Identification of polymorphism in ABCG2 gene in Rathi cattle

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Abstract
In present study, Rathi cattle were selected to analyze ABCG2 genes for polymorphism. Total 160 lactating animals of Rathi cattle from livestock research station (LRS), Nohar and Bikaner were selected. Blood samples were collected and DNA isolated by kit method. For Exon 7-partial intronic region, Exon 14 and Intron 15 region of ABCG2 gene primers were constructed based on available sequences of ABCG2 gene in the NCBI GenBank database. Fragments of 393, 207 and 323-bp of exon 7-partial intronic region, exon 14 and intron 15 region of ABCG2 gene, respectively were amplified by a polymerase chain reaction in a final reaction volume of 25μl. The PCR-RFLP and PCR-SSCP method was used according to region of ABCG2 gene. In exon 7-partial intronic region and exon 14 of ABCG2 gene shows monomorphism. In intron 15 region of ABCG2 gene two SSCP patterns were observed and it shows polymorphic nature of this region. In intron 15 region of ABCG2 gene observed two alleles and two genotypes with frequency 0.85, 0.15, 0.70 and 0.30, respectively, were observed. In present study, two regions of ABCG2 gene observed monomorphic nature and one region, intron 15 region of ABCG2 gene revealed polymorphic nature.

Keywords: Rathi cattle, ABCG2 gene, polymorphism and PCR

Introduction
In the world, India holds the first rank in milk production with 22 percent of global production [1]. Total milk production in-country is 187.75 million tonnes which are increased by 6.5% than the previous year. India has great diversity in livestock population and possesses 50 registered, well-recognized breeds of cattle [2]. Native milk cattle breed Rathi has breeding tract in Hanumangarh, Loonkaran sar tehsil of Bikaner, Shriganganagar districts of the state [3]. Rathi cattle contribute 0.83 percentage to the total population of indigenous cattle in India [4]. Rathi cattle breed is known for its hardiness to withstand the harsh agro-climatic conditions in the arid and semi-arid zone of Rajasthan. Even in dry conditions, Rathi animals have good potential for milk production. Rathi cattle produce good lactation milk yield in the semi-arid region of Rajasthan [5]. Conservation and improvement can be possible by the use of molecular tools in selecting animals for breeding. Milk production-related gene i.e. ABCG2 is located on bovine chromosome 6. This is responsible for genetic variation in milk production traits, milk fat and its composition of bovine milk [6, 7] affect absorption and distribution, altering the effectiveness and toxicity of drugs in animals and human [8, 9]. During lactation, in the mammary gland, the ABCG2 efflux transporter has induced the secretion of different compounds into milk. ABCG2 gene mapping and characterization of genes controlling important milk performance, production, health and quality traits have become an important field of research in livestock [10, 11]. Therefore, the excellent candidate genes, ABCG2 gene having a crucial role in milk production and milk composition were included in the present investigation.

Materials and Methods
An overall 160 Rathi animals were selected from Livestock Research Station Nohar (LRS), Hanumangarh and Bikaner (80 animals from each farm). Only milking cows with a minimum of 120 days of lactation were included in the study. After approval of ethical committee, Blood samples were collected aseptically from jugular vein puncture into the anticoagulant EDTA containing vacutainers tube and were transported to Molecular Genetics Laboratory in an...
icebox. Genomic DNA from the whole blood sample was extracted through the spin column method as per standard method [13]. Three primers according to the Exon 7, Exon 14 and Intron 15 region of ABCG2 gene were constructed based on available sequences of ABCG2 gene in the NCBI GenBank database. The sequences of primers, the accession number of the reference sequence and expected fragment length of the different selected regions are represented in Table 1.

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

| Selected Region     | Primer Sequences                  | Gen Bank Accession No. | Expected Fragment Length | References |
|---------------------|-----------------------------------|------------------------|--------------------------|------------|
| ABCG2 Exon 7 and partial Intron region | Forward 5'-TAAAGGCAGGAGTATAAAG-3'  | NC007304.4            | 393                      | [13]       |
|                     | Reverse 5'-TAA CAC CAA ACT AAC CGA AG-3' |                        |                          |            |
| ABCG2 Exon 14       | Forward 5' CACGAGACTGTACGGGAATTT 3' | AJ871176              | 207                      | [14]       |
|                     | Reverse 5' GGACATGAAACCAGCACGGT 3'  |                        |                          |            |
| ABCG2 Intron 15     | Forward 5'-TGGATACGAGCCCAATCCC-3'  | AJ871176              | 323                      | This study |
|                     | Reverse 5'-AGCCACTGTACGTGAACG-3'   |                        |                          |            |

PCR reaction mixture (25μl) used for amplification of genomic DNA of different region of ABCG2 gene used in present study are presented in Table 2.

Table 2: PCR reaction mixture used for amplification of genomic DNA of different region of ABCG2 gene

| S. No. | Content                              | Volume
|--------|--------------------------------------|---------|
|        |                                      | Exon 7 | Exon 14 | Intron 15 |
| 1.     | 5X PCR buffer                        | 5μl     | 5μl     | 5μl       |
| 2.     | 1.5 mM MgCl2                         | 3μl     | 3μl     | 2.5μl     |
| 3.     | 10 Mm dNTP’s mix                     | 1μl     | 1μl     | 1μl       |
| 4.     | forward primer (70pmol/μl, 80pmol/μl, 70pmol/μl) | 1μl | 0.75μl | 0.75μl |
| 5.     | reverse primer 70 pmol/μl (70pmol/μl, 80pmol/μl, 70pmol/μl) | 1μl | 0.75μl | 0.75μl |
| 6.     | Genomic DNA 25 ng/μl                 | 4μl     | 4μl     | 5μl       |
| 7.     | Taq DNA polymerase 5U/μl             | 0.2μl   | 0.3μl   | 0.3μl     |
| 8.     | DNAase free water                    | 10.8μl  | 9.7μl   | 9.7μl     |

Amplification was carried out in PCR thermocycler with a program illustrated in Table 3. After the amplification, the PCR products were stored at -20°C for further analysis.

Table 3: PCR programming for amplification of regions of ABCG2 gene

| parameters | Steps | Initial denaturation | Cycle Denaturation | Cycle Annealing | Exon 7 | Exon 14 | Intron 15 | Synthesis | Final extension | Hold |
|------------|-------|----------------------|--------------------|-----------------|--------|---------|-----------|-----------|----------------|------|
|            |       | Temperature          | 95 °C              | 95 °C           | 95 °C  | 95 °C   | 95 °C     | 54 °C     | 54 °C          | 54 °C |
|            |       | Time                 | 4 min              | 45 sec          | 45 sec | 1 min   | 10 min    | 10 min    | 10 min         | 10 min |
|            |       | No. of Cycle         | 1 cycle            | 35 cycles       | 1 cycle| 1 cycle | 1 cycle   | 1 cycle   | 1 cycle        | 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) by electrophoresis method. The genetic variation in the selected genomic regions of ABCG2 gene was identified through two different approaches RFLP and SSCP methods. The digestion of the amplified fragment of ABCG2 exon-14 region was carried out through Hinf1 restriction enzyme. Restriction digestion of the amplified products of exon 14 of the ABCG2 gene was carried out separately in a 30 μl reaction mixture containing 10X buffer (2μl), amplified product (10μl), Hinf1 10 units (1μl) and nuclease-free water (17 μl). Uniform mixing of the reaction mixture was assured through the spinning of samples for a few seconds before incubation at 37°C for 6 hr in a water bath. The separation of the restricted products was carried out on 8% polyacrylamide gel at 120 V to obtain a clear picture of the digested samples. The polyacrylamide gel after 2/3 migration of the digested sample, was stained in a gel tray containing 1% ethidium bromide solution. The digested bands were visualized under UV light and documented by a gel documentation system. Aliquots of 5μL PCR products were mixed with 5μL 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. The genetic structure of the studied population at two locations (LRS, Nohar and Bikaner) for gene and genotypic frequencies, observed heterozygosity (Ho), expected heterozygosity (HE) and expected unbiased heterozygosity (HE unbiased), an effective number of alleles and Nei’s genetic distances were analyzed through POPGENE program (version 3.1) [15].
Results and Discussion

Variation in regions of ABCG2 gene

Single SSCP bands pattern (‘AA’) was observed for the 393 fragment of exon-7-partial intronic region of the ABCG2 gene in Rathi cattle (Fig 1). Thus monomorphism was observed in all studied animals for this region. Three genotypes for exon 7 of the ABCG2 gene were observed that dissimilarity in Chinese Holstein cows with present study [19].

The PCR-RFLP analysis of the 207-bp exon 14 of ABCG2 gene PCR product with Hinf I enzyme resulted in three restriction fragments of 207, 151 and 56 bp for the AC fragment (Fig. 2). The present study revealed the presence of single genotypic SSCP pattern. Similar results were reported in Modicana cow [18], in German dairy cow [18] and indigenous cattle of Turley [19].

The PCR-SSCP analysis of the 323-bp product of the ABCG2 gene in the Rathi breed revealed the presence of two unique patterns reflecting their respective SSCP genotypic pattern (Fig. 3). The SSCP pattern resolved for animals of Rathi cattle was considered as AA to AB.

Gene and genotypic frequency of regions of ABCG2 gene

In our study, all animals at both locations have the same single SSCP pattern in exon-7 and exon 14 of ABCG2 gene and monomorphism observed in these regions. As per available literature, in Chinese Holstein cows identified three genotypes for exon 7 of the ABCG2 gene [17]. As per available literature, a study show dissimilarities with the present work, in two population SAR and EAR cattle [19], in Chinese Holstein cattle [18, 20] identified polymorphism and found A and B allele and three genotypes AA, AB and BB for exon-7 of ABCG2.

In White Fulani and Muturu cattle breeds [21], in Sahiwal and Hariana cattle breed [20], in Dutch Holstein–Friesian [23], also observed similar results with our present study and reported monomorphism for exon-14 of ABCG2. Some workers show dissimilarities with present work, in SAR and EAR cattle [19]. The group-wise gene and genotypic frequency observed for SSCP genotypic patterns of intron 15 of ABCG2 gene are represented in Table 4.

Table 4: Gene and genotypic frequencies of intron 15 of ABCG2 gene detected through SSCP analysis

| Group | N  | Genotypic Pattern | Gene frequency |
|-------|----|-------------------|----------------|
|       |    | AA                | AB             | A      | B      |
| 1     | 80 | 0.55 (44)         | 0.45 (36)      | 0.775  | 0.225  |
| 2     | 80 | 0.85 (68)         | 0.15 (12)      | 0.925  | 0.075  |
| Overall | 160 | 0.7 (112)     | 0.3 (48)       | 0.85   | 0.15   |

Note: group 1=LRS, Nohar; group 2 =LRS, Bikaner, Number in parenthesis are number of observations

The overall studied population of Rathi cattle revealed non-significant deviation from Hardy-Weinberg equilibrium (p 0.05) that indicates that animals similar in their genotypic distribution for gene frequency. However, in group 2 Chi square and G square test value observed for the ABCG2 intron 15 gene showed significant deviation from Hardy-Weinberg equilibrium (p 0.05) that showed that animals differ in their genotypic distribution concerning gene frequency. The results indicate the presence of sufficient genetic variation at the intron 15 locus though in the form of heterozygote combination (Table 5).

Table 5: Within-population heterozygosity estimates, PIC and FIS values of Rathi variant of intron 15 of ABCG2 gene

| Group | Sample size (N)* | Observed Heterozygosity (H₀) | Expected Heterozygosity (Hₑ) | Nei’s unbiased Heterozygosity (Hₑ) | PIC | Fixation index (Fₛ) |
|-------|-----------------|-------------------------------|-----------------------------|-----------------------------------|-----|---------------------|
| 1     | 80              | 0.1500                        | 0.1396                      | 0.1387                            | 0.2437 | -0.0811           |
| 2     | 80              | 0.4500                        | 0.3509                      | 0.3487                            | 0.2437 | -0.2903           |
| Overall | 160   | 0.3000                        | 0.2558                      | 0.2550                            | 0.2437 | -0.1765           |
The Shannon index for LRS, Nohar cattle was observed lowest among all studied animals with a value of 0.2664 (Table 5). Shannon index indicate the heterozygosity in LRS, Bikaner cattle, higher index value was recorded.

| Group | Sample size of alleles | Observed number of alleles | Effective number of alleles | Shannon’s Information Index |
|-------|------------------------|---------------------------|-----------------------------|----------------------------|
| 1     | 160                    | 2.0000                    | 1.1611                      | 0.2664                     |
| 2     | 160                    | 2.0000                    | 1.5355                      | 0.5332                     |
| Overall | 320                  | 2.0000                    | 1.3423                      | 0.4227                     |

Conclusion
The present study concluded polymorphic nature of intron 15 region of ABCG2 gene and monomorphic nature of exon 7-partial intronic region, exon 14 of ABCG2 gene in Rathi cattle of studied cattle.

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