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Using PCR-PIRA based genotyping for identifying complex vertebral malformation allele in Frieswal young bulls in India

Alyethodi, R. R.[1*]**; Kumar, S.**[1]**; Deb, R.**[1]**; Alex, R.**[1]**; Singh, U.**[1]**; Sharma, S.**[2]**; Ashish,**[2]**; Choudhary, J.**[2]**; Sengar, G.**[2]**; Singh, R.**[1]**; Tyagi, S.**[3] **and Birham, P.**[1]

[1]Animal Genetics and Breeding Section, ICAR-Central Institute for Research on Cattle, Meerut Cantt, Meerut, 250001, Uttar Pradesh, India; [2]MSc, Animal Genetics and Breeding Section, ICAR-Central Institute for Research on Cattle, Meerut Cantt, Meerut, 250001, Uttar Pradesh, India; [3]Animal Physiology Section, ICAR-Central Institute for Research on Cattle, Meerut Cantt, Meerut, 250001, Uttar Pradesh, India

*Correspondence: R. R. Alyethodi, Animal Genetics and Breeding Section, ICAR-Central Institute for Research on Cattle, Meerut Cantt, Meerut, 250001, Uttar Pradesh, India. E-mail: rfq_rahman@yahoo.co.in

Summary

Complex vertebral malformation (CVM) has considerable economic impact on dairy cattle breeding due to extensive use of artificial insemination (AI). Identifying the carrier is an important factor to reduce the incidence of the genetic disorder. The study was conducted to identify the carriers of CVM in Frieswal cattle by polymerase chain reaction-primer-introduced restriction analysis (PCR-PIRA) method, which was further confirmed by sequencing. Carrier prevalence of 1% was observed in the Frieswal cattle. The results of the study clearly demonstrated the existence of carriers of CVM among Frieswal bull calves. Due to the widespread use of AI it is recommended to screen young bulls at early stages for this defective allele in order to avoid its rapid spread within the population.

Key words: CVM, Frieswal, PCR-PIRA

Introduction

Complex vertebral malformation (CVM) is a lethal syndrome in Holstein population leading to malformed calves that are either spontaneously aborted or die shortly after birth (Kanae *et al*., 2005). At the molecular level, it is due to a point mutation of the bovine solute carrier family 35 member 3 (SLC35A3) gene that results in the substitution of Valine by Phenylalanine (V180F) which impairs the function of transporter membrane protein [uridine diphosphate (UDP)-Nacetylglucosamine] (Thomsen *et al*., 2006; Agerholm 2007). This mutation is been reported associated with milk traits, persistency of lactation and somatic cells score (Chu *et al*., 2010). Since the single point mutation in SLC35A3 gene does not create or abolish any restriction site, it cannot be identified by restriction fragment length polymorphism (RFLP) method. Alternative molecular detection approaches including allele specific-polymerase chain reaction (AS-PCR) (International Patent WO 02/40709 A2, 2002), high resolution melting analysis (Gabor *et al*., 2012), PCR single stranded conformation polymorphism (Rusc and Kaminski, 2007) and real time based genotyping (Zhang *et al*., 2012) have been proposed.

Frieswal cattle is a synthetic breed of Holstein-Friesian (HF) (5/8) and Sahiwal (3/8) developed by ICAR-Central Institute for Research on Cattle (ICAR-

CIRC), Meerut, India in collaboration with Ministry of Defence. As imported HF semen has been used for the development of the breed, it is vulnerable to the genetic disorders reported in HF. Hence, the present study was carried out to identify the carriers for CVM in Frieswal bull calves in order to eliminate them from the population.

Materials and Methods

Frieswal bull calves received from 37 military farms throughout India, reared at Bull Rearing Unit, ICAR-CIRC were used in the study. Genomic DNA was extracted by conventional phenol-chloroform method as described by Sambrook *et al*. (1989) with minor modifications. Genotyping of CVM was done using PCR-primer-introduced restriction analysis (PCR-PIRA) method (Kanae *et al*., 2005). In brief, PCR-PIRA method employs two sets of primers with a common reverse primer, the generated amplicon sizes for both primer sets are 287 bp. Forward primers of each set create a specific restriction site depending on the nucleotide at the single nucleotide polymorphism (SNP) location. Both forward primers were complementary to sequence from nucleotides 537 to 554 of the bovine SLC35A3 gene, but 2 nucleotides at the fourth and fifth positions from the 3´ ends of the primers were different. Three nucleotides at the 3´ ends of both primers were similar to nucleotides

556-558 of both the wild-type and CVM alleles. The set I forward primer (5´CAC AAT TTG TAG GTC TCA ATG CA) creates *NsiI* restriction site if CVM mutant allele is present at a 559th position (T allele) while set II forward primer (5´CAC AAT TTG TAG GTC TCA CTG CA) creates *PstI* restriction site in the wild-type allele (G allele). The schematic representation of PIRA has been shown in Fig. 1. The primer sequences and digested product sizes are presented in Table 1.
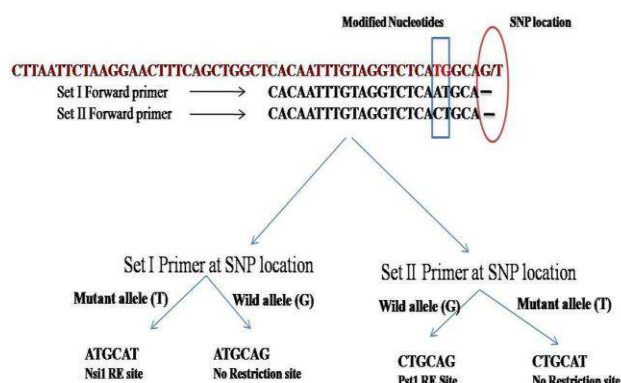

Fig. 1: Schematic representation of PCR-PIRA method

Polymerase chain reaction was carried out in a final reaction volume of 10 µL. Two PCRs were set for each sample, one with the set I primer and the second with set II primers. PCR cocktail consisted of 50 to 100 ng of genomic DNA, 200 µM of each dNTP, 5 Pico moles of each primer, 1 unit of Taq DNA polymerase and Taq buffer with 1.5 mM MgCl$_2$ for each reaction. DNA was amplified with an initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, primer annealing of 60°C for 45 s, extension at 72°C for 35 s, with final extension 72°C for 10 min. PCR products generated with the set I and II primers were digested with *NsiI* and *PstI* restriction enzymes (RE), respectively. The digested products were separated by 10% native PAGE, and stained by silver nitrate.

For sequencing of CVM alleles, primers were designed (Table 1) and PCR products were sequenced using ABI 3100 (Applied Biosystems, USA) Automated DNA Sequencer. Raw sequence data were analysed using Chromas (Ver. 1.45, http://www.technelysium.com). The BLAST algorithm was used to analyze the mutation in the generated sequence against the sequences in the NCBI GenBank (http://www.ncbi.nlm.nih.gov) databases. The mutant gene frequency of the CVM was estimated by counting the number of alleles (Nei, 1987).

Results

Both sets of primers (Set I and II) were successfully amplified DNA fragment of 287 bp (Fig. 2). The DNA samples amplified with Set I primers did not yield any digested products with *PstI* restriction enzyme and vice versa making the PCR-PIRA a suitable technology for screening of CVM alleles. When amplified and digested with the set I primers and *NsiI* enzyme, the mutant allele showed complete digestion resulting in amplicon sizes of 262 bp and 25 bp while wild allele showed undigested band pattern of 287 bp. In the case of primer set II and *PstI* digestion, similar pattern was observed for a wild allele with 262 and 25 bp bands and a mutant allele with 287 bp patterns. The carrier animals showed both bands of 287 and 262 bp with both the enzymes (Fig. 3). In our observations, chances of false positive are high if set II primers and *PstI* are used due for incomplete digestion while set I primers always yielded correct genotyping pattern as they can cut only the mutant allele. Hence, the final screening of bull calves was done with *NsiI* which yielded 3 carrier genotypes out of 300 bull calves screened (Fig. 4). The amplicon sizes were found higher than expected sizes with 10% PAGE which may be due to secondary structures which are common in higher percentage PAGE. The resolution was better with PAGE hence it was switched over to AGE for separation of products with products of similar sizes. Chromatograph analysis confirmed the presence of a mutated allele.
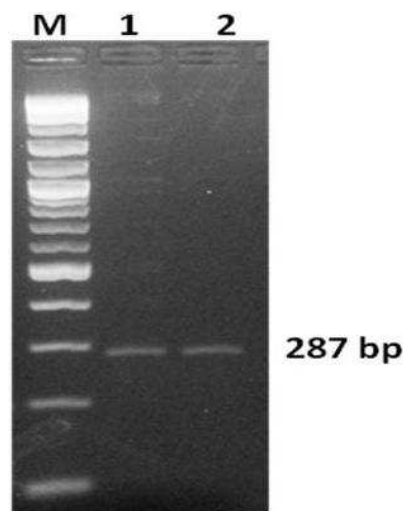

Fig. 2: Amplification of CVM with both primer sets. Both sets of primers showed amplified DNA fragment of 287 bp

Table 1: Primers, PCR product size and RE, digested product sizes used for identification of CVM in Frieswal bulls

| Primer sets	Primer sequence (5'→3')	PCR product size (bp)	RE used	Digested product sizes		
				CVM allele	Wild allele	Heterozygous
Set I (*NsiI*)	F-5´CACAATTTGTAGGTCTCAATGCA R-5´CGATGAAAAAGGAACCAAAAGGG	287	*NsiI*	262, 25	287	287, 262, 25
Set II (*PstI*)	F-5´CACAATTTGTAGGTCTCACTGCA R-5´CGATGAAAAAGGAACCAAAAGGG	287	*PstI*	287	262, 25	287, 262, 25
Sequencing primers	F-5´TGGAAATGGTTGCATTTTTACCTTAAG R-5´TTTGCAACAACAAGCAGTTTTTATAAGG	389				
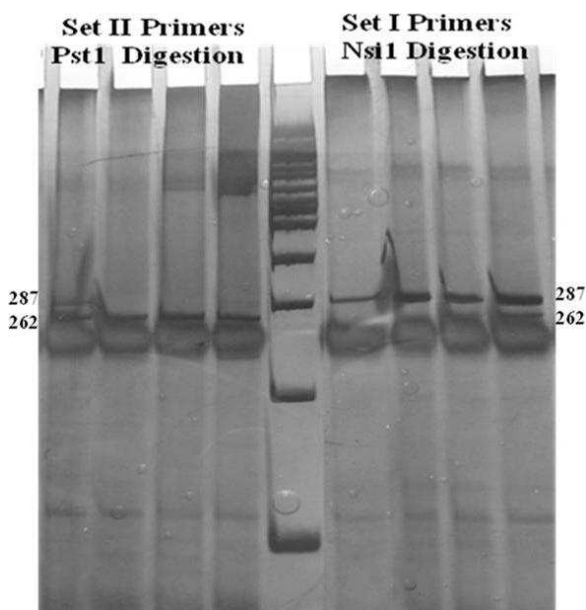
Fig. 3: Illustration of CVM genotypes on native PAGE. Lane 1 and Lane 9 shows heterozygous genotype pattern
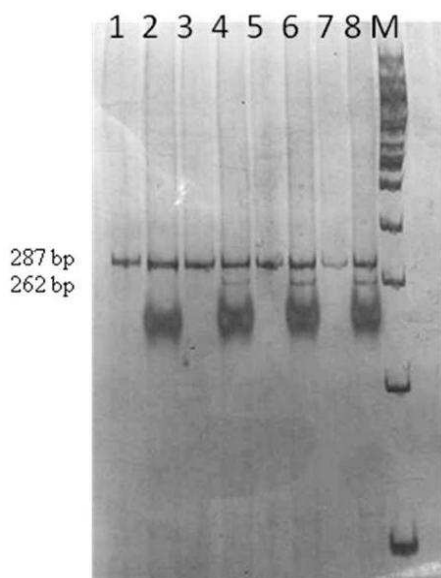

Fig. 4: CVM genotype pattern with *NsiI* enzyme. M: 100 bp Molecular Marker. Lane 2: Normal genotype, Lanes 4, 6, 8: Carrier genotype pattern, and Lanes 1, 3, 5, 7: Non-digested PCR products

Discussion

In our investigation, out of 300 bull calves, three were heterozygous for CVM allele. A carrier prevalence of 1.0% was thus noticed for CVM with an allelic frequency of CVM mutant 0.01. The carrier prevalence in Frieswal bull calves is far less than what was reported earlier (Mahdi *et al.*, 2010) in Karan Fries cattle (23.1%) of India. The occurrence of CVM has so far reported in several countries. The prevalence rate was higher in the initial stages due to lack of efficient methods for identifying such type of genetic disorders and it was

13.2% in Germany (Konersmann *et al.*, 2003), 31% in Denmark (Thomsen *et al.*, 2006) and 32.5% in Japan (Nagahata *et al.*, 2002). In China a high frequency of carriers in at risk female population (46.7%) of confirmed bulls (Chu *et al.*, 2008) while random population analysis showed a lower frequency of 2.92% (Wang *et al.*, 2011). This disorder has also been reported from Turkey (Meydan *et al.*, 2010) and Poland (Rusc *et al.*, 2013) with frequencies, 3.4 and 16.6%, respectively. Lower frequencies of the mutant allele in the later studies are due to extensive culling of carriers from the breeding population. Recently two new mutations in SLC35A3 gene at positions 554 and 555 have been reported by sequence analysis in Indian Holstein population. It needs to be evaluated as the reported mutation causes missense mutation leading to methionine to threonine substitution, yet no phenotypic changes could be identified (Kotikalapudi *et al.*, 2013). But in the present study none of these mutant alleles were detected in the Frieswal population. It may be due to the difference in the Holstein inheritance between studied populations or maybe the reported mutation is a *de novo* mutation.

Rapid spreads of genetic diseases are feasible in herds due to the extensive use of a few elite sires through artificial insemination (AI). The inability to distinguish carrier and normal calves morphologically and early death of affected animals due to recurrent infections demand rapid screening of breeding populations using molecular tools in order to eliminate the carriers from the population and thus to reduce the frequency of affected animals. This study shows that PCR-PIRA method can be used as a reliable method for identification of CVM in animals.

Based on the results, the prevalence of recessive disorder of CVM is low in Frieswal bulls in India. It has to be taken with precaution as the actual number of clinical cases of CVM is unknown in India. Hence, the monitoring of CVM in young bulls should be performed to keep the breeding population free from known genetic diseases.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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