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SHORT COMMUNICATION

Detection of Silent Homozygous Polymorphism in Exon 4 of *SLC35A3* Gene in a Holstein Cattle Carrier for Complex Vertebral Malformation

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ABSTRACT

The complex vertebral malformation (CVM) syndrome is a congenital autosomal recessively inherited disorder first observed in Danish Holsteins. It is caused by a point mutation (G→T) at nucleotide position 559 of the gene, bovine solute carrier family 35 member 3 (SLC35A3). Bovine SLC35A3 plays a vital role in the development of the axial skeleton. The aim of this study was to detect carriers of CVM in Holstein population using Polymerase Chain Reaction- Primer-introduced restriction analysis and Restriction Fragment Length Polymorphism (PCR-PIRA and RFLP) methods. Our results show that one out of 60 bulls tested exhibited polymorphism (G→T) at position 559 in exon 4 of *SLC35A3* gene. To confirm this polymorphism, the PCR product was purified using ExoSAP-IT followed by sequencing by Applied Biosystems 3130XL Automated Sequencer using the ABI BigDye Ver 3.1. Gene sequences from normal and carrier animals were compared using the software, codon code Aligner 4.0.4. Surprisingly, the sequence analysis of PCR product also revealed the presence of two previously unknown homozygous mutations (TG→CT) at nucleotide positions 554 and 555 in addition to the previously reported heterozygous mutation at position 559. The bull was immediately culled from the breeding programme. To the our best of knowledge, this is the first study to report the existence of homozygous and heterozygous mutations at positions 554, 555 and 559 in exon 4 of *SLC35A3* gene in Indian Holstein cattle. However, it is surprising that no phenotypic effects were observed in the carrier bull, necessitating further studies to fully elucidate the effects of these novel homozygous mutations.

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INTRODUCTION

The complex vertebral malformation (CVM) syndrome is a congenital autosomal recessively inherited disorder in Holstein cattle (Agerholm *et al*., 2001). Studies of Danish Holsteins with CVM have shown that the extent of foetal mortality prior to gestation day 260 is approximately 77% (Nielsen *et al.,* 2003). Majority of the calves that survive until the end of the gestation period are stillborn and are phenotypically characterized by retarded growth and mild bilateral flexion of the carpal and pastern joints with rotation of the digits. Additionally, most of the animals have vertebral malformation, malformed ribs, and arthrogryposis of the tarsal and posterior pastern joints (Agerhoim et al., 2004). Extensive malformation of the cervical and thoracic vertebrae is observed in typical cases, causing a shortening of the neck. Other malformations have been reported as a part of this syndrome, including cardiac interventricular septal defects, malformation of the great vessels and myocardial hypertrophy (Agerholm *et al*., 2001, Nielsen *et al*., 2003). The syndrome was first discovered in the Danish Holstein population in 1999 (Agerholm et al., 2001), but shortly thereafter reported in the United States (Duncan et al., 2001, Holstein Association, USA, 2006), the United Kingdom (Revell, 2001), Netherlands (Wouda *et al*., 2000), Japan (Nagahata *et al*., 2002), Germany (Konersmann *et al*., 2003), Sweden (Berglund *et al*., 2004), Denmark (Thomsen *et al*., 2006), and India (Mahdipour *et al*., 2010). It is important to recognize that CVM is not newly identified disease. It has been present in the Holstein breed since many generations, and that

only the DNA test for its diagnosis is a new advancement. Genealogical records traced the origin of the disease-causing allele to a common ancestral bull, Carlin-M Ivanhoe Bell, which has been extensively used in dairy cattle breeding worldwide for over two decades due to the superior lactation performance of his daughters (Mahdipour et al., 2010). Coincidently, Carlin-M Ivanhoe Bell was a carrier for two genetic disorders, CVM and Bovine leukocyte adhesion deficiency (BLAD). The BLAD and CVM genes are mapped to chromosomes 1 (Shuster *et al*., 1992) and 3 (Thomsen *et al*., 2006), respectively. When the sire (father) of Carlin-M Ivanhoe Bell, a bull named Pennstate Ivanhoe Star, was tested he was found to be a carrier of both CVM and BLAD (Thomsen et al., 2006). Carlin-M Ivanhoe Bell's grandsire Osborndale Ivanhoe, however, carried only BLAD. Scientists therefore believe that the mutation responsible for CVM occurred either in Pennstate Ivanhoe Star (Sire) or somewhere in his maternal family.

Biochemical aspect of the CVM reveals that this disease results from an impaired protein molecule, *Uridine diphosphate N-acetylglucosamine* (UDP-N-acetylglucosamine) transporter or Golgi UDP-GlcNAc transporter in the Golgi apparatus membrane (Patel, 2012) . These transporter proteins transport a nucleotide sugar, UDP-N-acetylglucosamine *or UDP-GlcNAc* and coenzyme during metabolism from cytosol (site of synthesis) into the Golgi lumen before these can be substrates for the glycosylation of proteins, lipids, and proteoglycans (Patel, 2012). The *UDP-GlcNAc* plays an important role in the structure of the cytoskeleton. The molecular basis of CVM is a substitution of guanine by thymine (G→T) in a solute carrier family 35 member 3 gene (*SLC35A3*) which encodes a UDP-N-acetylglucosamine transporter. The gene is located on bovine chromosome BTA3 (Thomsen *et al*., 2006). This mutation results in the substitution of Valine by Phenylalanine at position 180 (V180F), impairing the function of the transporter membrane protein. This single point mutation in *SLC35A3* gene with no restriction site can be analysed using single-stranded conformation polymorphism (PCR-SSCP) (Orita *et al*., 1989). An alternate method is PCR-Primer Introduced Restriction Analysis (PCR-PIRA) which creates a *Pst* I restriction site in the wild-type gene during PCR (Kanae *et al*., 2005). Once the restriction site is created, restriction fragment length polymorphism (RFLP) analysis can be performed. This paper describes a unique case of CVM carrier which was diagnosed by creating a restriction site for RFLP and further confirmed by gene sequencing.

MATERIALS AND METHODS

Blood samples were collected into EDTA blood collecting vials, from 60 apparently healthy Holstein bulls maintained at different frozen semen banks for routine investigation of autosomal diseases including CVM. DNA was extracted from blood cells by phenol-chloroform method (Sambrook *et al*., 1989) which was followed by qualitative and quantitative verification of DNA. For detection of point mutation in *SLC35A3* gene, polymerase chain reaction (PCR) was performed to create a restriction site for *Pst* I (PCR-PIRA). As described by Kanae et al., (2005), the 233 bp DNA fragment was amplified by PCR, which was set by adding sense primer consisting of 23 bases each Forward- 5'- CAC AAT TTG TAG GTC TCA CTG CA -3' and an antisense primer 5'- CGA TGA AAA AGG AAC CAA AAG GG -3'. The forward primer introduced a *PstI* site in the amplified product from the wild-type allele. PCR mix contained 10X PCR buffer, 10 mM dNTPs, 5U/ µl of *Taq* DNA polymerase (Kapa Bio systems), 5 pM each of sense and antisense primer (MWG-Biotech AG), 1.5 mM $MgCl_2$, 80 ng genomic DNA and sterilized distilled water to make a final volume of 20µl. The following PCR conditions were used: predenaturation for 5 min at 95°C, denaturation for 60s at 95°C, annealing at 56°C for 60s, extension at 72°C for 90s followed by 35 cycles of: 60s at 95°C, 60s at 56°C, 90s at 72°C, and ending with 10 min at 72°C for final extension. The PCR products were subjected to electrophoresis on a 1.5 % agarose gel with ethidium bromide and visualized under a UV transilluminator to verify the amplified products. Amplified PCR product was digested with *Pst* 1 (Fermentas life science, India) restriction enzyme as per the standard protocol provided by the supplier. The digested fragments were electrophoresed on a 3% agarose gel stained with ethidium bromide and observed under an UV transilluminator. In order to confirm the polymorphism in exon 4 of *SLC35A3* gene, the PCR product was subsequently sequenced. As described by Jonathan (2008), a simple method to treat PCR products prior to sequencing using ExoSAP-IT was performed. After the purification step of the PCR product, it was sequenced in an Applied Biosystems 3130XL Automated Sequencer using the ABI BigDye Ver 3.1. Sequence analysis comparison of the gene sequences was performed using the Codon Code Aligner 4.0.4 Software.

RESULTS AND DISCUSSION

The PCR-PIRA was performed with genomic DNA samples extracted from the blood of 60 Holstein bulls prior to RFLP analysis. The size of the PCR product was 233bp and it was subjected to further RFLP analysis using *Pst* 1 restriction enzyme. In normal bulls, the PCR product could be digested by the restriction enzyme yielding one fragment of 212bp whereas in the carrier (heterozygous) two fragments of 233 (uncut) and 212 bp were observed (Fig. 1). One bull exhibited polymorphism (G→T) at position 559 in exon 4 of *SLC35A3* gene out of the 60 bulls tested (1.67%), a result that was far less than what was reported earlier by Mahdipour *et al*., (2010) in Karan Friesian cattle (23.1%) in India. The genotype and gene frequency of heterozygous allele were estimated to be 0.0167 and 0.008, respectively. In order to confirm the mutation, the PCR product was sequenced which confirmed the polymorphism at position 559 (see the chromatogram in Fig. 2). However, while comparing the obtained sequence with NCBI sequence (Accession No. NC_007301.5) and the documented sequence (Kanae *et al*. 2005), two homozygous mutations (TG→CT) at 554 and 555 nucleotide positions were also found as indicated in figure 2. To our surprise, these two mutations have no obvious phenotypic effects on the bull as indicated by the fact that the bull is apparently healthy at 18 months of

age. There are many reports of polymorphism at position 559 but none of them reported mutations at positions 554 and 555 as observed in our studies. In India, the studies on Karan Fries, a breed that was developed by crossbreeding between Holstein and Tharparkar (*Bos indicus*), also exhibited polymorphism (T/G) at 559 in 12 out of 52 bulls tested (Mahdipour *et al*., 2010). However, mutations at positions 554 and 555, as observed by us, were not reported by them.

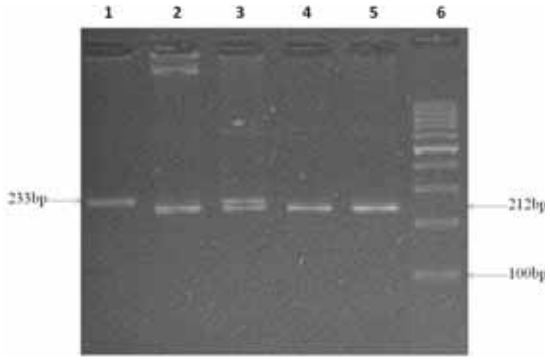

Fig. 1: Analysis of CVM allele by the PCR-PIRA method. Electrophoretogram of *PstI* digested PCR product generated by amplification of genomic DNA using CVM specific primers. Lane # 1: PCR product of 233bp, Lanes # 2, 4 & 5: 212bp band for normal animals, Lane #3: 233bp and 212 bp bands for heterozygous carrier animal. Lane # 6: O'Range Ruler™ 100bp DNA ladder (Fermentas).

The defective allele for CVM had spread in Holstein populations worldwide due to an extensive use of breeding bulls that were later turned out to be carriers of the defective gene. Konersmann *et al*., (2003) reported that 13.2 % of 957 sires used for insemination in Germany were diagnosed as carriers of CVM, while a prevalence of 31%, 32.5% and 23.07% was found in Denmark (Thomsen *et al*., 2006), Japan (Nagahata *et al*., 2002) and India (Mahdipour *et al*., 2010), respectively. Also, the Holstein Association of the USA reported in 2006 that of 11868 bulls examined, 2108 were found to be carriers for CVM i.e., 17.76 %, which was higher than our studies. In their studies, no productive and reproductive differences between carrier and normal animals were reported. The only difference which was very obvious was the increase in the rate of intra-uterine mortality. However, the risk of return to service was also significantly higher in carrier animals (Berglund *et al*., 2004). In this context, various methods have been used for identification of single nucleotide polymorphism in SLC35A3 gene. Agreholm *et al*., (2001) performed genotyping of the CVM locus in a template directed single-base extension assay and Kanae *et al*., (2005) introduced PCR-primer introduced restriction analysis (PCR-PIRA) for detecting a single nucleotide mutation in any gene that lacks a restriction site. Rusc and Kaminski (2007) used PCR-Single Strand Confirmation Polymorphism (SSCP) method. Regardless of the method used to detect CVM, the presence of CVM carriers in population is of great concern to the breeders. Our present study also revealed the prevalence of the mutant gene in Indian Holsteins; it is therefore, advisable to screen for all the possible autosomal recessive diseases, especially in Holstein bulls before they are added to any breeding programmes to avoid the risk of spread of any such diseases.

In conclusion, the sequence comparison of PCR products revealed two novel homozygous mutations (TG→CT) at 554 and 555 nucleotide positions in addition to the previously known heterozygous mutation at position 559. To the best of our knowledge, this is the first study to report the existence of both homozygous and heterozygous mutations (TG→CT) at positions 554, 555, and 559 in Indian Holstein cattle. Therefore, our study reveals the presence of novel polymorphism in exon 4 of SLC35A3 gene of Holstein which surprisingly has no aberrant phenotypic effects. Hopefully, our novel findings will alert animal scientists to look for new polymorphisms despite a lack of effects on the phenotype of the animals. Further studies are required to fully elucidate the impact of these novel mutations on the productive and reproductive performance of Holsteins.

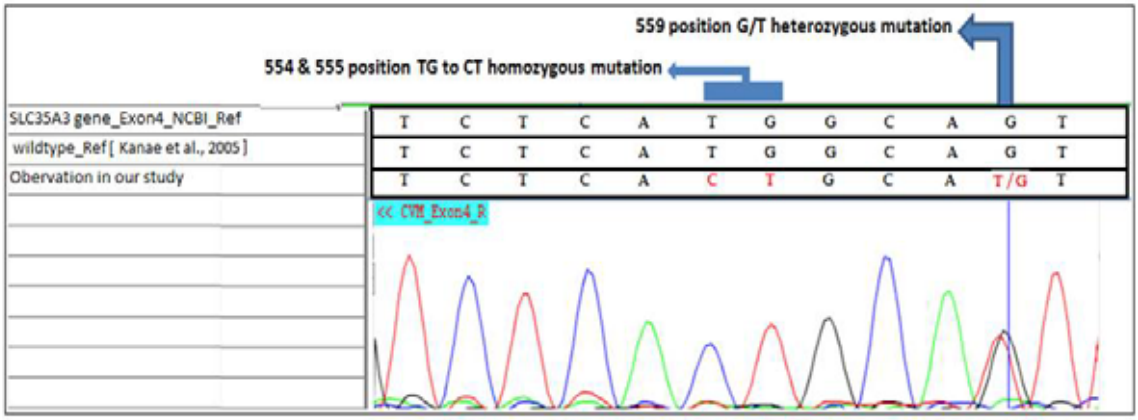

Fig. 2: Comparing partial sequences of exon 4 of SLC35A3 gene obtained in our study (3rd line) with the sequences available on NCBI site (1st line) and that documented by Kanae et al (2nd line), reveals the presence of two novel homozygous mutations (TG→CT) at positions 554 & 555 together with a previously known heterozygous mutation (G→T) at position 559.

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