Research paper

Development of allele-specific PCR based DNA test for detection of syndactylism (mule foot) related missense mutation in Holstein Friesian cattle

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ABSTRACT

Syndactylism or 'mule foot' is an autosomal recessive disorder of cattle, characterized by fusion of digits resulting in painful hooves and reduced mobility. The genetic defect has been reported in Holstein cattle populations, worldwide. Molecular studies have indicated the substitution of 2 nucleotides (delCGinsAT) in reading frame belonging to exon 33 of low-density lipoprotein-receptor-related protein 4 gene (LRP4) at chromosome 15 as the cause of mule foot. In this study, an allele specific polymerase chain reaction (AS-PCR) based method was developed for detecting the mule foot allele in cattle. In order to identify normal (wild-type) and mutant alleles at mule foot disease locus separately, two separate allele-specific forward primers were designed for each of the allele along with a common reverse primer. PCR reactions and conditions were standardized for amplification of each of loci separately. The wild-type and mutant alleles could be easily distinguished by detection of amplification from PCR of wild allele (CG) primer set, whereas no amplification by primer set for mutant allele (AT). Using the protocol, total 24 samples of Holstein Friesian (HF) bulls were screened, which were found to possess normal genotype at mule foot locus in all of the animals. This allele-specific PCR based DNA test was found suitable for the detection of mule foot mutation in cattle population specifically of Holstein lineages.

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INTRODUCTION

In cattle, syndactylism, also called "mule foot," refers to the fusion or non-division of the two functional digits of the bovine foot, consisting synostotic phalanges (Hart-Elcock et al., 1987; Charlier et al., 1996). Affected calf can have 1-4 fused hooves with varying degree of lameness and reduced mobility due to painful fusion of the hooves; and stiff and insecure joints. Interestingly, this abnormality is subjected to a right–left and a front–rear gradient i.e. right front foot is always first and most severely affected; if two feet are affected, it will be the two front feet; the right hind foot is next and the left hind foot is last and least likely to be fused. The condition can be detected as early as 42 days post conception with autoradiography (Gruneberg and Huston, 1968). Although, the disease is simple autosomal recessive defect and reported in a number of cattle breeds however, it is ost prevalent in Holsteins. The disease also shows incomplete penetrance (79% in Holstein cattle) and variable expressivity (Huston, 1967, Millar et al., 2000). It is estimated that almost one-fifth of animals with homozygous condition may not show the fused hooves. Mulefoot carriers also had higher somatic cell score and lower productive life (Cole et al., 2016).

Earlier through genetic analysis, the locus of the disease trait was mapped to chromosome 15 (BTA 15) (Charlier et al., 1996; Drogemuller and Distl, 2006). Later, based on recent findings concerning the mouse mutants *dan* and *mdig* and a mouse knockout experiments, doublet missense substitution (c.4863_4864delCGinsAT) in exon 33 of

low-density lipoprotein-receptor-related protein 4 (LRP4) gene was identified as a strong candidate causal mutation for syndactylism in Holstein cattle (Duchesne et al., 2006, Drogemuller et al., 2007). However, four other *LRP4* non-synonymous point mutations were further reported in Holstein (c.4940C>T exon 33), Simmental (c.241G>A exon 3, c.3595G>A exon 26) and Simmental-Charolais crossbred (c.2719G>A exon 20) breeds of cattle. The LRP4 gene encodes a member of the multifunctional low-density lipoprotein (LDL) receptor molecule and has a critical role in limb development, involved in the control of limb development (Nykjaer et al., 2002). The gene alternatively called multiple epidermal growth factor-like domains 7 (MEGF7) is also involved in the control of bone morphogenetic protein and fibroblast-like growth factor. The mutation (c.4863_4864delCGinsAT) leads to amino acid changes at two *LRP4* codons (p. Asn1621Lys; Gly1622Cys) affecting a conserved EGF like protein domain in Holstein cattle. However, other mutations at different locations of LRP4 coding sequence were also found responsible for the similar disease in Simmental cattle (Drogemuller and Distl, 2006).

In the 1970s, a peak in the frequency of mule foot animals was observed in the bovine population, however with the development of progeny testing (i.e., mating of tested individuals with affected animals) and of microsatellite marker genetic testing the frequency of syndactylism has considerably declined (Johnson et al., 1980) and is about 0.07% in U.S Holstein cattle at present (Cole et al., 2016). However, the fact that mule foot animals are observed in the current Holstein population reveals that carrier animals, especially bulls, are still used in cattle breeding in some parts of the world. Thus, eradication of this disorder specially in cattle population with Holstein lineages requires detection methods that are simple, accurate and economical. In this paper we describe a simple and cost effective allele specific PCR protocol to screen mule foot i.e. syndactylism disease in cattle.

MATERIALS AND METHODS

Samples and DNA extraction

The blood samples of HF bulls were used for the standardization of PCR as well as further screening. DNA isolation was carried out as per standard phenol– chloroform procedure (Sambrook and Russel, 2001). The isolated DNA was checked for purity and quantity using Nanodrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, USA) and the integrity was assessed by running in 0.8 % agarose gel. The isolated genomic DNA was stored at -20 °C until further use.

Primer designing

Primers were designed manually to amplify flanking region of syndactylism locus (15:g.77675516CG>AT) at LRP4 gene (GenBank accession number, AC_000172.1). According to the principle of allele-specific PCR, two allele specific forward primers were designed for CG-wild type (F-WT) and AT- mutant allele (F-M). Since, the two nucleotides at their 3 terminus corresponded to the two nucleotide substitutions (CG>AT) in forward primers (Table 1). The reverse primer was designed as the common primer at downstream of the sequence. The primer sequences were searched for uniqueness using the NCBI BLASTW search engine.

PCR amplification

Two separate PCR reactions were prepared in the final volume of 20 µL in two tubes, each containing separate forward primer along with other common ingredients. The final concentrations of the PCR ingredients were: 14.35 µl of distilled water, 2 µl of 10×PCR buffer, 0.5 µl of 10mM dNTP mix (Thermo Fisher Scientific), 0.5 µl of each primer (10 pmol/ µl), 2.0 µl of genomic DNA (50 ng/ µl concentration), and 0.15 µl of Taq DNA polymerase

Table 1: Primer sequences and PCR parameter for genotyping mulefoot disease locus in cattle

Primer	Primer Sequences (5' x 3')	Optimal primer concentration (µM)	Amplicon size (bp)
F(WT)	5'-CTTGTGGCGTGAACAA**CG**G-3'	0.25	932
F(M)	5'-CTTGTGGCGTGAACAA**AT**G-3'	0.25	
R	5'-CGCAGGGTAGTCGGTAATGT-3'	0.25	

(5 U/ µl) (New England Biolabs). Gradient PCR cycling were followed for both the reactions. The PCR conditions were: initial denaturation at 95 °C for 2 min 30 sec and 32 cycles of denaturation at 94 °C for 30 sec, annealing at 55 to 61 °C for 30 sec, and extension at 72 °C for 60 sec and a final extension at 72 °C for 10 min. PCR products were analyzed on 2% agarose gel with ethidium bromide in TAE buffer for 50 min at 80V. After the electrophoresis, the PCR products were differentiated for allele identification as well as for their intensity of amplified fragments. The PCR conditions with most intense amplicon were chosen for further screening of 24 DNA samples of HF bulls.

RESULTS AND DISCUSSION

In order to screen out normal (wild-type) and mutant alleles at mulefoot disease locus in cattle of exotic inheritance specifically of Holstein, an allele-specific PCR based protocol was developed in this study. We designed two allele-specific forward primers for each of the allele and a common reverse primer to discriminate both of the alleles. We designed two 19-nucleotide-long allele-specific forward primers, F(M) and F(WT), complementary to the 5' end of the mule foot disease and normal locus, respectively while the oligonucleotide primers differ from each other by a single nucleotide at the 3' end. These primers were used in two separate tubes along with common reverse primer. The primers were selected because these fulfilled the criteria for highly-efficient allele-specific amplification as there were two nucleotides differences in wild and mutant genotypes.

Results of PCR cycles at different annealing temperature showed the amplifications at all of the annealing temperatures with almost equal intensity of bands of 932 nucleotide long for primers set of wild allele (Fig. 1). Among these, PCR reaction with initial denaturation at 95 °C for 2 min 30 sec and 32 cycles of denaturation at 94 °C for 30 sec, annealing at 58.5 °C for 30 sec, and extension at 72 °C for 60 sec and a final extension at 72 °C for 10 min was taken as standard. Final volume and concentrations of PCR ingredients - 2 µl of 10×PCR buffer, 0.5 µl of 10mM dNTPs, 0.5 µl of each primer (10 pmol/ µl), 2.0 µl of genomic DNA (50 ng/ µl), and

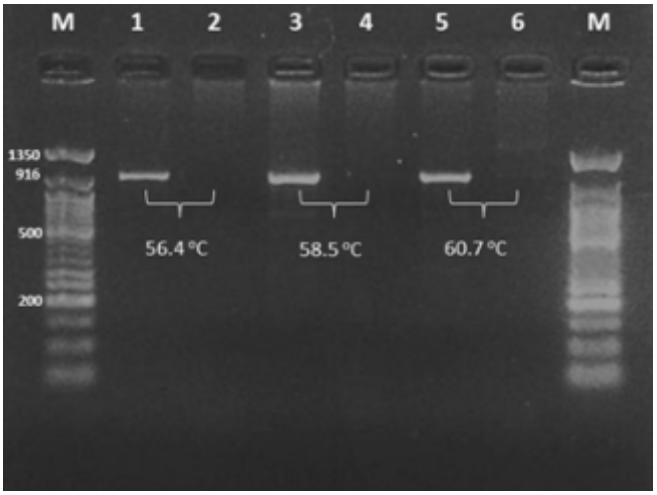

Fig. 1. Gradient PCR reaction for amplification of mulefoot disease locus. Ladder marker 50 bp (M), wild type allele (Lanes nos. 1,3,5), mutant allele (Lanes nos. 2, 4, 6).

0.15 µl of *Taq* DNA polymerase (5 U/ µl) in 20 µl reaction were also optimized with the PCR conditions. There was no amplification for the primers set of mutant allele which indicated absence of mutation in the samples. In carrier animals, both CG- and AT-specific primer pairs give rise to two PCR fragments of same length. However, in animals free from mulefoot disease, one DNA band could be recognized only from CG specific primer pair (wild type). Non amplification of PCR from primers specific to mutant allele also indicated about not yielding any false positive result by the protocol. This AS-PCR test was applied to the 24 samples of HF bulls, which also showed the presence of normal allele through observing CG allele specific PCR products (Fig. 2).

The AS-PCR, also known as amplification refractory mutation system (ARMS) or PCR amplification of specific alleles (PASA) is deemed to be a simpler, faster and reliable method for detecting the mutation involving single base change or small deletions (Kwok and Chen, 2003). It works on the principle of sequence-specific PCR primers that allow amplification of test DNA only. AS-PCR method works efficiently, if the nucleotide at the 3'-end of the primer perfectly complements the base at the mutant or wild-type sequences and there will be no amplification if target allele is not present in the DNA. The LRP4 c.4863_4864delCGinsAT mutation is particularly suitable for the development

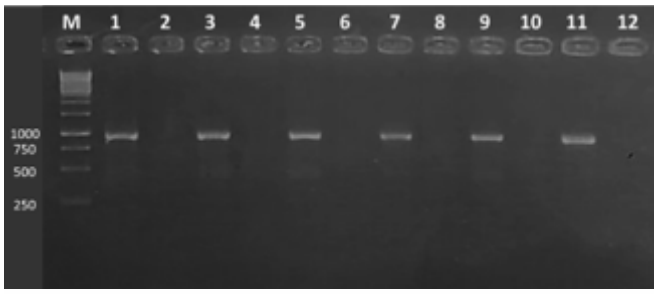

Fig. 2. Allele specific PCR reaction (AS-PCR) for diagnosis of cattle for mule foot.
Ladder marker 1 Kb (M), wild type cattle (Lanes nos. 1–2, 3-4, 5-6, 7–8, 9-10, 11-12).

of a allele specific PCR system because it is produced by a double substitution (CG>AT). Therefore, the use of an additional mismatch is not necessary.

The technique has been successfully applied previously for noninvasive prenatal screening of single-gene disorders like sickle cell anaemia and β-thalassaemia in human (Wu et al., 1989; Chiu et al., 2002); and Complex vertebral malformation in animals (Ghanem et al., 2008). The allele-specific PCR protocol is a time-efficient and cost-effective method for screening. It also reduces the risk for experimental errors since it involves fewer steps.

CONCLUSION

In this study, an allele-specific PCR based screening protocol was developed to screen out at mule foot disease mutation in cattle of exotic inheritance specifically of Holstein. By this protocol, wild-type and mule foot disease alleles could be easily distinguished by detection of amplification from PCR of wild allele (CG) primer set and no amplification by primer set for mutant allele (AT). This allele-specific PCR based DNA test was found simple, accurate and inexpensive and suitable for the detection of syndactylism related mutation in cattle population with Holstein lineages.

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