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ORIGINAL ARTICLE **OPEN ACCESS**

Aquaporin-3 (AQP3) Gene - Novel Molecular Characterisation in *Bos indicus*

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ABSTRACT
Aquaporins are integral membrane proteins from a larger family of major intrinsic proteins (MIP) that form pores in the membrane of biological cells. Till now more than 200 members of the Aquaporin family have been found in plants, microorganisms, invertebrates and vertebrates. Total 12 types of AQP genes have been discovered in mammals but their importance in the physiology of these organisms is still being uncovered. In the present investigation, novel molecular characterization of the 368 bp long fragment of Exon-6 of AQP3 gene was performed in Indian cattle through PCR amplification, sequencing, SSCP and bioinformatics analysis. The sequencing results revealed one novel SNP in Exon-6 at 276th position (G>C). At this particular locus DNA sequences of animals with single and double peaks in the sequence chromatograms were observed. The sequence variations within the Rathi breed of Indian cattle were also observed. Accordingly, two alleles (G, C) and two genotypes (GG and GC) were identified in the Rathi breed. Genomic identity and variations between Bos indicus sequenced result and predicted/submitted AQP3 cDNA Sequences of Bos taurus, Bos mutus, Bison bison, Bubalis bubalis, Bubalis carabanesis, Capra hircus, Cervus elaphus, Oryx dammah, Cervus canadensis, Ovis aries etc. were calculated at GenBank, NCBI. It ranged between 100% to 76.24%. and was 99.17 %. Query coverage was 100% with most of other related mammalian species. The evolutionary relationship between sequences showed a close relationship between bos mutus (XM_005903022.2), Bison (XM_010840119.1), Bos indicus (XM_019966412.1), Bos taurus and also with cross breed Bos indicus x Bos taurus (XM_027550016.1).

Key words: *AQP3 gene, Bos indicus, Rathi breed, Genomic characterisation, SSCP, SNP, Phylogeny tree, Multiple Sequence alignment*

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INTRODUCTION

The livestock sector of India has emerged as a vital sector for ensuring a sustainable agriculture system. We can see and understand evidence from the 77th round survey of the National Sample Survey Office (NSSO-2019) that more than one-fifth (23 per cent) of agricultural households with very small areas of land (less than 0.01 hectare) reported livestock as their principal source of income. Farming households who are rearing cattle are better able to withstand distress during the extreme weather conditions, that's why we need to increase milk production in India. The 36.04 % of the total livestock population (193.46 million) of India is Cattle. In which pure indigenous cattle share only 22 % part and non-descript cattle and cross-bred sequentially share 52 % and 26.54 % which shows high level of dilution of indigenous cattle (BAHS-2022 [1].Over the span of three decades, India has transformed from a country of acute milk shortage to the world's leading milk producer. India has also been the largest producer of milk in the world since 1998. According to BAHS-2022, the total milk production of the country is 221.06 million tonnes, but combine share of indigenous and non-descript cattle is only 20.17% of total milk produced in India. So, we need to increase milk production of both descript and non-descript indigenous cattle.

Many genes are associated with milk production traits due to polygenic inheritance nature. CSN1S1, CSN1S2, CSN2, CSN3, ACACA, DGAT1, DGAT2, ME1, SCD, LPL, LIPE, BTN1A, MFGE, GH, PRLR, PITX2, POUF1, and STAT5 genes are candidate genes whose polymorphism is linked with milk yield and composition traits [2]. Polymorphism in Aquaporin (AQP) gene members and its association with milk production traits in cattle has not been investigated yet. The AQP are members of the MIP super family which function as membrane channels that selectively transport water, small neutral molecules and ions out of and between cells. Thus, it is associated with different process like water transport, cellular response to hypoxia, cellular response to oxygen-glucose deprivation, glycerol transport, odontogenesis,

positive regulation of immune system process, renal water absorption, transmembrane transport and urea transport. In this context, genetic profiling of AQP3 gene in Rathi cattle of Rajasthan, India was performed and expected polymorphism was identified as genetic markers to increase milk production.AQP3 was first cloned from the rat cDNA library [3-5]. The cattle AQP3 gene extends over about 1777 bp in *Bos taurus* (NCBI Reference Sequence: NM_001079794.1). This gene is present on chromosome number 8 in zebu cattle which have 6 Exons and 5 Introns [6]. It is predicted to be about 1809 bp in *Bos indicus* (NCBI Reference Sequence: XM_019966412.1).Prediction of *Bos indicus* AQP3 was based upon automated computational analysis and it was derived from a genomic sequence (NC_032657.1) of *Bos indicus* (isolate QUIL7308 breed Nelore chromosome 8, Bos_indicus_1.0) whole genome shotgun sequence. It was annotated using gene prediction method: "Gnomon", which is supported by mRNA and EST evidence.

The present investigation was undertaken for the very first partial characterization of Bos indicus AQP3 gene and to study the polymorphism in it. Partial genomic region of Exon-6 of AQP3 gene was selected because of the predicted SNP present in it, which was identified according to the gene data bank of NCBI(NM_001079794.1). The genetic variation or polymorphism in selected genomic regions was identified through the PCR-SSCP method. To identify and confirm different genotypic variants and SNP, sequencing of the selected gene products was carried out.

MATERIAL AND METHODS

Genomic DNA extraction:

A total of 104 Rathi cattle were selected from the LRS farm of RAJUVAS, Bikaner for the collection of blood samples. The genomic DNA was isolated (with DNA isolation kit supplied by Himedia Pvt. Ltd.) from whole blood by using standard phenol-chloroform extraction protocol [7]. Some slight modifications were used according to requirement in the centrifuge machine. Various steps followed were Binding, Washing and Elution. The isolated genomic DNA was checked for quality by using Horizontal Agarose gel electrophoresis using 1% w/v Agarose.

PCR Amplification of the Exon-6 of AQP3 gene:

PCR amplification of 368 bp AQP3 gene fragment was performed utilizing primers designed with the help of online Primer-3 Software, also and by manual method. Primer sequence (5'-3') in Forword direction was TACTGAAGCCTATAAGAGCC whereas reverse primer was TTTTCCATCCTGAAGGAAAT. The PCR reaction was carried out in 25µl of total volume, containing ready to use Primers synthesized from Eurofins genomics. dNTP's, $MgCl_2$, primers, Taq DNA polymerase, Nuclease free water and approximately 50 ng sample of genomic DNA were used for amplification. After purification of the amplified fragment, bidirectional sequencing using forward and reverse primers was done using Sanger Dideoxy Chain termination method (GeneOmBio Technologies Pvt. Ltd.). Components of PCR were used according to Table 1.

Table 1: Components of PCR for each reaction and their function

S.N.	Reaction component	Use	Volume (µl)
1.	**5X PCR buffer**	For maintaining the pH	5.0
2.	**$MgCl_2$**	Cofactor and catalyzer for Taq DNA polymerase	2.0
3.	**DNTPs**	Functions as substrate and energy source	1.0
4.	**Forward Primer**	Attachment to the initial point to flank the amplicon region	0.5
5.	**Reverse Primer**	Attachment to the end point to flank the amplicon region	0.5
6.	**Target DNA**	Substrate for PCR reaction	2.0
7.	**Taq DNA polymerase**	Responsible for polymerisation	0.25
8.	**Nuclease free water**	Solvent	13.75
9.	**Total Volume**		25 µl

Detection of Genetic polymorphism by SSCP (denaturing urea PAGE) method:

The single strand conformation polymorphism (SSCP) method was used to identify mutations within the amplicons. SSCP analysis was performed according to guidelines described by Hayashi and Yandell [8] and Summer *et al.*[9], with slight modifications according to requirement and lab conditions.

Genotyping of the PCR Amplicons for sequence analysis:

Forward and reverse raw sequences were generated through the Sanger sequencing method. Sequenced data were edited for accuracy through Codoncode Aligner software (USA) and different sequence patterns were generated. The pair wise and multiple alignment of the different sequence pattern was

done to analyse the differences and relationship between Indigenous cattle AQP3 gene sequences and other various species.

Study of evolutionary and genetic relationship:

Obtained sequence results were compared with predicted Sequences of *Bos taurus, Bos Mutus, Bison bison, Bubalis bubalis, Bubalis carabanesis, Capra hircus, Cervus elaphus, Oryx dammah, Cervus canadensis, Ovis aries, Rangifer tarandus, Balaenoptera musculus* AQP3 sequences available at National Centre for Biotechnology Information (NCBI) database using BLAST software program (http://www.ncbi.nlm.nih.gov/) to study the sequence variation and relationship. The estimation of evolutionary relationship between different species was inferred by the neighbour joining method of phylogenetic trees using the Blast Tree View system of online BLAST® software of NCBI and also by NCBI Multiple Sequence Alignment Viewer, Version 1.24.0.

RESULTS AND DISCUSSION

Extraction of Genomic DNA

All samples employed in isolation of genomic DNA were found to be fragmentation free as evidenced by the absence of smearing on gel and presence of intact bright genomic DNA band (Fig. 1).The DNA concentration among samples varied between 50 to 75 ng/µl which indicated the suitability of the extracted DNA samples for hassle free in vitro amplification by PCR.

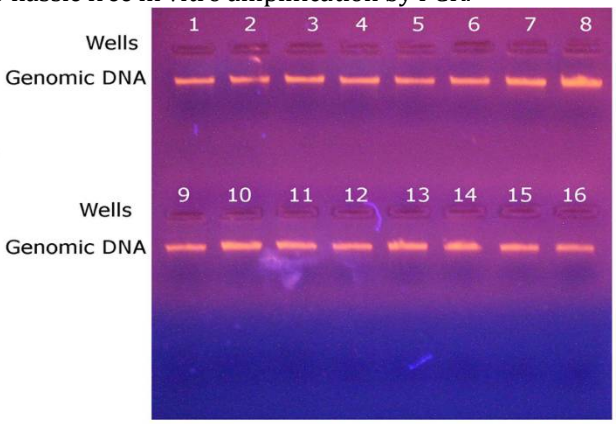

Fig 1.Isolation of Genomic DNA from blood Sample of Rathi cattle, visualized under UV Illuminator stained with EtBr

PCR Amplification of the Exon-6 of AQP3 gene:

An annealing temperature of 56°C for 45 secondswas found optimal for amplification of the targeted AQP3 gene fragment. 40 cycles were found to be optimal for the desired amplification of the 368 bp PCR product. The PCR products were checked for amplification by electrophoresis on 1.0 % agarose gel in parallel with 100 bp DNA marker (Fig 2) and visualized under UV Illuminator. After this procedure, amplified Amplicons were stored at -20°C till further use.

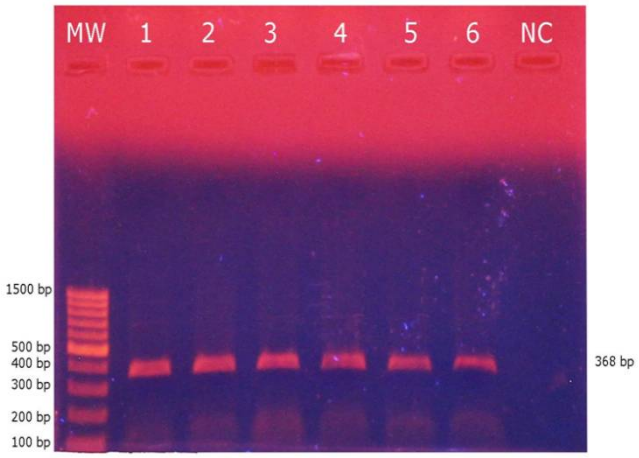

Fig 2: PCR amplified product of 368 bp fragment of Exon-6 of AQP3 gene in Rathi cattle, visualized under UV Illuminator stained with EtBr
(MW: Molecular weight marker, Lane 1-6: PCR Amplicons of 368 bp, NC: Negative)

Detection of Genetic polymorphism in Exon-6 of AQP3 gene by SSCP method:
Two different SSCP bands patterns (P1 and P2) were observed for 368-bp fragment of Exon-6 of AQP3 gene in Rathi cattle. This result is suggestive of dimorphic allelic nature of this region. The polymorphic variants revealed through urea PAGE based single strand conformation polymorphism (SSCP) are shown in Fig 3,4.

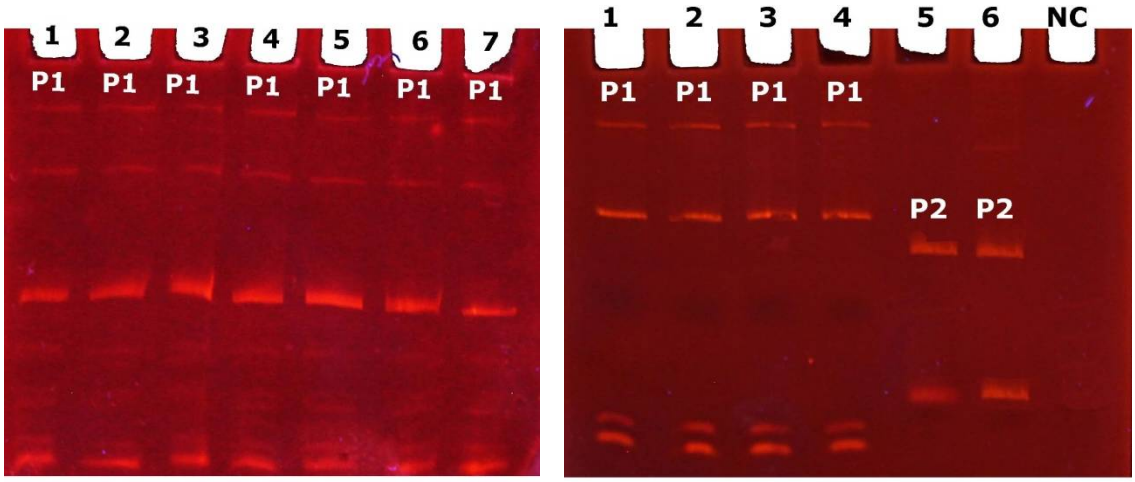

Fig 3,4: SSCP denaturized DNA products of Exon-6 of AQP3 gene in Rathi cattle, visualized under the gel doc system, stained with EtBr

Generation of gene sequences:
To confirm the genotype of pattern P1 and P2 of Exon-6 of AQP3 gene, forward and reverse raw sequences were generated through Sanger sequencing method. Sequenced data were edited for accuracy through Codoncode Aligner software (USA). The sequences with 368 bp size were assembled with the help of reference sequence already available at NCBI database (GenBank Accession No. NC_032657.1). Presence of amplified AQP3 gene fragment in the exon-6 region was confirmed in this step. Represented alignment also revealed that one novel SNP is present in target fragment of Exon-6 of AQP3 gene at 276th position(G>C). Representative sequenced data of the animals having SSCP banding pattern 'P1' revealed GC genotype, whereas sequenced data of the animals having SSCP banding pattern 'P2' revealed GG genotype in Rathi cattle. The sequence variation within the Rathi breed is depicted in table 2.

Table 2. Gene sequence variation in 6th exonic region of *AQP3* gene detected through Sequence analysis

Name	Nucleotide position –276
Bos indicus - G allele	G
Bos indicus - C allele	C

Evolutionary relationship studies with phylogenetic tree and Multiple Sequence alignment:
Genomic identity between novel Bos indicus AQP3 sequence and predicted AQP3 genomic Sequences of Bos indicus was found to be 99.46%, it was 100% same for Bos taurus. Genomic identity ranged between 99.06% to 76.24% for different species like *Bos Mutus, Bison bison, Bubalis bubalis, Bubalis carabanesis, Capra hircus, Cervus elaphus, Oryx dammah, Cervus canadensis, Ovis aries, Rangifer tarandus, Balaenoptera musculus.* Query coverage was 100% with most of other related mammalian species. Number of Miss Matched Nitrogen bases, Accession Length together with the genomic identity and query coverage are represented in Table-3.

Table 3. Percentage identity between 368 bp long AQP3 gene sequence of Bos indicus with AQP3 gene sequences of another 14 species

Accession i.d.	Organism and type of Reference Sequence	Query Cover	% identity	Miss Match	Acc. Length
XM_027550016.1	PREDICTED: *Bos indicus x Bos taurus* aquaporin 3 (AQP3), mRNA	100%	100%	0	1796
NM_001079794.1	*Bos taurus* aquaporin 3 (AQP3), mRNA	100%	100%	0	1777
XM_019966412.1	PREDICTED: *Bos indicus* aquaporin 3 (Gill blood group) (AQP3), mRNA	100%	99.46%	2	1809
XM_005903022.2	PREDICTED: *Bos mutus* aquaporin 3 (Gill blood group) (AQP3), mRNA	100%	99.06%	2	1697
XM_010840119.1	PREDICTED: *Bison bison* aquaporin 3 (Gill blood group), mRNA	100%	95.80%	3	1808
XM_006066697.4	PREDICTED: *Bubalus bubalis* aquaporin 3 (Gill blood group) (AQP3), mRNA	100%	94.85%	9	1787
XM_055578825.1	PREDICTED: *Bubalus carabanensis* AQP3, transcript variant X2, mRNA	100%	94.58%	10	1562
XM_018052367.1	PREDICTED: *Capra hircus* aquaporin 3 (Gill blood group) (AQP3), mRNA	100%	93.78%	20	1812
OU343093.1	*Cervus elaphus* genome assembly, chromosome: 16	100%	93.24%	19	47758366
XM_040236133.1	PREDICTED: *Oryx dammah* AQP3, (LOC120860332), mRNA variant X2,	100%	93.24%	19	1549
XM_043453775.1	PREDICTED: *Cervus canadensis* aquaporin 3 (Gill blood group), mRNA	100%	92.97%	20	1794
XM_027964294.2	PREDICTED: *Ovis aries* aquaporin 3 (Gill blood group) (AQP3), mRNA	100%	92.70%	22	1781
OX596113.1	*Rangifer tarandus platyrhyncus* genome assembly, chromosome: 29	64%	92.92%	13	52056294
XM_036856574.1	PREDICTED: *Balaenoptera musculus* aquaporin 3 (Gill blood group) (AQP3), transcript variant X2, mRNA	98%	76.24%	55	1796

The evolutionary relationship between sequences showed a close relationship between *bos mutus* (XM_005903022.2), Bison (XM_010840119.1), *Bos indicus* (XM_019966412.1), *Bos taurus* and also with cross breed *Bos indicus x Bos taurus* (XM_027550016.1). The sequence variation and identity percentage were determined on the basis of pairwise nucleotide BLAST of Indian The evolutionary relationship between AQP3 genes of different species was also inferred using Neighbour joining method of phylogeny tree construction (Fig 5).

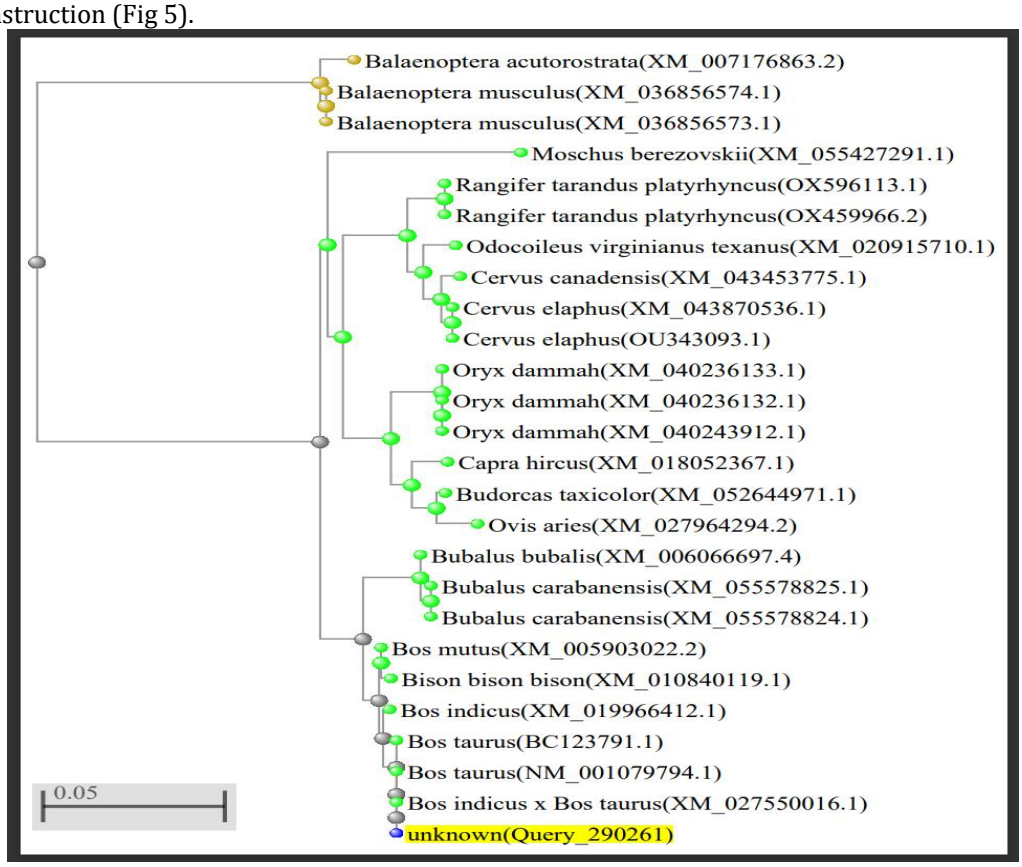

Fig. 5:Phylogeny tree (Neighbour joining method) based upon AQP3 gene

Multiple Sequence alignment was prepared between AQP3 gene and cDNA sequences from different species with NCBI MSA programme (Ver: 1.22.0), which show all possible genetic similarity and differences between targeted 14 species (Fig.6). Grey bands represent genetical similarity whereas red colour of bands shows genetical variations.

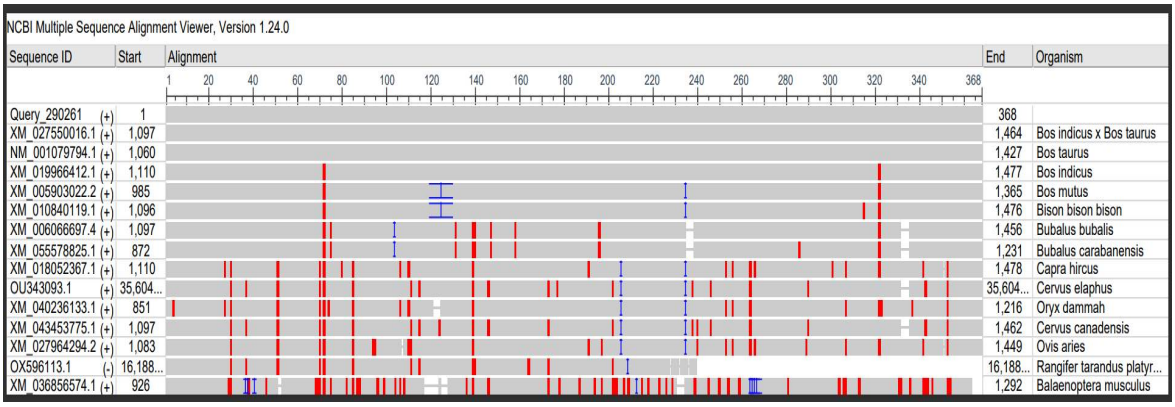

Fig 6. Multiple Sequence alignment between AQP3 gene and cDNA sequences from different species. (At extreme left of Fig Sequence ID is denoted as accession number, at extreme right of Fig zoological name of species is mentioned)

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