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Allele Specific PCR Based Genotyping Reveals Lack of Bovine Leukocyte Adhesion Deficiency, Bovine Citrullinaemia and Complex Vertebral Malformation Carriers in Indian Cattle Population

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A B S T R A C T

Bovine Leukocyte Adhesion Deficiency (BLAD), Bovine Citrullinaemia (BC), and Complex Vertebral Malformation (CVM) are the common autosomal recessive disorders in cattle affecting dairy industry. The present study was designed to standardize allele-specific PCR based genotyping method for examining the occurrence of these disorders in Indian cattle population. A total of 327 indigenous (*Bos indicus*), exotic (HF) cattle breeds and HF crossbreds (*Bos taurus* × *Bos indicus*) from various parts of India were screened. An allele specific PCR technique was applied to detect mutation in specific DNA target sequences. The amplified products were purified and sequenced to confirm the change in nucleotide in the selected regions. Allele-specific PCR-based screening methodology was optimized for genotyping. No animals were found as carrier of these genetic abnormalities in the present study. Allele-specific PCR-based screening methodology may be efficiently employed to screen the animal population for these genetic defects. All tested animals were free from the aforementioned genetic disorders. But this may not provide us conclusively cattle herds maintained at the study area are free from these genetic disorders. Therefore, it is highly recommended to continue screening procedure to check undesirable transmission mutated version of alleles in Indian cattle population.

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. From the perspective of animal breeders genetic diseases are of grave concern as health and productivity of farm animals are badly compromised due to these disorders. Inherited disorders in cattle are mostly caused by autosomal recessively inherited genes i.e. they are only expressed as a diseased phenotype if both alleles are present. These autosomal recessive genetic diseases are breed-specific in cattle. For instances genetic disorders like Factor XI deficiency syndrome (Brush *et al.*, 1987), Complex Vertebral Malformation (Steffen, 2001), Bovine

Leukocyte Adhesion Deficiency (Kerhli *et al.*, 1990), Bovine Citrullinaemia (Harper *et al.*, 1986), and Deficiency of Uridine Monophosphate Synthase (Robinson *et al.*, 1993) occur exclusively in Holstein-Friesian.

Bovine Leukocyte Adhesion Deficiency (BLAD) is a recessive autosomal genetic disorder in Holstein cattle including several species of mammals. The molecular genetic basis of this disorder is a point mutation (A→G) at position 383 of bovine CD18 gene at bovine chromosome 1 causing aspartic acid to glycine substitution at amino acid position 128 (D128G) (Nagahata *et al.*, 1997). BLAD, which is characterized by lack of expression of adhesion molecules of the CD11/CD18 family on the leukocyte surface, results in death of the homozygous animal that is unable to defend itself from pathogens (Shuster *et al.*, 1992).

Bovine Citrullinamia (BC) is an inherited disorder of Holstein cattle due to deficiency of the urea cycle enzyme argininosuccinate synthetase [L-citrulline: L-aspartate ligase (AMP forming), EC 6.3.4.5] (Pedeeri *et al.*, 199). It is caused by a transition (C→T) at codon 86 of the gene Argininosuccinate Synthetase at 11th chromosome converting a CGA codon to translation-terminating TGA codon.

This conversion results in a truncated peptide product (85 amino acids long rather than the normal 412 amino acids) that lacks enzymatic activity. As urea cycle is non-functional in BC affected animal elevated citrulline is found in blood and tissues and neurological disorders result from accumulation of ammonia. Initially depression is observable within a day, followed by unsteady gait, aimless wandering, apparent blindness, head pressing, collapse, convulsions, and death within 1 week (Robinson *et al.*, 1993).

Complex Vertebral Malformation (CVM) is a recessively inherited lethal genetic disorder caused by a point mutation (G→T) at nucleotide position 559 in the gene *SLC35A3* (solute carrier family 35 member 3) located in bovine chromosome 3, which leads to frequent abortion of fetuses, vertebral anomalies and prenatal death (Agreholm *et al.*, 2001; Nielsen *et al.*, 2003).

Crossbreeding of nondescript low milk producing zebu cattle with exotic germplasm of Holstein, Jersey and Brown Swiss breeds by artificial insemination (AI) is very popular among Indian dairy farmers. A huge leap in milk production in India throughout last few decades have become possible due to widespread implication of crossbreeding program at grass root level up to the farmer's doorstep. While disseminating superior germplasm through crossbreeding with elite HF bull semen the chance of silent propagation of autosomal recessive genetic disorders is very common. As these genetic disorders are homozygous recessive in nature they remain unnoticed in heterozygous carrier animals. Hence screening of animals to single out the genetically defective, carrier one is of utmost importance to prevent spread of these defects in Indian dairy herd. Moreover, regular screening of the offspring is required as the disorders are naturally inherited. Therefore, this study has been designed to standardize PCR based diagnostic test for identifying three genetic disorders namely BLAD, BC and CVM in Indian dairy herd.

Materials and Methods

Ethical committee approval

Since we did not perform any trial by keeping experimental animals, ethical committee approval is not required as per our Institutional ethical committee. It means such work is ethically approved one which does

not require specific submission of proposal and approval.

Blood collection and isolation of genomic DNA

Blood samples were randomly collected from a total of 327 indigenous (*Bos indicus*), exotic (HF) cattle breeds and HF crossbreds (*Bos taurus* × *Bos indicus*) from 7 different states of India vis-à-vis NDRI cattle yard unit at Haryana, Punjab, Tamil Nadu, Kerala, Uttar Pradesh and Karnataka state. Whole blood was collected from jugular vein into a tube containing 5% EDTA. The samples were transported to Animal Genomics Laboratory in Animal Biotechnology Center at NDRI, and was stored at -20°C until genomic DNA extraction were done. The genomic DNA was isolated from white blood cells using standard phenol-chloroform procedure (Sambrook and Russell, 2001). The quality and quantity of DNA samples were analyzed by Agarose gel (0.8%) and measurement of optical density at 260 nm using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at -20°C for further use. The working DNA concentration of 50 ng/ml was used for all the subsequent analyses.

PCR optimization and product visualization

For genotyping the animals under study on the basis of 3 individual point mutations located on 3 different genes Allele-specific PCR was developed as described by Ye *et al.*, (Ye *et al.*, 2001). For identifying BLAD and CVM, PCR amplification of mutation spanning regions of CD18 and SLC35A3 genes were amplified in 25µl reaction volume containing 2.5µl10X PCR buffer, 1.6 mM $MgCl_2$, 200µM of dNTPs (Thermo Fisher Scientific), 0.12µM each of outer and inner forward, outer and inner reverse primers, 1 U Dream Taq DNA polymerase (Thermo Fisher Scientific), 50 ng of template DNA and nuclease free water.

For identifying BC, PCR amplification of ASS gene was carried out in 25µl reaction volume containing 2.5µl 10X PCR buffer, 1.6 mM $MgCl_2$, 200µM of dNTPs (Thermo Fisher Scientific), 0.06 µM each of outer and inner forward, outer and inner reverse primers, 1 U DreamTaq DNA polymerase (Thermo Fisher Scientific), 50 ng of template DNA and nuclease free water.

Following amplification reaction PCR products were separated in a horizontal submarine gel electrophoresis unit (DNAse and RNAse free agarose 2%; 1× TAE buffer; 100 V). The gel was stained with Ethidium bromide solution (0.5 µg/ml) for 10 min in dark and visualized using a molecular imager (Gel Doc XR, BIO-RAD). Amplicon sizes were checked with reference to DNA size markers to identify any animal carrying mutated allele. Details of primers, PCR cycling parameters of all 3 genes under this study are mentioned bellow (Table 1).

Purification and sequencing of PCR products

For confirmation the PCR products were purified using ethanol precipitation and sequenced using outer forward primer automated ABI 377 sequencer (PerkinElmer Applied Bio system, Foster city, CA, USA) by Xcelris Private limited (Ahmadabad, India). The raw sequence data was edited by Bioedit (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Results and Discussion

Allele-specific PCR was successfully used to genotype the DNA fragments of CD18, SLC35A3 and ASS genes for BLAD, CVM

and BC respectively. For CD18 gene the outer primers (OF and OR) produced a 326 bp amplicon (Fig. 1A). The inner forward (IF) primer detected normal allele (Adenine) at position 383 of CD18 gene with an amplicon size of 174 bp (Fig. 1A). Although inner reverse (IR) primer was designed to detect mutated allele with G (Guanine) at same position no animal was detected to carry the mutated allele for CD18 gene (Fig. 1A). Allele specific PCR genotyping result on gel photo was confirmed by sequencing the corresponding mutation containing a region for CD18 gene. The result on the chromatograph was similar to that of allele specific PCR result on gel photo (Fig. 1B).

Similarly for *SLC35A3* gene a common PCR product for both normal and mutant type of amplicon size 140 bp was produced by the outer primers (OF and OR). The inner reverse (IR) primer detected normal allele G (Guanine) at 559^{th} position with product size 114 bp (Fig. 2A). It is also evident from Figure 2 that no defective allele was present among the animals under study as Inner forward (IF) primer did not produce any amplicon of 81 bp size. The result on the chromatograph was similar to that of allele specific PCR result on gel photo (Fig. 2B).

Similar results were found for BC also. The amplified PCR product of *ASS* gene locus yielded two bands of 199 bp and 317 bp of common product for normal animal. However, none of the animals showed affected i.e. 170 bp fragments on the gel photo for bovine citrullinaemia (Fig. 3 A&B).

The dense set of single nucleotide polymorphisms (SNPs) across the genome and mutational stability of SNPs have made them useful DNA markers for population genetic study and for mapping susceptibility genes for complex diseases. Here we have genotyped 327 animals for the presence of defective alleles of BLAD, CVM and BC with Allele-specific PCR, a simple and economical SNP genotyping method involving a single PCR reaction followed by gel electrophoresis. Two outer primers and two allele-specific inner primers are used in a single PCR reaction to ensure generation of allele specific amplicons (Ye *et al.,* 2001). The outer forward (OF) and outer reverse (OR) primers are strategically designed in such a way so that they (OF vs. OR) can amplify a specific amplicon of the target gene, thereby ensuring gene specificity and PCR efficiency. Simultaneously amplification reaction with inner forward (IF) and inner reverse (IR) with OR and OF primers respectively (OF vs. IR and IF vs. OR) confirm the allele specificity. A mismatch is intentionally inserted near the 3'-end of inner primers to improve allele specificity. Although the mechanism of Allele-specific PCR is simple it needs rigorous standardization procedure compared to conventional PCR. The successful outcome of Allele-specific PCR is influenced by various factors like primer concentration, Inner to outer primer ratio, $MgCl_2$ and dNTPs concentration, annealing temperature, Taq polymerase concentration and preparation of template DNA (Medrano and Oliveira, 2014). We successfully optimized all these factors to develop Allele-specific PCR for all three genes specific for three important genetic disorders of bovine.

BLAD is a homozygous recessive genetic disorder that prevails globally among Holstein breed of cattle. In USA, 80% of the 10 million dairy cows are Holsteins. It is estimated that 16000 calves are born with BLAD each year. The average economic loss per calf is roughly 300 USD annually (Patel and Patel *et al.,* 2014). Semen from Holstein bulls is widely used in India for crossbreeding purpose to upgrade poor milk yielders zebu cattle. So chance of genetic transmission BLAD

producing defective allele across the lineages of crossbred animals is very high. BLAD carriers were first reported in two young Holstein bulls (1.33%) which were born through artificial insemination with the imported semen (Pedeeri *et al.,* 199). After that strict screening procedure is followed in semen stations, bull mother farms and embryo transfer centers across the country to check the presence of BLAD causing mutation in CD 18 gene. A study on a group of 55 Karan Fries (HF × Tharparkar) calves revealed 2 (3.64%) were BLAD carriers and 1 (1.82%) was BLAD affected (Yathish *et al.,* 2010). In a separate investigation, out of 42 HF and HF crossbred bulls, 2 bulls (4.76%) of HF were found to be heterozygous for BLAD (Patel *et al.*, 2011). The percentage of animals carrying CVM is higher worldwide. The syndrome was first discovered in the Danish Holstein population in 1999 (Agreholm *et al.,* 2001).The analysis of a total of 957 sires showed that the frequency of CVM affected animals was 13.2% (Konersmann *et al.,* 2003), of which the possibility of a CVM-homozygote calf was between 0.3 to 0.45% in German Holsteins. In Sweden, 228 bulls were genotyped for CVM during 1995-1999, of which 53 bulls, i.e. 23%, were confirmed CVM carriers (Konersmann *et al.,* 2003). In India, a study revealed very high occurrence (23.08%) of CVM disorders within 52 Karan Fries bulls (Yathish *et al.,* 2011).

Table.1 Mutation points, the primer pairs, anticipated amplicon sizes and PCR cycling parameters of *CD18, SLC35A3* and *ASS* genes

Genetic disorder	Gene	Mutation	Primer	Primer sequence (5'→3')	Product size	Cycling parameters
BLAD	*CD18* (*DQ344626.1*)	A→G	IF	CGGAGGGCCAAGGGCTACCCCATAGA	Mut 207bp, WT174 bp Co 326 bp	Annealing temp.55˚C; 34 cycles
			IR	GTAGGAGAGGTCCATCAGGTAGTACATGC		
			OF	CCAGCATAAGAGAATGGGGAGAGTCCTG		
			OR	GTTTCAGGGGAAGATGGAGTAGCTGCCT		
CVM	*SLC35A3 (AY160683.1)*	G→T	IF	TGGCTCACAATTTGTAGGTCTCATGGTAG	Mut81 bp, WT 114bp Co 140 bp	Annealing temp.55˚C; 34 cycles
			IR	CCACTGGAAAAACATGCTGTGAGGAA		
			OF	GTTTCTTTTTGTTCAGTGGCCCTCAGAT		
			OR	ATTTTCTCAAAGTAAACCCCAGCAAAGC		
BC	*ASS (FJ853494)*	C→T	IF	CCAGTCCAGCGCACTGTACGAGGCCT	Mut 170bp, WT 199bp Co 317 bp	Annealing temp.57˚C; 30 cycles
			IR	GCGAGAGAGGTGCCCAGGAGGTAGCG		
			OF	CGTCTGCCTTAGACACAGGCCATCGGAG		
			OR	CTCCTCACCCTCTCCAGCCACACATGCT		

Fig.1 A Agarose gel electrophoresis (2%) of*CD18* allele specific polymerase chain reaction product for detecting Bovine Leukocyte Adhesion Deficiency (BLAD). Lane 1-3: Homozygous normal animals showing two bands (174 bp and 326 bp); N: PCR without genomic DNA (negative template control); M:DNA molecular weight marker (O'RangeRular 100 bp DNA Ladder, Thermo Fisher Scientific). **B.** Chromatogram showing presence of nucleotide Adenine (A) at 383 position of *CD18* gene in homozygous normal animals

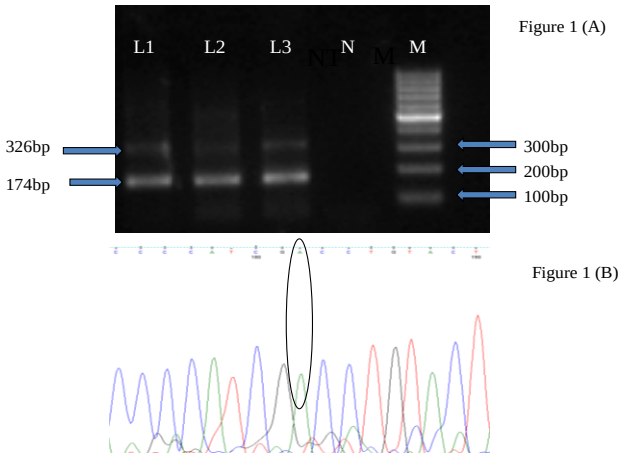


Fig.2 A Agarose gel electrophoresis (2%) of*SLC35A3* allele specific polymerase chain reaction product for detecting Complex Vertebral Malformation (CVM). Lane 1-8: Homozygous normal animals showing two bands (114 bp and 140 bp); N: PCR without genomic DNA (negative template control); M:DNA molecular weight marker (O'RangeRular 50 bp DNA Ladder, Thermo Fisher Scientific). **B.** Chromatogram showing presence of nucleotide Guanine (G) at 559 position of *SLC35A3* gene in homozygous normal animals

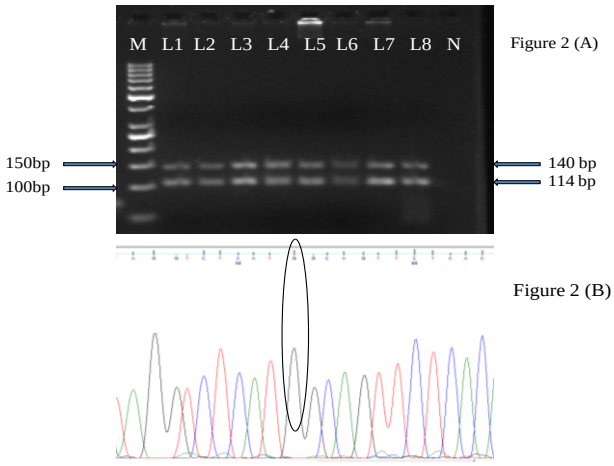
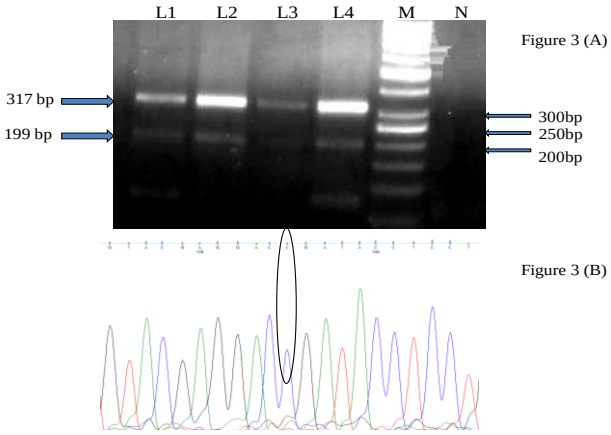


Fig.3 A Agarose gel electrophoresis (2%) of *ASS* allele specific polymerase chain reaction product for detecting Bovine Citrullinamia. Lane 1-4: Homozygous normal animals showing two bands (199 bp and 317 bp); N: PCR without genomic DNA (negative template control); M:DNA molecular weight marker (O'RangeRular 50 bp DNA Ladder, Thermo Fisher Scientific). **B.** Chromatogram showing presence of nucleotide Cytocine (C) at 86th codon of *ASS* gene in homozygous normal animals



The occurrence of bovine Citrullinaemia was high in Australia. Healy *et al.*, reported that 50% of Australian national Holstein herds and 30% of breeding bulls in AI centers were descendants of Linmack Kriss King (LMKK), which was carrier for Citrullinaemia. In India, one Holstein carrier bull for citrullinaemia was observed during screening a group of various breeds of *Bos taurus* (n=200), *Bos indicus* (n=80), *Bos taurus* × *Bos indicus* crossbreds (n=50) and *Bubalus bubalis* (n=135) (8). A case of a carrier bull has been recently identified during routine investigation (Kotikalapudi *et al.*, 2014).

The present investigation did not reveal any of these genetic disorders in the study population. Earlier also very low incidence (3.23%) of BLAD has been observed in Holstein Friesian population in Iran (Rahimi *et al.*, 2006). In a separate study Patel *et al.*, found lack of carriers of BC in Indian Holstein population (Patel *et al.*, 2006). No carrier animal of CVM was found in an investigation carried out by Rezaee *et al.*, in an experimental population of Iranian Holstein bulls (Rezaee *et al.*, 2008). Rigorous genetic screening procedure of young bulls and semen samples followed in semen station may be the cause of lack of carrier animals in the population under study.

In conclusion, the genetic disease causes the heavy losses because of poor animal performance, loss of production and compromised reproductive potential of the animal. If genetic disease is not diagnosed it will be transmitted from generation to generation silently in the breeding population affecting animal productivity negatively. Allele specific PCR can be employed routinely to detect these genetic disorders in cattle especially in Holstein and crossbred Holstein population. Although no carrier of BLAD, BC and CVM has been observed in the study population incessant monitoring is required in animals reared in semen stations, bull mother farm and embryo transfer center

to check the spread of genetic disorders through ongoing breeding program and the full benefit of crossbreeding program in Indian dairy industry can be reaped.

Conflict of interest

There is no conflict of interest among the authors to declare.

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