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First report of cholesterol deficiency associated *APOB* mutation causing calf mortality in Indian Holstein Friesian population

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For cattle farmers, it is highly desired to rear healthy calves for persistent supply of highly productive cow in the herd. A diseased calf and its death is associated with high costs for the farmer, such as the value of animal, the costs for veterinary health care and the raising costs (Mohd Nor *et al*. 2012). Apart from economic losses, calf morbidity and mortality are serious animal welfare issues (Mee 2013). Cholesterol deficiency, a new monogenic autosomal recessive inherited defect in Holstein calves is a lipid metabolism disorder and is named Cholesterol Deficiency Haplotype (CDH). Recessive homozygote calves suffer from incurable idiopathic chronic or recurrent diarrhea and usually die at the age from 3 weeks to 6 months (Kipp *et al*. 2015, Menzi *et al*. 2016). The estimated economic loss due to CDH is approximately 1.3 million euros in Germany (Kipp *et al*. 2015) and 1.7 million dollars in the United States (Cole *et al*. 2016).

A case-control genome-wide association study (GWAS) on 23 affected (chronic diarrhea and underdevelopment followed by death) and 11,177 normal (survived first year of life) German Holstein calves followed by homozygosity mapping and haplotype analysis revealed a ~2.7 Mb haplotype region on BTA 11 associated with calf mortality in homozygous state (Kipp *et al*. 2015). Upon subsequent whole genome re-sequencing of an affected calf and a healthy partially inbred male carrying one copy of the critical segment on chromosome 11 in its ancestral state and one copy of the same segment with the cholesterol deficiency mutation, Menzi *et al*. (2016) detected a 1.3 kbp (1299 bp) insertion of a transposable LTR element in the coding sequence of the *APOB* (apolipoprotein B) gene. Schütz *et al*. (2016) also confirmed the 1.3 kbp insertion of an endogenous retrovirus (ERV21LTR_BT) into exon 5 of the *APOB* gene next to genome position 77958994 on BTA 11, as the causative mutation of cholesterol deficiency in Holstein. This 1299 bp insertion on the APOB transcript cause a frame-shift at amino acid residue 135 in the bovine APOB protein sequence resulting in premature termination of the 4,567 amino acid long protein (p.Gly135ValfsX10 (Menzi *et al*. 2016). Since, APOB is an essential compound of chylomicrons and low-density lipoproteins, the truncated APOB protein cause an inability of chylomicron excretion from intestinal cells, leading to malabsorption of cholesterol from the intestine and transport to circulation and liver in homozygous calves of CDH (Kipp *et al*. 2015). Consequently, the synthesis of LDL and VLDL particles in the liver is affected, which altogether result in severe hypocholesterolemia (reduced cholesterol serum levels). The disease-associated haplotype was traced to the Canadian Holstein sire Maughlin Storm born in 1991 (Kipp *et al*. 2015, VanRaden and Null 2015). Because, the Storm and his progenies have been used globally by means of transport of semen and embryos, it is important to investigate whether this deleterious mutation has been transmitted in the Indian cattle population also. The objective of the present study was to screen the Cholesterol Deficiency haplotype in the Indian Holstein population.

In this study, a total of 303 animals (228 bulls, 40 cows and 35 calves) were screened for the *APOB* mutation. Breeds of bulls included were, Holstein-Friesian (n=60), Jersey (n=30), Holstein-Friesian crossbreds (n=40), Jersey crossbreds (n=30), Karan Fries (n=18) and Indigenous (n=50, 10 each of Sahiwal, Gir, Kankrej, Tharparkar and Red Sindhi). In addition to bulls, 40 Karan Fries cows and 35 Karan Fries calves were also included in the study. The experimental samples for the present study were taken from the herd of Karan Fries cattle maintained at Cattle Yard and Artificial Breeding Research Centre of ICAR-National Dairy Research Institute, Karnal. For other breeds (exotic, crossbreds and indicine), the DNA samples available at Animal Genetics Lab-3 of ICAR-National Bureau of Animal Genetic Resources, Karnal (Haryana) for genetic testing from various semen stations across India were used.

Genomic DNA was extracted from blood by following protocol of Sambrook and Russell (2001). Quality and quantity of the isolated genomic DNA were evaluated using Spectrophotometer and by using 0.8% agarose gel electrophoresis prior to polymerase chain reaction (PCR)

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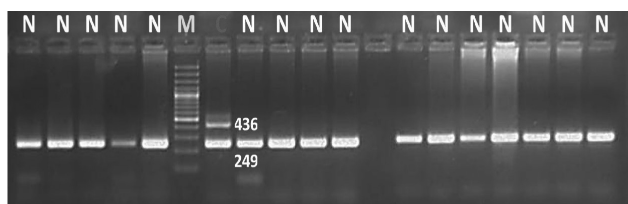
Table 1. Oligonucleotide sequence and genotyping pattern after PCR amplification in carrier, normal and affected animals of Cholesterol Deficiency Haplotype (CDH)

	Primer Sequence (5′–3′)	T_a (°C)	Genotype and fragment size
Forward (Wild)	GGTGACCATCCTCTCTCTGC	59	Normal: 249
Forward (Mutant)	CACCTTCCGCTATTCGAGAG		Carrier: 249, 436
Reverse	AGTGGAACCCAGCTCCATTA		Affected: 436

*T_a = Annealing temperature

amplification. The diagnostic test described by Menzi *et al*. (2016) was used to screen for CDH mutation. In this test a combination of three allele-specific primers were allowed for genotype differentiation (Table 1): reverse common primer starting from wild sequence 5′ AGTGGAACCCA GCTCCATTA 3′ and two forward primers discriminating wild sequence from mutant. The wild forward primer 5′ GGTGACCATCCTCTCTCTGC 3′ ensured amplification of 249 bp, but mutant forward primer 5'CACCTTCCGCTA TTCGAGAG 3' starting from inserted LTR element produced larger amplicon of 436 bp.

The single tube PCR amplification using each of three primers was standardized. PCR was conducted in a total volume of 10 µl with 1.5 µl of template DNA (50 ng/µl concentration), 0.4 µl forward (wild), 0.4 µl forward (mutant) and 0.4 µl reverse primer (10 pmole/µl concentration of each), 5 µl of 2 × Dream Taq green master mix (Thermo Fisher Scientific, US) and 2.3 µl of water. PCR was performed in thermal cycler (T-100 Bio Rad) as follows: initial denaturation at 95°C for 3 min and 35 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for 1 min, with a final extension of 72°C for 10 min. The PCR products were separated on 2% agarose gel including 0.5 µg/ml of ethidium bromide, and photographed under UV light. CDH carrier yielded fragment of 436 bp size along with 249 bp product, whereas normal animals revealed a single band of 249 bp (Fig. 1).



C = 436, 249 bp; N = 249 bp; M = 100 bp ladder

Fig. 1. Screening of the CDH mutation. PCR products were electrophoresed through 2% agrose gel.

The mutant *APOB* gene was found in one of the Holstein Friesian bull; while it was absent in other genetic groups. The incidence of the mutation was 1.67% (1 out of 60) in Holstein Friesian bulls. The presence of mutant allele was confirmed by DNA sequencing. PCR amplification of carrier in two separate tubes, one with forward (wild) and reverse primer and another with forward (mutant) and reverse primer was done giving product lengths of 249 and

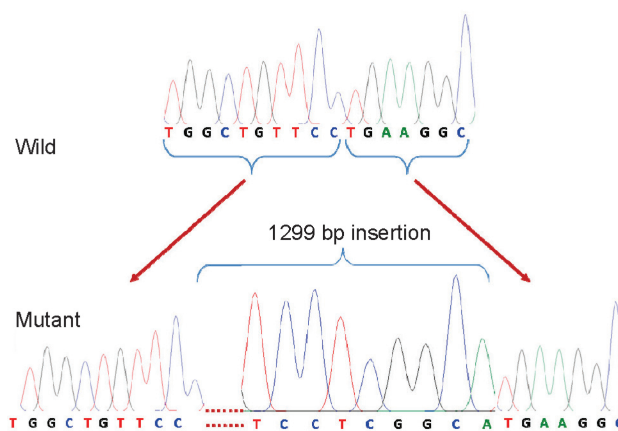


Fig. 2. Sequencing of PCR products of 249 bp (in normal) and 436 bp (in mutant) showing insertion of 1,299 bp in CDH carrier.

436 bp, respectively. Both these samples were sequenced by First Base INT (Malaysia), which confirmed the insertion of nucleotides in heterozygous condition (Fig. 2).

The carrier frequency (1.67%) of CDH genetic defect in Indian Holstein Friesian population is lower than the average literature values in other countries (Kipp *et al*. 2015, Kipp *et al*. 2016, Schütz *et al*. 2016, Kamiñski and Ruœæ 2016, Cole *et al*. 2016, Li *et al*. 2018). Kipp *et al*. (2015) calculated a carrier frequency of about 8.7% from the estimated 3,400 Holstein calves born each year homozygous for CD haplotype in Germany. However, Kipp *et al*. (2016) reported maximum carrier frequency of 12.25% in German Holstein population during 2012 which declined to 7.87% in 2016 due to screening strategies over the year. In another study, Schütz *et al*. (2016) found 12.5% carriers among Holstein bulls born during 2012 to 2015 in Germany. Kamiñski and Ruœæ (2016) reported substantially much higher frequency (33.33%) of CDH carriers in Polish Holstein cattle. However, mean carrier frequency of 2.5% for CDH haplotype was observed in U.S Holstein population (Cole *et al*. 2016). The percentages of CDH carriers in Chinese Holstein were found to be around 5.07% and 1.11% for bulls and cows, respectively (Li *et al*. 2018).

SUMMARY

Cholesterol deficiency is a new monogenic autosomal recessive inherited defect in Holstein cattle and is associated with chronic and recurrent diarrhea followed by death of calves during the first six months of life. We have

successfully screened the causative mutation for cholesterol deficiency in indigenous, exotic and crossbred cattle of India. This is the first report about the presence of *APOB* mutation causing calf mortality in the Indian Holstein Friesian population. The mutation associated with cholesterol deficiency was not detected in any of indigenous breeds of cattle suggesting that the mutation could be specific to Holstein Friesian breed only. The occurrence of causal mutation for cholesterol deficiency in one of the Holstein Friesian bull under study (with carrier frequency of 1.67%) clearly indicates that mutation has already been transmitted into Indian Holstein-Friesian population. In our opinion it is the right time to take stringent actions in order to check its further spread. Further, this study accentuates the need for regular screening of existing as well as importing breeding bulls, especially of Holstein-Friesian lineage, for CDH genetic defect before introduction of bulls into genetic improvement programs. It is also highly recommended that the screening for CDH mutation must be made mandatory for all the semen producing/importing agencies under the minimum standards for production of bovine semen.

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