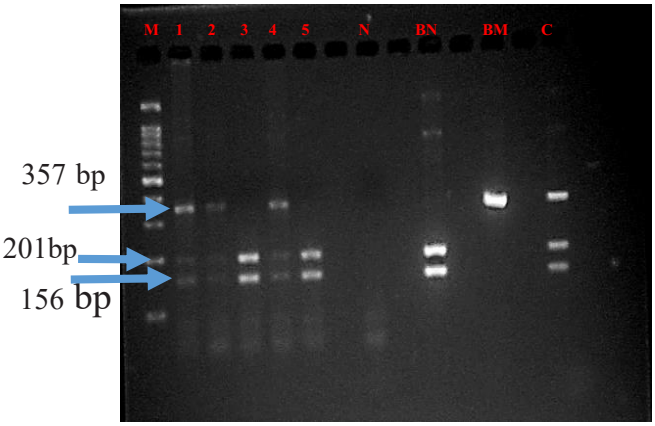

Fig. 9: Illustration of PCR -RFLP for BLAD in cattle on 3% agarose gel electrophoresis.M- 100 bp ladder; Lane 3 and 5 Taqα1 RE digested products shows two band pattern (201 bp and 156 bp Normal wild genotype)] and Lane 1, 2 and 4 suspected samples for BLAD shows three band pattern (201 bp, 156 bp,357 bp) for carrier genotype N=Negative; BN=Normal wild type control; BM=Mutant control; C=Carrier control

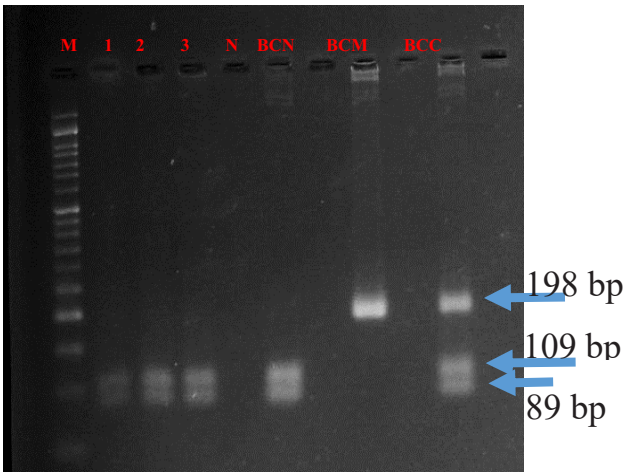

Fig. 10: Illustration of PCR-RFLP for BC in cattle on 3% agarose gel electrophoresis. Lane M- 100 bp ladder; Lane 1 to 3 *AvaII* RE digested products shows two band pattern (109 bp and 89 bp Normal wild genotype) N=Negative contol; BCN=Normal wild type control; BM=Mutant control(198bp); Lane BCC shows Positive carrier control (198 bp, 109 bp,89 bp)

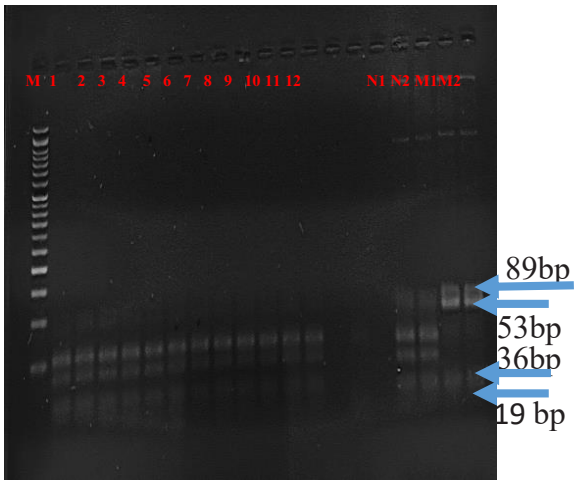

Fig. 11: Resolution of AvaI digested fragments of DUMPS specific PCR products on 4% agarose gel electrophoresis M=50 bp ladder; Lanes 1-12=*AvaI* digested fragments for samples (53 bp, 36 bp and 19 bp); N= Negative control; N1,N2=Normal Wild type control (53 bp, 36 bp and 19 bp) M1,M2=Mutant control (89 bp, 19 bp)

We initially focused on farms where controlled breeding practices were followed and high-risk breeding populations like Holstein Friesian were present. The study was further expanded to other cattle populations, including indigenous and crossbreds.

This paper reveals the presence of heterozygous carriers for the BLAD mutation in HF. The identified BLAD carrier frequency in the HF population was 1.76%, while no animals exhibited mutations for BC, DUMPS, and FXI. The finding for BLAD carrier frequency was higher than previously reported frequencies in India, 1.33% (Padeeri *et al.*, 1999) and 0.04% (Meydan *et al.* 2010); however, it was lower than the reported frequency of 3.23% (Patel *et al.*, 2007). It is important to know that we found zero cases of these disorders in the Indigenous cattle population, which differs from previous studies by Sudhakar *et al.* (2023), which showed the introduction of mutant genes into indigenous cattle populations through AI. The absence of genetic disorders may be due to differences in the indigenous cattle populations tested across studies and variations in AI practices or genetic resilience in the breeds. The presence of BLAD carriers in the HF population requires careful breeding management to prevent the spread of deleterious gene within the cattle population. Genetic disorder testing and informed breeding decisions help to minimize the risk of BLAD affected offspring. The positive findings of this study are the absence of BC, DUMPS, and FXI mutations suggest that current breeding strategies may be effective in eliminating these mutations.

Additionally, the use of synthetic genes as positive controls, which we have designed in our study, significantly enhanced the reliability of genetic screening methods by providing a reference for comparison of positive and unknown samples, which helps to avoid false-positive results interpretation.

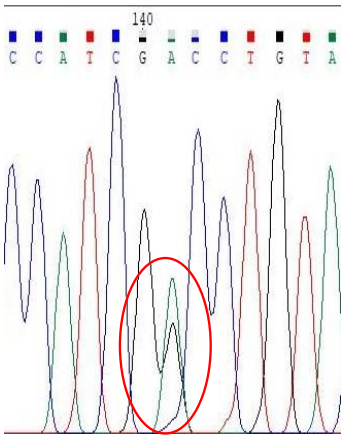

Fig.12

Fig.13

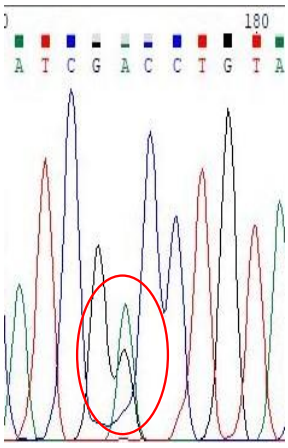

Fig. 14

Fig.12, 13 & 14: chromatograph analysis showed the presence of a double peak for Adenine (Green peak) and Guanine (Black peak) indicating carrier mutation site. Oval highlights the mutation.

Conclusion

This study confirms the genetic status of cattle populations and shows BLAD prevalence in the studied population as 0.6% with 0.006% genotype frequency, whereas heterozygous genotype frequency of 1.76% in the HF breed, confirming the absence of other genetic disorders BC, DUMPS, and FXI in crossbreds and indigenous populations. These findings emphasize the necessity of comprehensive genetic screening. Additionally, the genetic integrity of indigenous breeds indicates the need for targeted selective breeding strategies to maintain a disease-free population. The incorporation of synthetic genes in our study helps to enhance the accuracy and ensure reliable detection of mutant and carrier animals and minimize false positive results. Considering these results, future research should explore the genetic background and breeding history of carrier animals through pedigree analysis, which helps to understand the transmission dynamics of these genetic disorders.

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