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Genotyping bovine leukocyte adhesion deficiency and bovine Citrullinemia in Sahiwal and Holstein cross bred animals of Durg district of Chhattisgarh state

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Abstract
The present study was carried out at College of Veterinary Science and A.H., Anjora, Durg (C.G.) to screen the Sahiwal animals and HF cross bred animals for BLAD and Bovine Citrullinemia genetic disorders by using PCR-RFLP method. The genomic DNA obtained from blood samples of 50 Sahiwal animals maintained at Bull Mother Experimental Farm, Dairy Unit, College of Veterinary Science and A.H, Anjora, Durg and 100 Holstein Friesian crossbred animals maintained at, ABIS farm, Durg were screened in the present study. An allele specific PCR-RFLP technique was applied to detect mutation in specific DNA target sequences and was standardized for Sahiwal and HF crossbred animals. The amplified products were purified and sequenced to confirm the change in nucleotide in the selected regions. None of the Sahiwal animals and HF crossbred animals was found to be carriers of BLAD and BC gene. Thus the gene frequency of normal allele is 1 and that of BLAD/BC gene is 0. The new methods of molecular genetics enable us to find the cause at gene level. Screening of all the animals in an organized farm, the breeding bulls and the prospective bulls is very important to check the spread of genetic defect genes in the population.

Keywords: BLAD, bovine Citrullimenia, Chhattisgarh, genetic disorders, Sahiwal, HF

Introduction
A genetic disease is an illness caused by congenital abnormalities in chromosome or gene. Genetic disease may be inherited from parents. Alternatively, it may be caused by de novo mutations or new alterations in offspring genome. (Gebreselassie *et al.*, 2019)[1]. BLAD is a lethal, autosomal recessive genetic disease and is caused by the point mutation (A to G) at the position 383 of CD18 gene located on the first chromosome of bovine. Bovine Citrullinemia is an inborn error of metabolism due to deficiency of the enzyme *Arginine succinate Synthetase* which is critical for urea cycle. (TGA/stop codon) at codon 86 of the gene ASS (*Arginine succinate Synthetase*) located in the 11th chromosome of bovine coding for *Arginine succinate Synthetase*, leading to impaired urea cycle.

As a result of crossbreeding some genetic disorders have been introduced in Indian herd by the in-discriminate use of foreign breeds. In animal breeding, genetic disorders are one of the most important issues for breeders. A defect in a single gene in bulls can lead number of offsprings to die permanently or suffer life-long deformities and they can significantly influence the economy of animal husbandry (Cakmak and Yardibi, 2019) [2]. However, the new methods of molecular genetics enable us to find the cause at gene level. Screening of all the animals in an organised farm, the breeding bulls and the prospective bulls is very important to check the spread of genetic defect genes in the population. In view of the above consideration the present study has been designed to screen the Sahiwal animals of Bull Mother Experimental Farm dairy unit and Holstein Friesian/Holstein Friesian cross bred animals for BLAD and Bovine Citrullinemia genetic defect and to determine the gene and genotype frequency of BLAD and Bovine Citrullinemia.

Material s and methods
The screening study was carried out at College of Veterinary Science and A.H., Durg, Chhattisgarh. All procedures, in animal experiments, were approved by the Institutional

Animal Ethics Committee. The blood samples from 50 pure Sahiwal animals and 100 Holstein Friesian crossbred animals maintained at Bull Mother Experimental Farm, Dairy Unit, College of Veterinary Science and A.H, Anjora, Durg and ABIS Dairy Farm, Rajnandgaon respectively were collected for this study.

Isolation of DNA and Amplification of BLAD and BC gene: Genomic DNA was extracted by DNA extraction kit as per manufacturer instructions. HiPurA Blood Genomic DNA Miniprep Purification Kit by Hi-Media company was used for DNA isolation. Primer pair (forward and reverse) used to amplify the target regions of BLAD (Bovine CD 18 Chromosome 1 nucleotide 383) and Bovine Citrullimemia gene (ASS gene Codon 86 chromosome 11) is given in Table 1.The primers designed were inspected for their properties using oligoanalyzer software. The PCR reaction components used for each reaction volume are given in Table 2.

Table 1: Primer sequences, restriction enzymes and allele sizes of CD18 gene and ASS genes

Gene	Primer sequence	PCR product size (bp)	Restriction enzyme	Reference
BLAD-F BLAD- R	AGGTCAGGCAGTTGCCTTCAA GGGGAGCACCGTCTTGTCCAC	367 bp	Taq I	Yatish *et al* (2010) [3]
ASS1 FP ASS1 RP	GTGTTCATTGAGGACATC CCGTGAGACACATACTTG	198 bp	Ava II	Meydan *et al*. (2010) [4]

Table 2: PCR reaction components used for each reaction volume

PCR component	Volume	Concentration
Master Mix	5µl	5X
Reverse primer	1µl	10pmole/µl
Forward primer	1µl	10pmole/µl
Nuclease free water	13µl	_
Template DNA	5µl	50ng
Total	25 µl	_

The PCR cycling parameters were optimized separately on PCR machine for detecting allele specific amplifications of two genes under the present study as described below:

Table 3: Standardization of PCR reaction for BLAD and BC

Steps	Process	Temperature and Time	
		Bovine Citrullinamia	Bovine Leukocyte Adhesion Deficiency
1	Initiation	95 ^{0}C, 3 minutes	95 ^{0}C, 5 minutes
2	Denaturation	95 ^{0}C, 30 seconds	95 ^{0}C, 45 seconds
3	Annealing	53 ^{0}C, 30 seconds	56 ^{0}C, 45 seconds
4	Extension	72 ^{0}C, 30 seconds	72 ^{0}C, 1 minute
Repeat steps 2 to 4 for 35 cycles			
5.	Final Extension	72 ^{0}C, 5 minutes	72 ^{0}C, 10 minutes
6.	Holding	4 ^{0}C	4 ^{0}C

The amplified PCR products band pattern was electrophoresed on 2.0% per cent agarose gel. The reaction mixture for restriction digestion of PCR product is given in Table 4. The PCR products were digested with the following fast digest restriction endonuclease. *Taq I* for BLAD and *Ava II* for BC. The PCR products for BLAD and BC were digested with *Taq I Restriction Enzyme* and *Ava II Restriction Enzyme* respectively at 65^{0}C in dry water bath but the RE process was standardized with time duration of 3.5 hours for BLAD and 3 hours for BC. The restriction digestion band pattern was electrophoresed on 2.5% per cent agarose gel.

Table 4: Reaction Mixture for Restriction Digestion of PCR Product

Component	Stock	Volume/reaction	Conc. /Reaction
PCR Product		10.0 µl	
Digestion buffer	10 X	2.0µl	1 X
Restriction endonuclease	10U/µl	0.5 µl	5U/Reaction
Milli Q water		7.5 µl	
Total		20µl	

The PCR products were amplified from genomic DNA using outer forward primer and outer reverse primer of each locus. After purification of PCR product of genes namely CD18 and ASS were sequenced for confirmation purpose of the change of the nucleotide. The purified PCR products were sequenced by Eurofins Company, Bangalore.

Statistical analysis: The gene and genotype frequencies and Hardy-Weinberg equilibrium were tested by Chi-square method by standard procedure given by Snedecor and Cochran (1994) [5] using SPSS statistical software.

Results and Discussion

In the present study, two different sets of primers were used for CD18 and ASS genes and PCR reactions were standardized for each gene for Sahiwal and HF crossbred animals. The normal or unaffected allele for BLAD exhibits two fragments of 313 bp and 54 bp whereas the carriers demonstrate three fragments of 367 bp, 313bp and 54 bp fragments. The normal BC allele consists of two fragments in healthy cows 109 bp and 89 bp where as cattle of BC carrier exhibits three fragments of 198 bp, 109 bp and 89 bp fragments.

The PCR products of BLAD gene visualized on 2% per cent agarose gel revealed a fragment of 367 bp (Fig 1). The PCR products of 367 bp digested with *TaqI Restriction Enzyme* showed that all the animals were normal i.e. unaffected (homozygous wild type) The PCR products of BC gene visualized on 2% per cent agarose gel revealed a fragment of 198 bp (Fig 2). The PCR products of 198 bp digested with *Ava II Restriction Enzyme*, all the animals produced two fragments of showed that all the animals were normal i.e. unaffected (homozygous wild type). The analysis for BLAD and BC revealed that all the 150 animals genotyped were identified as normal homozygous.

The allele specific PCR genotyping results were confirmed by amplifying and sequencing the corresponding mutation containing region (Fig 3 and 4). No carrier animals of BLAD and BC were noted in the present study. The frequency of BLAD and BC carriers estimated in the present study was zero per cent and the frequency of normal animal was found to be 100%.Thus the gene frequency of normal allele is 1 and that of BLAD gene is 0.

The allele specific PCR genotyping results were confirmed by amplifying and sequencing the corresponding mutation containing region. No carrier animals of BLAD and BC were noted in the present study and the herd under study is BLAD and BC free. The frequency of BLAD and BC carriers estimated in the present study was zero per cent and the frequency of normal animal was found to be 100%.Thus the gene frequency of normal allele is 1 and that of CD 18 and ASS gene is 0.

Like the findings of present results the no carriers animals for BLAD disorders in the different herds of cattle in India and abroad were also reported by Patel *et al.* (2006) [6], Valdes *et al.* (2009) [7], Oner *et al.* (2010) [8], Adamov *et al.* (2013) [9], Khade *et al.* (2014) [10], Debnath *et al.* (2016) [11], Hemati *et al.* (2015) [12] , Mondal *et al.* (2016) [13] , Dagong *et al.* (2018) [14], and Koshchaev *et al.* (2018) [15]. In contrary, the BLAD carriers were reported in different herds as 0.01% in Holstein by Cakmak and Yardibi (2019) [2], 3.64% in Karan fries by Yathish *et al.* (2010) [3], 0.02% in Holstein by Meydan *et al.* (2010) [4],0.0084% in Holstein cows by Akyuz and Etrugul (2006) [16], 18.9% in Simmental cows by Citez *et al.* (2006) [17], 0.8% by Czarnik *et al.* (2007) [18], 0.3% in Holstein cows by Schutz *et al.* (2008) [19], 0.01% in Holstein cows by Nasreen *et al.* (2009) [20], 0.49% in Chinese Holstein by Jianbin *et al.* (2006) [21], 0.49% in Chinese Holstein by Li *et al.* (2011) [22], 1.38% in Chinese dairy cattle by Sun *et al.* (2011) [23], 0.04% in Holstein by Patel *et al.* (2012) [24], 2% in Holstein by Sahin *et al.* (2013) [25], 2% in Holstein by Agaoglu *et al.* (2015) [26], 4.58% in Holstein cows by Akyuz *et al.* (2015) [27] , 0.01% in Holstein by Kaya *et al.* (2016) [28], 0.005% in Holando cattle by Sica *et al.* (2016) [29] and 2.2% in Holstein by Avanus and Altinel (2017) [30]

Similar to the findings of present study no carriers animals for BC disorders in the different herds of cattle in India and abroad were also reported by Meydan *et al.* (2010) [4], Patel *et al.* (2006) [6], Oner *et al.* (2010) [8], Debnath *et al.* (2016) [11], Hemati *et al.* (2015) [12], Koshchaev *et al.* (2018) [15] , Sahin *et al.* (2013) [25], Agaoglu *et al.* (2015) [26], Kaya *et al.* (2016) [28], Sica *et al.* (2016) [29], Avanus and Altinel (2017) [30], Ignetious *et al.* (2017) [31] and. In contrary to the results of our findings the BC carriers in different herds were reported by different workers as 2.3% in Holstein cows by Schutz *et al.* (2008) [19], 0.16% in Chinese Holstein by Jianbin *et al.* (2006) [21], 0.16% in Chinese Holstein by Li *et al.* (2011) [22], 7.72% in Chinese dairy cattle by Sun *et al.* (2011) [23].

In India, Holstein and its crossbred carriers of BLAD and BC were reported earlier in the studied population but in the present study these diseases were not found. The reason might be the small sample size. The farms where the study was carried out are well organized and has well equipped facilities to screen the breeding bulls for different diseases before service. Hence, the bulls would be healthy and free from these genetic diseases so that their offspring are also free from these genetic disorders. From the results it is recommended that for controlling BLAD and BC screening works on the disorders should continue further encompassing as many animals as possible.

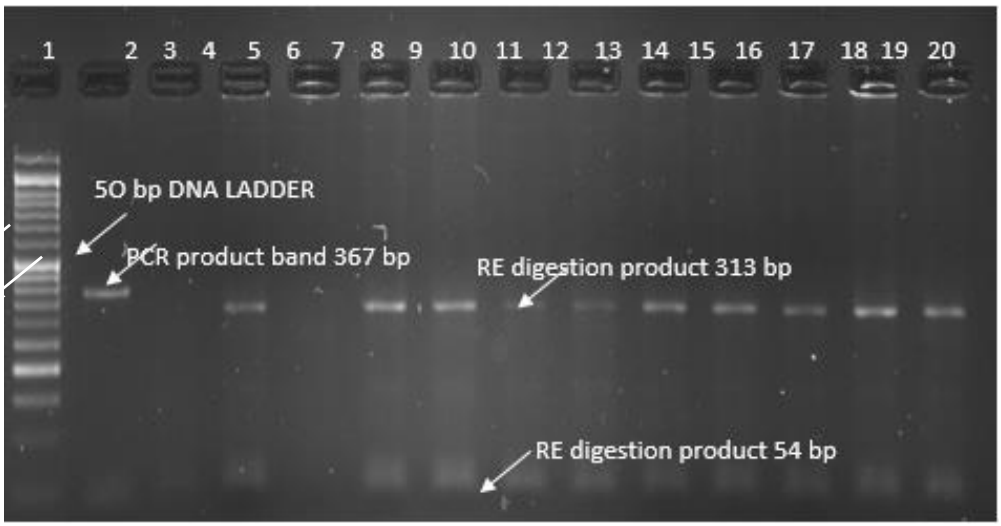

Fig 1: RFLP of CD-18 gene (BLAD) fragment using *Taq-I Restriction Enzyme*

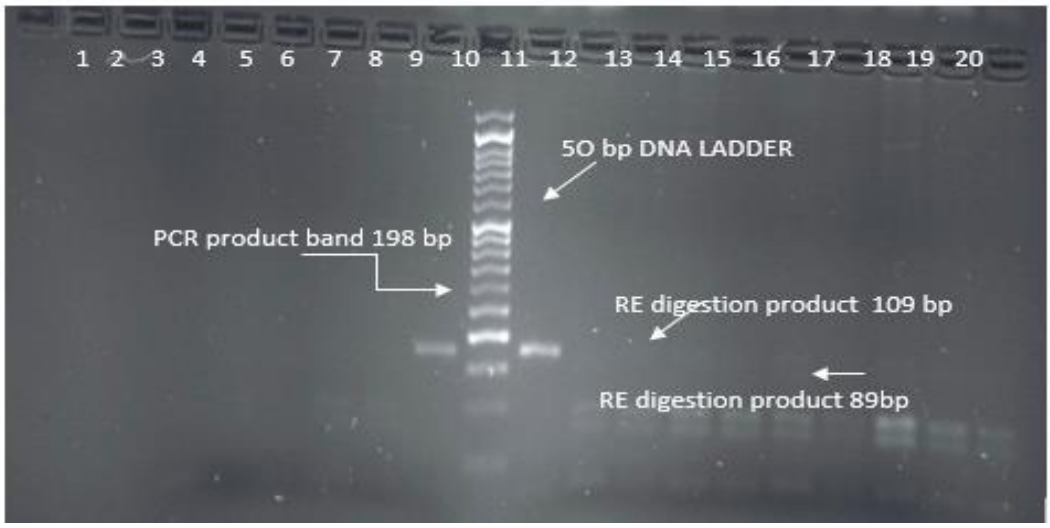

Fig 2: RFLP of ASS gene (BC) fragment using *Ava-II Restriction Enzyme*

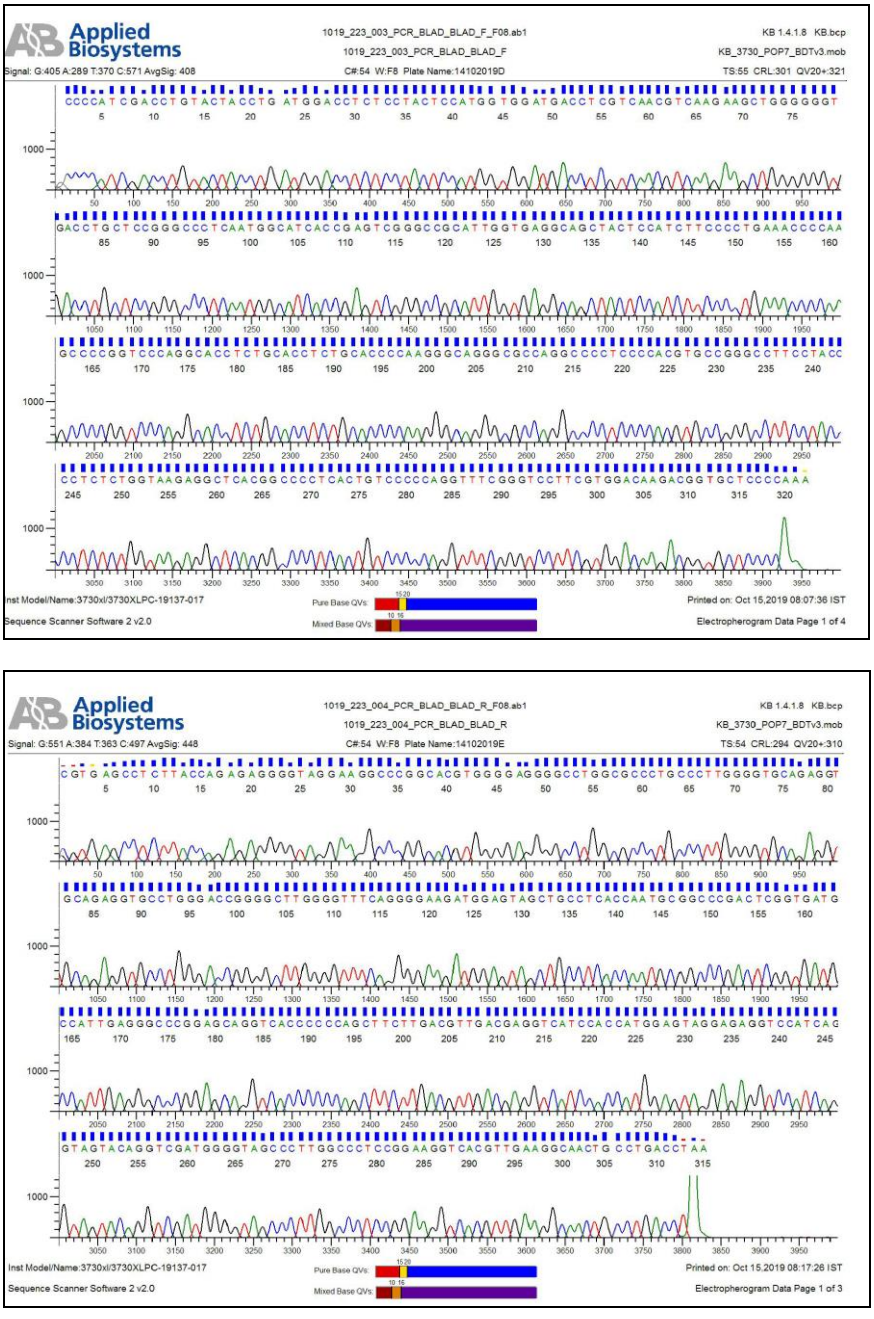

Fig 3: Sequencing of 367 bp for Normal sample from CD18 gene for BLAD. (Forward and Reverse). The present chromatogram data shows a normal nucleotide that is "A"

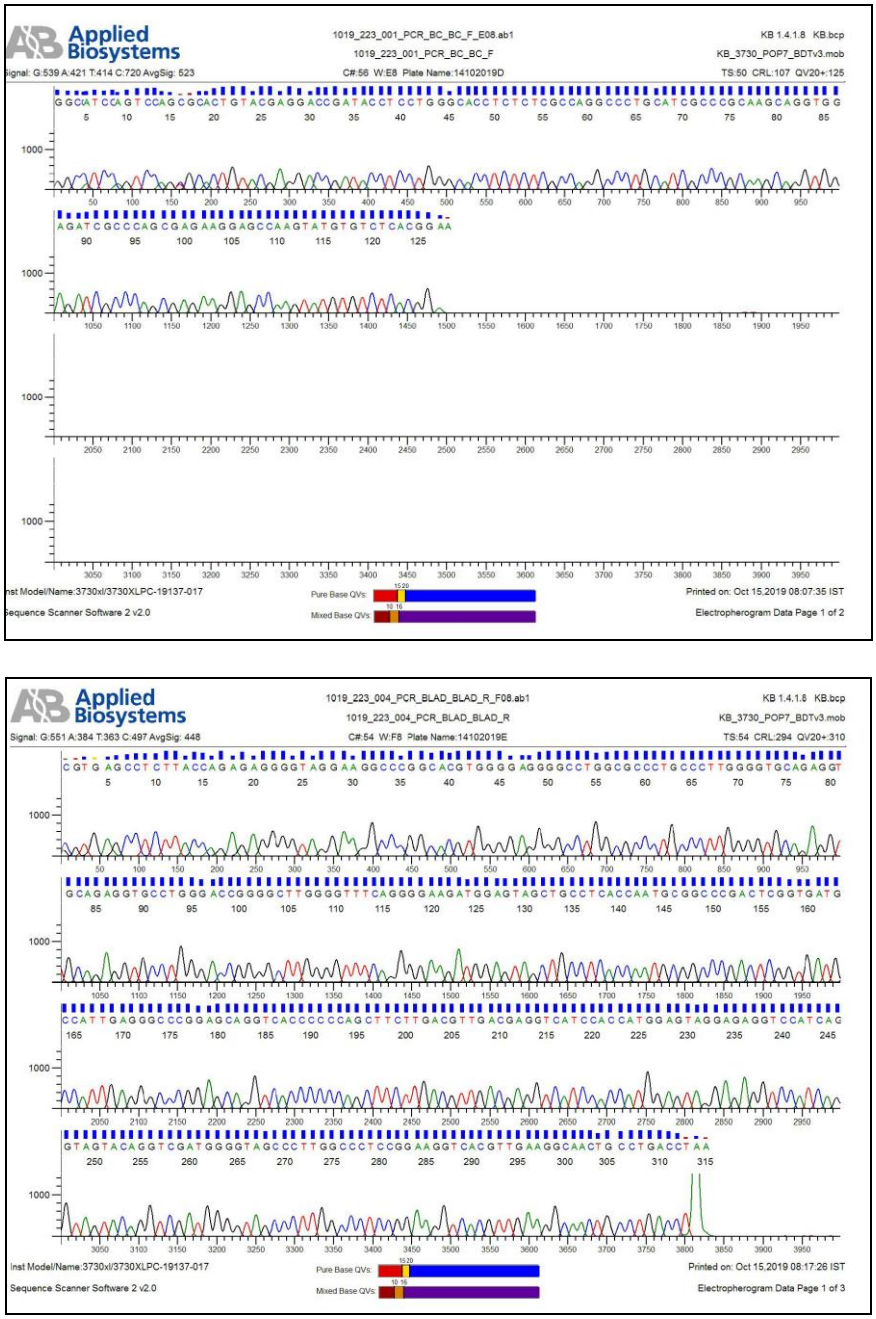

Fig 4: Sequencing of 198 bp for Normal sample from ASS gene for BC. (Forward and Reverse). The present chromatogram data shows a normal nucleotide that is "C".

Conclusion

Thus it is concluded that the breeding bulls should be mandatory screened time to time for inheritable genetic disorders before using them for breeding purpose. Therefore, it is highly suggested that screening works on the disorders should continue further encompassing as many animals as possible from the studied farms and other farms in India.

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