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Identification of polymorphism in *OPN* gene in Rathi cattle through SSCP method

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Abstract
The present study was undertaken to explore the genetic variability in part of intron-5 exon-5 portion of intron-6 region of *OPN* gene in 160 Rathi cattle. From each animal in selected population 2 ml blood were collected for DNA isolation by kit method. Species-specific primer was constructed based on available sequences of *OPN* gene in the NCBI GenBank database which used to amplify this region of *OPN* gene. A polymerase chain reaction in a final reaction volume of 25µl were prepared for region of *OPN* gene and after amplification fragments of 826 bp of part of intron-5 exon-5 portion of intron-6 region of *OPN* gene was obtained. Single-strand conformation polymorphism (SSCP) method was carried out to detect the genetic variation. Monomorphism was observed in selected region of the *OPN* gene. In this study this region of *OPN* gene not showed genetic polymorphism. Some studies shows polymorphism in this region of *OPN* gene. In this study presence of a single SSCP pattern was observed which indicates more commen pattern of this region of *OPN* gene. Further analysis of this gene in Rathi cattle is required to detect polymorphism and its association with milk production traits. Its variants would be used in the marker-assisted selection for the improvement of dairy traits in cattle.

Keywords: Polymorphism, *OPN* gene, Rathi cattle, PCR and SSCP

Introduction
Milk is characterized as secretion from mammary glands in mammals and it meets the complete requirement of infants (Fox, 2009) [5]. The number of female cattle is 145.12 million, which increased by 18.0% over the previous census Livestock census, 2012. According to BAHFS (2019) [3], Rajasthan produced 12.6 % of total milk production and holds the second rank in India. A Rathi cattle is native breed of Bikaner division (Anonymous, 2018) [2]. In dairy animals, milk production is an important economic trait. Rathi cattle produce good lactation milk yield in dry conditions and semi-arid region of Rajasthan (Dhaka *et al.*, 2015) [4]. Nowadays, breeding policies emphasized the conservation and improvement of the indigenous pure breed of cattle for improving milk production and productivity in a scientific manner (Anonymous, 2014) [1]. The *OPN* gene is located on bovine chromosome 6 along with six quantitative trait loci (QTL) influencing the milk production of dairy cattle (Schnabel *et al*., 2005) [18]. Molecular markers used for selecting superior animals for higher production and reproduction performance traits (Kumar *et al*., 2020) [10]. It is an acidic member of the small integrin-binding ligand N-linked glycoprotein (sibling) family (Johnson *et al*., 2003) [7]. Health, development, including the immune system and gut health, are also affected by *OPN* (Joung *et al*., 2005) [8]. Osteopontin (*OPN*), a phosphoprotein plays various roles in an immune response. *OPN* mRNA contained a protein-coding open reading frame of 834 bp which codes for amino acids (Karthikeyan *et al*., 2020) [9]. The present study was conducted to detect genetic variation in region of *OPN* genes through molecular marker approaches such as SSCP.

Material and Methods
An overall 160 animals were selected for the present study from a pure breeding population of Rathi. Only milking cows with a minimum of 120 days of lactation were included in the study. The Rathi cattle for the present trial were selected from Livestock Research Station of Rajasthan University of Veterinary and Animals Sciences, Bikaner (Rajasthan). About 2 ml of blood samples were collected aseptically from jugular vein puncture into the anticoagulant Ethylene Diamine Tetra Acetic acid (EDTA) containing vacutainers tube from all the animals of Rathi cattle. Genomic DNA from the whole blood sample was extracted through the spin column method as per standard method (Sambrook and Russell, 2001) [17]. The purity (OD ratio 260/280) and concentration (ng/µl) of extracted genomic DNA was determined by a

Nanodrop spectrophotometer and quality through 0.8 % agarose electrophoresis. The primers according to the Part of intron-5 exon-5 portion of intron-6 region of *OPN* gene was constructed on basis of available sequences of *OPN* gene in the NCBI GenBank database (Table 1)

Table 1: Primer sequences and expected fragment sizes of PCR products of selected genomic regions

Selected Region	Primer Sequences	GenBank Accession No.	Expected Fragment Length	References
OPN Part of Intron- 5 Exon-5 portion of Intron-6	Forward 5'CTGAGGAAACTGATGACAAC3'	AY878328.1	826	Salehi (2015) [15]
	Reverse 5' GCTTTCATTGGACTTACTTGG3'			
	Reverse 5'AGACCTCATCCTTGGGCC3'			

The allele-specific polymerase chain reaction was carried out to amplify selected genomic regions of *OPN* gene using specific desalted oligonucleotide primers custom synthesized in accordance with Leonard *et al*., 2005 [12] (Table 2). Amplification was carried out in PCR thermo cycler with programme illustrated in Table 3.

Table 2: PCR reaction mixture used for amplification of genomic DNA of Part of intron-5 exon-5 and portion of intron-6 of *OPN* gene

S. No.	Contents	Volume
1.	5X PCR buffer	5 µl
2.	1.5mM MgCl2	2 µl
3.	10 Mm dNTP's mix	1 µl
4.	Forward primer 70pmol/µl	1.5 µl
5.	Reverse primer 70pmol/µl	1.5 µl
6.	Genomic DNA 25 ng/µl	4.5µl
7.	Taq DNA polymerase 5U/µl	0.25 µl
8.	DNAase free water	9.25 µl

Table 3: PCR programming for amplification of part of intron-5 exon-5 and portion of intron-6 of *OPN* gene

Steps	Temperature	Time	No. of Cycle
I. Initial Denaturation	95ºC	5 min.	1 cycle
II. Cycle			
(i). Denaturation	95ºC	45sec	35 cycles
(ii). Annealing	55ºC	1min	
(iii). Synthesis	72ºC	1 min	
III. Final extension	72ºC	10min	1 cycle
IV. Hold	4ºC	5min	1 cycle

The quality and size of the PCR amplicons for different studied locus were assessed on 1.5% agarose gel containing ethidium bromide (1 % solution). 5 µl PCR product of each amplified sample was mixed with 1µl of 6X gel loading dye. The samples were then loaded on a precast 1.5 % agarose gel plate along with a standard molecular weight marker. Electrophoresis was carried out at a constant voltage of 120 V in 0.5X TBE buffer till the dye reaches opposite ends. The size of the amplified DNA fragments was assessed by comparison with a standard molecular weight marker.

Detection of Genetic Variation

The genetic variation in the selected genomic region of *OPN* gene was identified through SSCP method.

Single-strand conformation polymorphism (SSCP) analysis

To detect mutations, SSCP analysis (Orita *et al*., 1989) [13] was performed according to guidelines described by Hayashi and Yandell (1993) [6] with slight modifications. Aliquots of 5 µL PCR products were mixed with 5 µL denaturing solution (95 % formamide, 25 mM EDTA, 0.025 % xylene cyanole and 0.025 % bromophenol blue), denatured for 10 min at 95 °C followed by a rapid chill on ice for 10 min. Denatured PCR products were subjected to 8 % polyacrylamide gel electrophoresis in Tris-Borate-EDTA buffer and constant voltage (120 V) for 15 h at a constant temperature of 4 °C, and then gels were stained with 1% ethidium bromide solution and visualized with under UV light and documented by gel documentation system. Individual genotypes were defined according to band patterns. The frequencies of different electrophoretic patterns were recorded under each group.

Results

The present study was conducted to detect genetic variation in region of *OPN* gene through molecular marker approache such as SSCP. Osteopontin protein and is considered as key links in the gene networks constituting the hereditary component of milk productivity. *OPN* is located in the quantitative trait loci (QTL) for milk production traits and milk composition in BTA 6 (Lali *et al*., 2020) [11].

Isolation of Genomic DNA

Good quality genomic DNA was checked through 0.8% agarose and nano spectrophotometer. DNA was isolated from all blood samples of Rathi cattle. The DNA concentration varied between 50 to 75 ng/µl. The optical density (OD) 260/280 ratio, an indicator of purity was observed in the range of 1.6 to 1.8, which indicate the suitability of the extracted DNA samples for hassle-free in vitro amplification. All samples employed in the present investigation were found to

be devoid of fragmentation as evidenced by the absence of smearing on a gel and the presence of intact bright genomic DNA band.

Genetic Variation in part of intron-5 exon-5 portion of intron-6 of *OPN* gene

Amplification of part of intron-5 exon-5 portion of intron-6 of *OPN* gene

An annealing temperature of 55ºC was found to be optimal for the desired amplification of the 826-bp PCR product. Thirty-five cycles of amplification were required for complete and uniform amplification. A clear cut amplified band of 826-bp with no smearing was obtained in Rathi cattle (Fig. 1).

SSCP variations in part of intron-5 exon-5 portion of intron-6 of *OPN* gene

Single SSCP band pattern ('P1') was observed for the 826-bp fragment of intron-5 exon-5 portion of intron-6 of the *OPN* gene in Rathi cattle (Fig. 2). The SSCP analysis denotes the presence of only one allele ('A') of *OPN* gene for intron-5 exon-5 portion of intron-6 of locus in Rathi cattle.

Raschia *et al*., (2018) [14] shows dissimilarities with present study. In Argentinean dairy cattle, they investigated many genomic regions by genotyping of studied samples and observed thirty-nine SNPs of different genes; out of all markers in *OPN* gene have a SNP rs110930453 on intronic region and deletion in *OPN*3907 location. In an intronic region of the *OPN* gene with C/T variation in the sequence was observed.

Gene and genotypic frequency of part of intron-5 exon-5 portion of intron-6 of *OPN* gene

The variant in the analyzed locus was not found in the present investigation in Rathi cattle. In present study monomorphism was observed. In Iranian Holstein cattle, Salehi, (2015) [16] analyzed intron-5 exon-5 portion of intron-6 regions of the *OPN* gene and reported two allele with frequencies of T and C with frequencies as 0.59 ± 0.03 and 0.41 ± 0.03, respectively. Genotype frequencies of the three genotypes TT, TC and CC were 34.69, 48.62, and 16.69, respectively; work showed polymorphism in intron-5 exon-5 portion of intron-6 regions of the *OPN* gene. In this study single pattern was observed which shows higher number of this allelic form in this studied population.

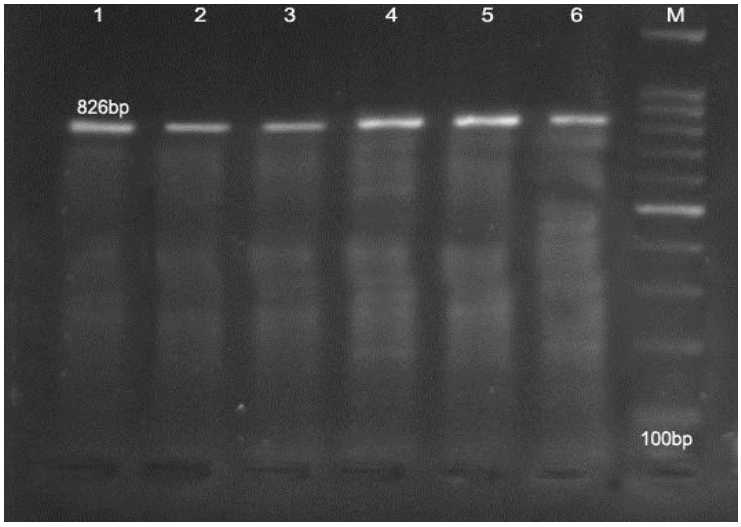

Fig 1: PCR amplified band of 826-bp of intron-5 exon-5 portion of intron-6 of *OPN* gene in Rathi cattle

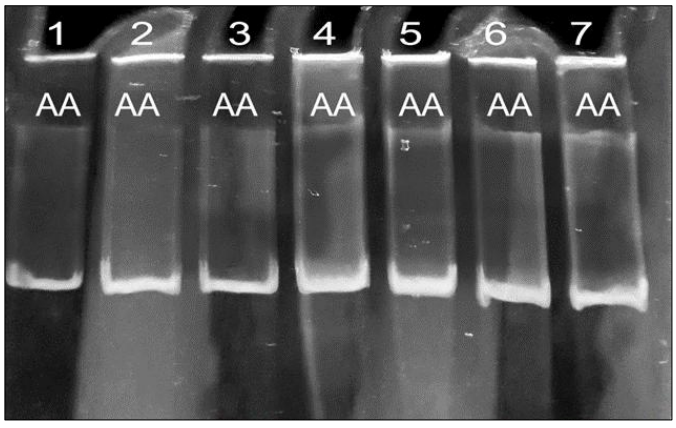

Fig 2: SSCP patterns of part of intron-5 exon-5 portion of intron-6 of *OPN* gene in Rathi cattle

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