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Novel association between polymorphism of the AQP3 gene and milk production traits in *Bos indicus*

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Abstract
Aquaporins are protein channels that passively transport the water in or outside the cells according the water potential gradient. Functions of aquaporin proteins are regulated by different AQP gene members. The present novel experimentation investigates polymorphism in AQP3 gene in indigenous (Rathi) breed of cattle and its association with different milk production traits. The study was performed on 104 Rathi cattle of LRS farm, RAJUVAS, Bikaner. DNA isolated from these samples were amplified by PCR using manually designed specific primers for 367 bp long fragment of exon 6 of AQP3 and subjected to Single Strand Conformation polymorphism (SSCP) analysis. Two genotypic patterns GC and GG were obtained by modified Sanger's di deoxy sequencing method. The genotypic frequencies of GC and GG were 0.90 (n=94), 0.10 (n=10) respectively. Gene frequencies for G and C were 0.55 and 0.45 respectively. The calculated genotypic frequency indicated that GG genotype is predominant in Rathi cattle. Analysis of variance (ANOVA) of linear model for dependent variable was non-significant ($p > 0.05$) for MY (Milk yield), LL (lactation length); significant for PDMY (per day milk yield) ($p \leq 0.05$) and highly significant for PMY (peak milk yield) ($p \leq 0.01$) in Rathi cattle at 5% level significance. It was also observed that PDMY, PMY and MY were higher for genotype GG than the genotype GC. Obtained results could open new possibilities to define AQP3 as candidate gene for their functional specificity to control the important economic traits related to the milk production in cattle.

Keywords: AQP3 gene, *Bos indicus*, Rathi breed, polymorphism, SNP, association studies, aquaporin, SSCP

Introduction

India is known for its rich diversity of cattle breeds, each with its own unique characteristics and adaptations to different regional climates and environments. The country is home to about 53 clearly defined cattle breeds (Annual report, NBAGR-2022) [2], which are categorized into milch (milk-producing), drought (draft), and dual-purpose breeds. These breeds have played a crucial role in the livelihood security of farmers. (Kumar *et al*., 2017) [5]. They are well known for their exceptional milk quality, ability to thrive in the hot and humid temperatures of the tropics, resilience to tropical illnesses, and adaptability to low-quality feed and scarce water resources. (Srivastava *et al*., 2019) [6] The indigenous cattle breeds contribute significantly to the milk production in India. The dairy industry in India holds a noteworthy position in the agricultural economy of the nation. It serves as a primary means of generating income and sustaining livelihoods for a vast number of rural households, specifically those belonging to small-scale and economically disadvantaged farmers. (Bhandari *et al*., 2021) [7]. India is ranked first in milk production, representing 24% of global production, as stated by the Food and Agriculture Organization (DAHD-2023) [3]. Per capita availability of milk in India significantly exceeds the average of worldwide PCA. Over the span of three decades, encompassing the 1980s, 1990s, and 2000s, the daily milk consumption within the country has experienced a substantial rise. Starting from a meager 107 grams per person in 1970, it has now reached an impressive 427 grams per person in the 2020-21 timeframe, in contrast to the global average of 322 grams per day during 2021 (BAHS-2022). Furthermore, India has witnessed a notable surge in its milk production, reaching a remarkable 210 million tonnes during the 2020-21 period.

This growth rate surpasses six percent, a significant improvement when compared to the mere two percent observed globally. The Indigenous and non-descript cattle combinedly contribute 20.17% of the total milk production in the country (BAHS-2022). The evident growth of India's dairy industry is accompanied by a limited increase in the participation of Indigenous cattle. Hence, the discovery of a novel marker gene for milk production would undoubtedly represent a significant accomplishment.

Many genes are associated with the traits related to milk production due to the complex and multifactorial nature of polygenic inheritance. Economically important production traits within the dairy industry such as milk yield traits have large phenotypic variation and high heritability (Brotherstone *et al.* 1997; Jamrozik and Schaeffer, 1997; Royal *et al*., 2002) [16, 18] thus are largely influenced by the genotype of the cow. The genetic control of milk production is a quantitative type of inheritance. It involves a wide range of genes that interact with each other and with the environment to determine the final phenotype. Among these genes, CSN_1S_1, CSN_1S_2, CSN_2, CSN_3, ACACA, $DGAT_1$, $DGAT_2$, ME_1, SCD, LPL, LIPE, BTN_1A, MFGE, GH, PRLR, $PITX_2$, $POUF_1$, and $STAT_5$ have been identified as candidate genes that play crucial roles in determining milk yield and composition traits. (Asim *et al.,* 2023) [8] These genes exhibit polymorphisms, which are variations in the DNA sequence, that are associated with differences in milk production and quality (Tesema *et al.,* 2018) [4]. Studying the polymorphisms in these candidate genes provides valuable insights into the genetic mechanisms underlying milk production traits, and can potentially be used for selective breeding programs to improve milk production in dairy animals. Overall, the identification and characterization of these candidate genes have opened up new avenues for understanding the genetic basis of milk production traits and for developing strategies to enhance milk production in livestock.

Most of candidate genes included under the MAS (marker assisted selection) for milk production are directly linked to the quality and quantity of fat, casein, globulin and lactose etc, component of the milk (Alsaftli, 2020) [9]. It is well known that milk yield is maximally increased by the water fraction. It also causes a huge difference between milk yield of exotic and indigenous breeds of cattle. More focused investigation is required to find out the candidate gene which regulates the water influx in the alveolar cells of mammary gland. The genes encoding for Aquaporins (AQP) can potentially be considered as one of the candidate genes for water fraction of milk. Thus we hypothesized AQP3 gene as influencing factor in milk production for this investigation. However, there has been a lack of research exploring the potential polymorphisms in Aquaporin (AQP) gene members and their potential associations with milk production traits in cattle. Aquaporins (AQP) are a group of proteins that belong to the MIP super family, which serve as vital membrane channels responsible for selectively transporting water, small neutral molecules, and ions both out of and between cells (Shivaraj *et al*., 2019) [19] As a result, these proteins are closely associated with various physiological processes, including water transport, cellular responses to hypoxia and oxygen-glucose deprivation, glycerol transport, positive regulation of the immune system process and transmembrane transport. (Seo *et al*., 2018, Zhu *et al.,* 2016) [10, 11] Given the complexity and importance of these processes, it becomes crucial to conduct genetic profiling and association studies focusing on the AQP3 gene. By investigating these potential associations, valuable insights can be gained regarding the genetic factors influencing milk production traits in Rathi cattle, A breed native to the Rajasthan region of India.

Materials and Methods

Animals and genomic DNA isolation

The study included total 104 lactating female cattle of Rathi breed. Animals were selected from the livestock research station farm of veterinary college, RAJUVAS, Bikaner. The blood samples (5 ml/cow) from the jugular vein were collected and immediately transported to the AGB departmental laboratory in a cool box containing ice and stored at −20 °C until further analysis. Standard phenol-chloroform extraction protocol was used for isolation of the genomic DNA (with DNA isolation kit supplied by Himedia Pvt. Ltd.) from the whole blood [Sambrook and Russell, 2001]. Various steps followed were Binding, Washing and Elution. Horizontal Agarose gel electrophoresis (using 1% w/v Agarose) was used for checking the quality of the isolated genomic DNA. The DNA concentration was determined using NanoDrop Thermo scientific spectrophotometer (260/280) and then diluted to the final working concentration of a 50 ng/µl.

PCR-SSCP - Sequence annotation Methods

PCR Amplification of the expected 368 bp sized fragment of Exon-6 of AQP_3 gene was carried out with primers designedby using the PRIMER3 program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Expected reference sequence was taken from NCBI database (Bos taurus aquaporin 3 (AQP3), mRNA, 1777 bp, Accession i.d.-NM_001079794.1). Primer sequence in Forword, reverse directions were TACTGAAGCCTATAAGAGCC and TTTTCCATCCTGAAGGAAAT respectively. Approximately 50 ng sample of genomic DNA, primers synthesized from Eurofins genomics, dNTP's, $MgCl_2$, primers, Taq DNA polymerase, Nuclease free water and were used for amplification. Standard protocol of Sanger Dideoxy Chain termination method (GeneOmBio Technologies Pvt. Ltd.) was employed to perform the polymerase chain reactions.

For the confirmation of mutation in amplicons SSCP (single strand conformation polymorphism) - denaturing urea PAGE method was used. SSCP analysis was performed according to guidelines described by Hayashi and Yandell, (1993) [13] and Summer *et al*. (2003) [14]. Different steps were preparing denaturing urea page gel in the vertical casting tray, empty run, heat treatment and cold shock, loading and final run of the DNA samples, vertical gel electrophoresis assembly system and visualisation of the DNA samples under UV trans-illuminator. Forward and reverse raw sequences of PCR products were generated through Sanger sequencing method to reveal the type of mutation in different patterns of SSCP and novel sequence of Exon-6 of AQP3 gene. Sequenced data were edited for accuracy through the "Codon-code Aligner" software.

Statistical Analysis

Animals with P_1 and P_2 patterns of SSCP were recorded for further statistical. Estimation of Gene and Genotypic frequency were carried out using Hardy-Weinberg equilibrium the genotypic and gene frequencies were estimated by standard procedure (Falconer and Mackey, 1998) [15]. Data of the milk production traits for Rathi cattle named 305-day milk yield (MY), lactation length (LL), per day milk yield (PDMY) and peak milk yield (PMY) were obtained from the data sheets of Rathi farm records (LRS). All the milking records of each animal were used to collect the required data. The lactation records from 104 cows were tested for significance of association of milk parameters with polymorphism in EXON-6 of AQP_3 gene by using least-squares method of SPSS ver. 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analysis was carried out under single gene model to estimate the effect of genotype on the traits.

Results and Discussion

Extraction of Genomic DNA & amplification of the Exon-6 of AQP3 gene by PCR

All samples utilized in the isolation of genomic DNA were determined to be devoid of impurity or fragmentation, as indicated by the lack of smearing on the gel and the presence of a coherent and vibrant genomic DNA band (see Fig 1).

The concentration of DNA varied between 50 to 75 ng/µl among the samples, thus signifying the appropriateness of the extracted DNA samples for seamless *in vitro* amplification via PCR. An annealing temperature of 56 °C for 45 seconds was found optimal for PCR amplification of the targeted AQP3 gene fragment. 40 cycles were found to be optimal for the desired amplification of the 368 bp Gene product. The PCR products were inspected for amplification by electrophoresis on 1.0% agarose gel in parallel with 100 bp DNA marker (Fig 2). After this procedure, amplified Amplicons were stored at -20 °C till further use.

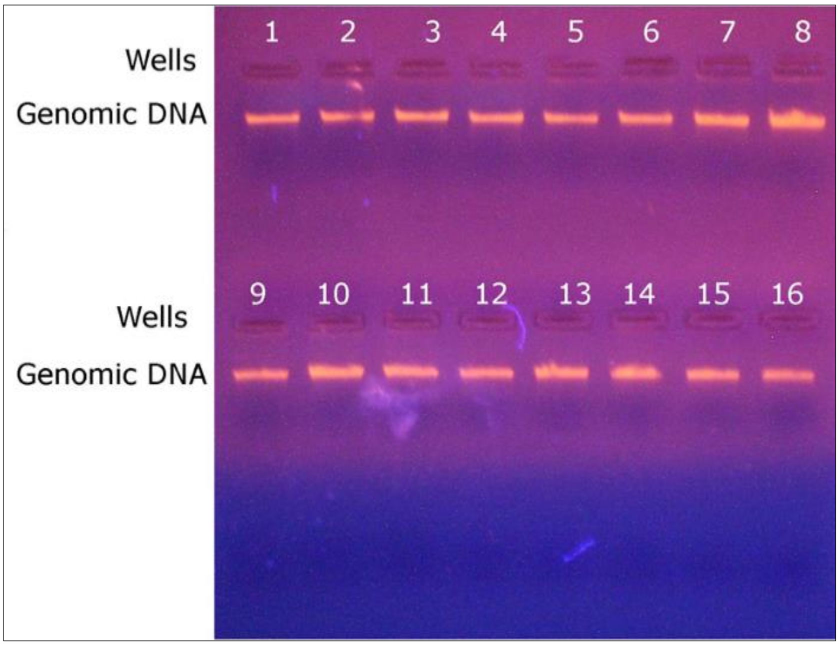

Fig 1: Isolation of Genomic DNA from blood Sample of Rathi cattle, visualized under UV Illuminator stained with EtBr

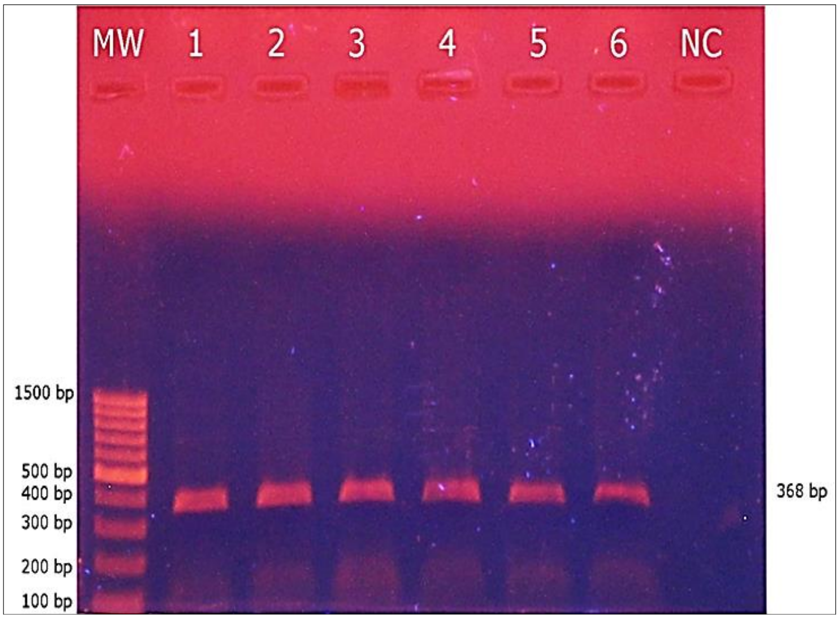

Fig 2: PCR amplified product of 368 bp fragment of Exon-6 of AQP3 gene in Rathi cattle, visualized under UV Illuminator stained with EtBr (MW: Molecular weight marker, Lane 1-6: PCR Amplicons of 368 bp, NC: Negative)

Detection of Genetic polymorphism in Exon-6 of AQP3 gene by SSCP method and SNP identification by gene sequences

Two distinct bands patterns, designated as P_1 and P_2, were observed for a 368-bp fragment of Exon-6 of the AQP3 gene in Rathi cattle. This finding suggests that this region exhibits a dimorphic allelic nature. The polymorphic variants P_1 and P_2 were obtained using the urea PAGE-based single strand conformation polymorphism (SSCP) method.

To verify the genotype of patterns P_1 and P_2 of Exon-6 of the AQP3 gene, forward and reverse raw sequences were generated using the Sanger sequencing method. The sequenced data were then subjected to editing for accuracy using the Codoncode Aligner software (USA). The resulting sequences, measuring 368 bp in size, were aligned with the aid of a reference sequence available at the NCBI database (GenBank Accession No. NC 032657.1). The alignment revealed the presence of a novel single nucleotide polymorphism (SNP) at the 276th position (G>C) within the target fragment of Exon-6 of the AQP3 gene. Representative sequenced data of the animals having SSCP banding pattern 'P_1' was revealed as GC genotype, whereas sequenced data of the animals having SSCP banding pattern 'P_2' was revealed as GG genotype.

Analysis for association of Genotype patterns of Exon-6 of AQP3 gene with Milk production traits

The lactation records from 104 cows were tested for significance of association of milk parameters with polymorphism found in the Exon-6 of AQP_3 gene by using least-squares method of SPSS ver. 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analysis was carried out under single gene model to estimate the effect of genotype on the traits. For association, study milk yield (MY), lactation length (LL), per day milk yield (PDMY) and peak milk yield (PMY) were taken as dependent variable. Relationship of each dependent variable with the two genotypic patterns 'GC' and 'GG' of 6th exonic region of AQP3 gene in Rathi cattle were analysed separately. Analysis of variance (ANOVA) of linear model for dependent variable was non-significant for MY, LL; significant for PDMY ($p\leq$ 0.05) and highly significant for PMY ($p\leq$ 0.01) in Rathi cattle at 5% level of significance. Results of the association study performed for the identified genotype are shown in the table1, fig 3 and fig 4.

Table 1: Association of GG ang GC genotypic patterns of Exon-6 of AQP3 gene with milk yield (MY), lactation length (LL), per day milk yield (PDMY) and peak milk yield (PMY) in Rathi Cattle

Genotype	Milk yield (MY)	lactation length (LL)	per day milk yield (PDMY)	peak milk yield (PMY)
GG	1656.18 ±230.70	246.26 ±17.03	6.71 ±0.726	12.04 ±4.23
GC	1350.86 ±59.77	247.30 ±5.37	5.39 ±0.18	08.93 ±0.32

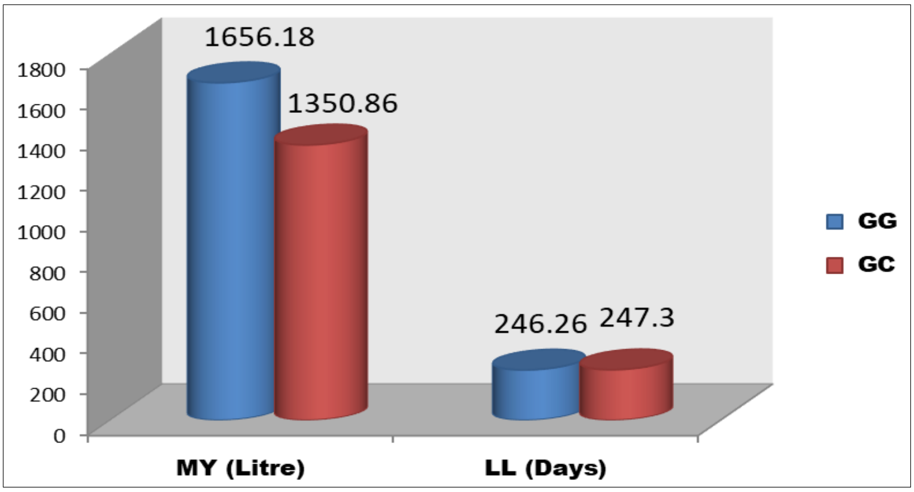

Fig 3: Association study of genotypic patterns of Exon-6 of AQP3 gene with milk yield (MY), lactation length (LL) in Rathi Cattle

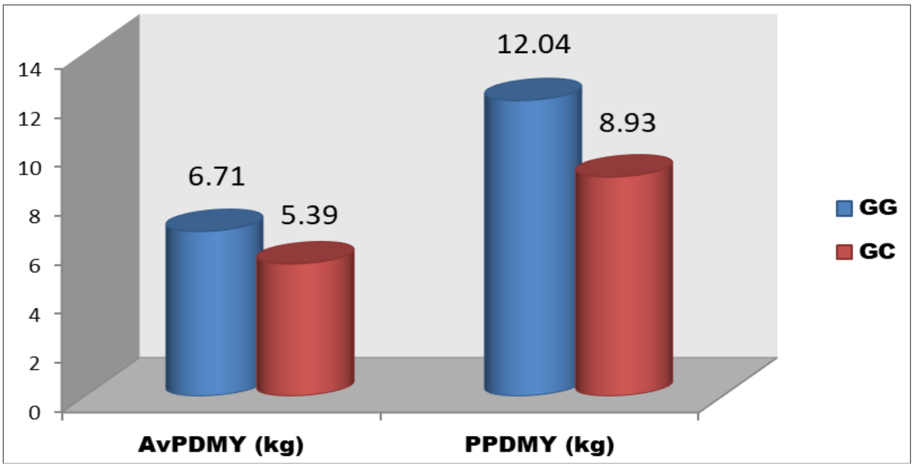

Fig 4: Association study of genotypic patterns of Exon-6 of AQP3 gene with per day milk yield (PDMY) and peak milk yield (PMY) in Rathi Cattle

Conclusion

It is important to highlight that the ANOVA did exhibit significance for PDMY (per day milk yield) ($p \leq 0.05$) and displayed a high level of significance for PMY (peak milk yield) ($p \leq 0.01$) in Rathi cattle, specifically at a 5% level of significance. Moreover, it was observed that the PDMY, PMY, and MY values were significantly higher for the GG genotype in comparison to the GC genotype. Thus, On the basis of present investigation, it can be concluded that animals with 'GG' genotype are high milk producing when comparing with 'GC' genotype. Clearly this is novel work that was needed to be performed, but yet more detailed research with including all the gene members of Aquaporin family is still required. To get the accuracy in association study with milk production, all the Aquaporin genes including all possible SNPs should be included in future investigation.

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