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IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM IN EMX2 GENE OF CROSSBRED DAIRY COWS WITH ANATOMICAL DEFECTS IN GENITALIA

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ABSTRACT

Emx2 is expressed during the development of the urogenital system and its mRNA can be seen in the primordia that would eventually give rise to the kidney, gonads and genital tract at the earliest stages of differentiation. *Emx2* is thought to be indispensable for the formation of both Mullerian and Wolffian ducts. The objective of the present study was to identify the single nucleotide polymorphism of emx2 gene in the genitalia of dairy crossbred cows with anatomical defects. An intronic variant c. 38215870 A>G identified in the ddRADseq was determined in the PCR product of 14 animals with anatomical defects in the female genitalia. The coding region of emx2 gene was amplified with an annealing temperature of 63.6 °C for 35 amplification cycles. Then the PCR products were subjected to restriction enzyme digestion by using the enzyme *Hinfl*, which was incubated at 37°C for one hour. While subjecting this fragment to PCR- RFLP, single band pattern was observed. All the animals exhibited a similar pattern, inferring that the population was monomorphic for this locus. None of the animals with anatomical defects in the female genitalia showed an emx2 mutation. According to this study, Mullerian duct defects in crossbred dairy animals were not frequently caused by emx2 gene.

Keywords: Emx2 gene, Mullerian duct, PCR-RFLP, dairy cows

INTRODUCTION

Mullerian duct developmental abnormality is a congenital malformation of the female reproductive tract and is characterised by abnormal Mullerian duct genesis, differentiation and development. Agenesis and abnormal Mullerian duct fusion are the causes of congenital uterine abnormalities (Troy *et al*., 2003). Several genes have been identified as crucial for

the growth of the Mullerian duct based on research using both human and animal models. Single nucleotide polymorphisms (SNPs) are used to predict breeding values. Genomic prediction holds great potential for dairy cattle breeding (Barkema *et al*., 2015; Boichard *et al*., 2016). The emx2 gene encodes a homeobox-containing transcription factor necessary for reproductive tract development. It is expressed in the early primordia of the reproductive systems, such as the Mullerian ducts. According to several researchers, *emx2* is expressed in the mouse uterus right before implantation, indicating that it is a crucial component in the development of the mouse female reproductive system (Pellegrini *et al*., 1996).

Emx2 is expressed during the development of the urogenital tissues and its mRNA can be seen in the primordia that would eventually give rise to the kidney, gonads and genital tract at the earliest stages of differentiation. *Emx2* is also expressed in the adult uterine endometrium and suggested a role for this gene in suppression of endometrial epithelial proliferation (Liu *et al*., 2015). In mice, *emx2* is thought to be indispensable for the formation of both Mullerian and Wolffian ducts. Mice deficient in *emx2* lack reproductive tracts, gonads and kidneys. Developmental abnormalities in the Mullerian duct derivatives are seen in mice with a homozygous null mutation for *emx2*. Zhu *et al*. (2016) studied the role of *emx2* in women with partially septate uterus and observed a significant higher concentration of *emx2* mRNA and protein levels in patients with septate uterus when compared with the control. Point mutations in human *emx2* result in severe schizencephaly. Its higher expression resulted in lower expression of *hoxa*10 and suggested that this might be the cause of anatomical infertility. Daftary and Taylor (2004) reported that *emx2* encoded a regulatory protein which favoured the growth and differentiation of reproductive tract. The studies on genetic factors relating to female genitalia of crossbred dairy cattle are rare. Hence, the present study was designed to identify the SNP of emx2 gene in the genitalia of crossbred dairy cows.

MATERIALS AND METHODS

In the present study, ovaries of 100 dairy cows / heifers were collected from the Meat Technology Unit, Mannuthy. This included six animals culled on account of factors other than infertility with a normal reproductive system and the remaining animals with a history of infertility.

Amplification and standardisation of gene

396bp region of emx2 gene was amplified using specific set of primers

which was designed by using "Primer 3 Plus" software (Table 1). The PCR conditions were optimised using different concentrations of reagents and different temperatures ranging from 58-65 ºC. The reaction mix and cycling conditions used are given in table 2. The PCR products were checked by gel electrophoresis using two per cent agarose gels along with 100 bp DNA Ladder (Thermo Scientific). Electrophoresis was performed and the gels were visualised under UV light and recorded in a gel documentation system.

Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP)

The methodology involved in PCR-RFLP included extraction of DNA, PCR amplification, restriction digestion and electrophoresis. The PCR products obtained were sequenced for confirmation and subjected to analysis using Primer Blast and Primer STAT. The amplicon sequence enclosing the selected variants was pasted in NEBcutter (http://nc2.neb.com/NEBcutter2/) and the restriction endonucleases which identified the polymorphisms were noted. The enzyme *HinfI* with the recognition sequence 5′G ANTC3′ was found to identify selected polymorphism.

The RFLP reactions were set up in 0.2 millilitre PCR tubes. All components except PCR products were mixed to prepare a master mix and then the required volume from the master mix was dispensed into each of the tubes which contained PCR products. The composition and condition of reactions are presented in table 3. The samples after restriction digestion were checked in two per cent agarose gels prepared in TBE along with a molecular weight marker (100 bp).

Table 1. Primers

Primer	Primer sequence (5'-3')
emx2 F	GCCAAGGGTCAGCTTCTATG
emx2 R	GGGGCATACTGTCCTCTCTG

Table 2. Standardised PCR protocol of *emx2*

Steps		Temperature	Time
Initial denaturation		95 °C	3 min
35 cycles of	Denaturation	95 °C	20 sec
	Annealing	63.6 °C	30 sec
	Extension	72 °C	30 sec
Final extension		72 °C	2 min

Table 3. Optimised concentration of PCR mixture for PCR-RFLP

Sl. No.	Constituents	Reaction volume
1	PCR product	2.5 µL
2	Enzyme	2 µL
3	Buffer	2 µL
4	Water (Millipore)	13.5 µL
	Incubation time and temperature	37 °C for 1 hour

RESULTS AND DISCUSSION

Among 100 animals studied, 14 animals showed anatomical defects in the genitalia. The anatomical defects noticed in the reproductive system were categorised as ovarian hypoplasia, tubal obstruction, uterus unicornis, bicornis bicorpus unicollis and kinked cervix.

An intronic variant c. 38215870 A>G was identified by ddRADseq. Amplification of gene fragments of 14 animals with anatomical defects and animals with normal genitalia was done as described in table 2. Polymerised chain reaction of *emx2* fragment produced a 396 bp product.

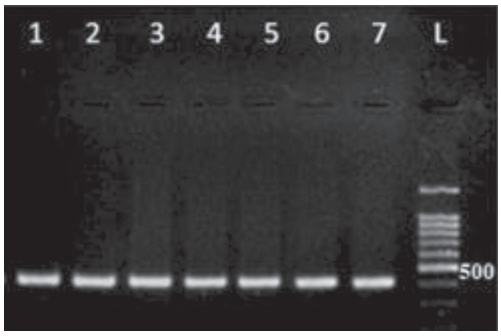

Fig. 1 PCR amplification of 396 bp fragment of emx2 gene
Lane 1-7: 396 bp product
Lane 8: 100 bp DNA Ladder

The products were subjected to restriction enzyme digestion as described in table 3. Visualisation of digested fragments in two per cent gel revealed single band pattern (Fig. 1), corresponding to homozygous AA. For further confirmation, pooled amplicons

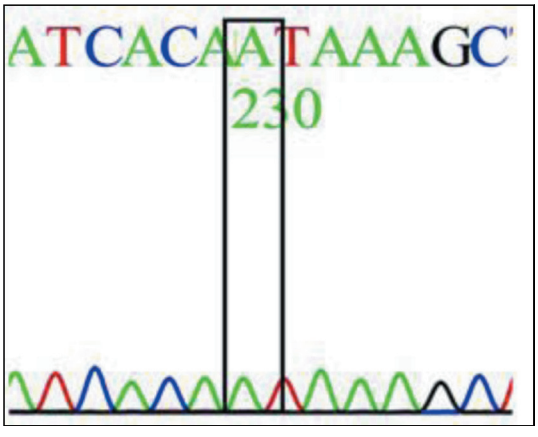

Fig. 2 Sequence map of AA genotype of group 1

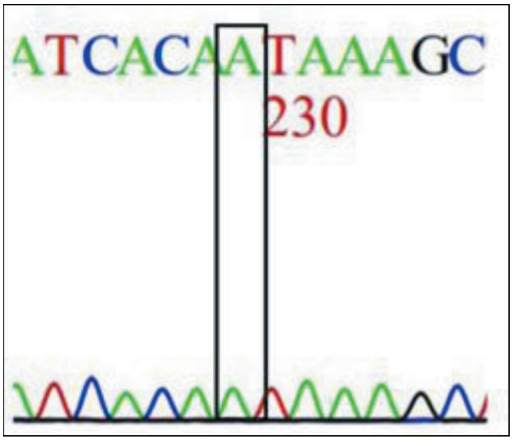

Fig. 3 Sequence map of AA genotype of group 2

from both the groups were sequenced and a snapshot of chromatogram depicting the AA genotypes in forward fragment is given in figures 2 and 3.

While subjecting this fragment to PCR- RFLP, single band pattern was observed. All the animals exhibited a similar pattern, inferring that the population was monomorphic for this locus. None of the 14 animals with anatomical defects in the female genitalia showed an *emx2* mutation at the particular site. Contrary to this, Liu *et al*. (2015) detected a mutation at c424 G>T by PCR analysis in woman with a didelphic uterus by the formation of premature stop codon at 142th position. Normally, wild type *emx2* encoded 252 amino acid, whereas this mutated *emx2* encoded only 141 amino acid with lacking homeodomain. Noonan *et al*. (2003) conducted *emx2* knock out gene study in mice and observed that in such mice, gonads and genital tract were not developed. This clearly indicated the role of *emx2* in Mullerian duct development.

This gene is the human ortholog of the Drosophila empty spiracles (ems) gene. In the fly, the ems gene regulates head and brain development, as well as the formation of the spiracles at the posterior end of the fly embryo. When *emx2* was specifically disrupted, it causes severe abnormalities in the reproductive system, kidneys and brain growth in mice. *Lim1*, *Pax2* and *Wnt4* were differentially expressed in the intermediate mesoderm in *emx2* mutant mice. (Miyamoto *et al*., 1997; Bazer, 2010). These related mechanisms suggest an underlying genetic pathway for the formation of the Mullerian ducts. Reduced *emx2* activity showed minimal impact; artificially enhanced *emx2* activity in mice restricts litter size. Similar to endometriosis, elevated *emx2* was observed in endometriosis and was thought to negatively impact endometrial development, allowing for greater proliferation and impaired decidualization (Daftary and Taylor, 2004). In the current study, there was no evidence of an *emx2* mutation in the animals with uterine abnormalities. According to this study, Mullerian duct abnormalities in dairy crossbred cows were not frequently caused by emx2 gene.

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