Indian J. Anim. Hlth. (2016), 55(1) : 77-86

Research Article

POLYMORPHISM AND NUCLEOTIDE SEQUENCING OF BMP-15 GENE IN AN ORGANISED HERD OF GAROLE SHEEP (*OVIS ARIES*)

RAJ KUMAR, S. TARAPHDER*, A. K. SAHOO, P. K. SENAPATI, R. PAUL, M. ROY, U. SARKAR, D. RAJA, I. SAMANTA[1] AND S. BAIDYA[2]

Department of Animal Genetics and Breeding
Faculty of Veterinary and Animal Sciences
West Bengal University of Animal and Fishery Sciences
Belgachia, Kolkata-700 037, West Bengal, India

The present study was carried out on thirty two ewes of Garole sheep with an aim to explore the DNA polymorphism of exon-2 of BMP-15 (Bone morphogenetic protein-15) gene using PCR-RFLP and Nucleotide sequencing technique. The obtained 141 bp PCR products were digested with *HinfI* restriction enzyme. This resulted in similar banding pattern with 110 and 31 bp length on 2.5 percent agarose gel. The basic finding of the current study was the absence of polymorphism at the exon-2 of BMP-15 gene in Garole sheep under study. The frequencies of wild genotype (++) and wild allele (+) of exon-2 of BMP-15 gene in 32 randomly selected Garole ewes were 1.00. Nucleotide sequence of PCR product (141 nt) revealed that the C nucleotide has not changed to T at this locus (C-T) for exon-2 of BMP-15 gene in all experimental animals. The BLAST analysis of present study showed 99-100 percent homology between species for the obtained sequence of exon-2 of BMP-15 gene of Garole sheep. The phylogenetic tree constructed on the basis of nucleotide sequences of different species revealed that Garole sheep form a cluster with Lohi and Bolochi sheep breed, Beetal, Boer and Markhoz goat breed indicating their closeness in the evolutionary time scale.

Key words: BMP gene, PCR-RFLP, BLAST, Nucleotide, Sheep, Garole

BMP-15 is a member of the transforming growth factor beta (TGFâ) superfamily which is specifically expressed in oocyte of the developing follicle. Sheep Bone morphogenetic protein 15 (BMP-15) has been mapped on the X chromosome. The full length coding sequence (1179 bp) is contained in two exons separated by an intron (5.4 kb). In some exotic sheep, it is clear that heterozygotes carrying inactivating mutations in only one copy of BMP-15 have an increased ovulation rate (Galloway *et al.*, 2004). The mutations in this gene cause increased ovulation rate and

**Corresponding Author*

[1]*Department of Veterinary Microbiology and Immunology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Belgachia, Kolkata-700 037, West Bengal, India*

[2] *Department of Veterinary Parasitology, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Belgachia, Kolkata-700 037, West Bengal, India*

twin and triplet births in heterozygotes. Ovulation rates in BMP-15 mutants are high in the heterozygotes while the homozygous mutants show a primary ovarian failure resulting in complete sterility in some prolific breeds of sheep (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004; Bodin *et al.*, 2007 and Monteagudo *et al.*, 2009). Such type of polymorphic studies conducted on exotic sheep open a new research arena to work on prolific Garole sheep available in eastern India. Hence, the present study was envisaged to explore the presence of polymorphism in exon-2 of BMP-15 gene in prolific Garole sheep using PCR-RFLP and Nucleotide sequencing technique.

MATERIALS AND METHODS

Experimental animals: The experimental sheep utilized for the present investigation were from the herd of Sheep and Goat Breeding Farm of West Bengal University of Animal and Fishery Sciences maintained at Mohanpur, Nadia, West Bengal. Sheep were selected at random irrespective of their age and so as to get representative sample of the population. In a total number of 32 ewes of Garole sheep belonging to the species *Ovis aries* were eventually covered under the present investigation.

Collection of blood samples: Blood samples (10 mL) were collected aseptically from jugular vein of each animal in a 15 mL disposable polypropylene centrifuge tube pre-added with an amount of 300-400 µL of 10 percent Ethylene diamine tetra acetic acid (EDTA) as anticoagulant. The tube was tightly capped and shaken gently to facilitate thorough mixing of blood with the anticoagulant. The samples were transported to the laboratory in an icebox containing ice packs and stored at 4^{o}C till further processing for the DNA isolation.

Isolation of genomic DNA: DNA samples were isolated from blood samples using a high-salt method as described by Miller *et al.* (1988) with few modifications. The quality of genomic DNA was checked on 0.8 percent agarose gel electrophoresis by spectrophotometry. The good quality samples were stored at -20°C after checking its purity till further use. Good quality DNA samples were selected for further study.

PCR Amplification : DNA samples were adjusted to a concentration of 50 ng/µL and exactly 2.5 µL of the DNA samples were used as template for polymerase chain reaction (PCR). Genomic DNA was amplified using primer sequences (F: 5'-CACIGICTICTIGITACIGTATTICAATGAGAG-3' and R: 5'-GATGCAATACTGCCTGCTTG-3') as described by Hanrahan *et al.* (2004). The amplification reaction conditions was carried out using 32 cycles at 94°C for 5 min, followed by 94°C for 45s, 63°C for 40s, 72°C for 45s, followed by 72°C for 10 min. The amplified PCR products were checked at 2.5% agarose gel. Electrophoresis was carried out @ 5-6

volts/cm. Standard 50 bp DNA ladder was also loaded into one of the wells as a marker. The bands were visualized on UV transilluminator and documented in Gel Documentation System (Bio Rad Laboratories, USA). The good quality PCR products were subjected to RFLP analysis for genotyping of the animals under study.

Restriction fragment length polymorphism (RFLP) : After confirmation of amplification of target region, variability of exon-2 of BMP-15 gene (C to T nucleotide change) was detected using *Hinf*1 restriction enzyme. A specific primer pair was used for *Hinf*I mutation (C to T nucleotide change) in the BMP-15 gene, whereas products from non-cariers of the mutation lack this site. The amplified PCR products were digested with *Hinf*I restriction enzyme. This reaction mixture (30 µL) for restriction enzyme was kept for incubation at 37°C for overnight. The banding patterns were checked on 2.5 percent agarose gel.

Custom DNA sequencing: Representative samples obtained by RFLP analysis were sequenced by automated sequencer (ABI Genetic Analyser 3700) using Sanger's dideoxy chain termination method from M/S Ascension Life Science, Kolkata, West Bengal. Amplified PCR products were subjected to custom DNA sequencing from both ends (5' and 3' ends) using the forward and reverse primer. The forward and reverse sequences for each PCR fragment were assembled to form contig of respective region. Nucleotide sequences were visualized using Chromas (Ver.1.45, http://www.technelysium.com.au/chromas.html).

Sequence Data Analysis : The obtained sequence was first blasted (www.ncbi.nlm.nih.gov/BLAST) to ascertain that sequences were of BMP-15 gene. Sequence data was edit using the Editseq program and Multiple sequence alignments were performed with MegAlign program of LASERGENE software respectively (DNASTAR, Inc, Madison WI, USA). The sequences were then aligned with that of the reported BMP-15 sequences of different species using MegAlign Programme of Lasergene Software (DNASTAR).

The exon-2 of BMP-15 gene of Garole sheep was compared with that of already submitted NCBI data sequence to annotate different exonic region to identify variation in respective region. The contiguous exon-2 nucleotide sequence was subjected to Basic logical alignment search (BLAST) at NCBI database to know the sequence homology with the corresponding regions of other species. Phylogenetic tree was constructed by MegAlign software program. Phylogenetic analysis was performed to determine the evolutionary relationship.

RESULTS

PCR-RFLP analysis: In this present investigation approximately 200-300 mg of genomic DNA were isolated from each blood samples of thirty two ewes of

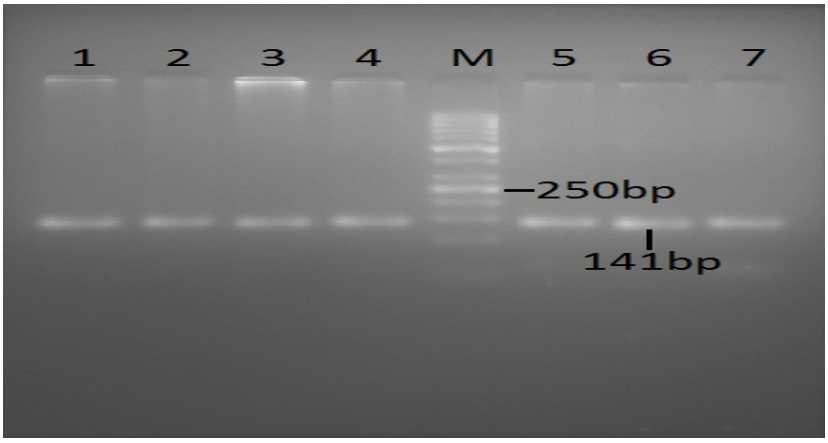
Garole sheep. All these samples under investigation showed a single genomic DNA band on 0.8 percent agarose gel electrophoresis. The amplification of exon-2 of BMP-15 gene resulted in a product size of 141 bp length on 2.5 percent agarose gel electrophoresis when visualized under the UV trans-illuminator in all the samples of Garole ewes of this University farm and depicted in Fig 1.

In all sheep studied, *Hinf*I detected a recognition sequence along the 141 bp length of PCR product of exon-2 of BMP-15 gene. After digesting with restriction endonuclease (*Hinf*I), the band pattern revealed homozygote wild type (++) was 110 and 31 bp fragment in this group of animal. Results obtained for PCR-RFLP analysis was presented in Fig. 2. The photographs showed same banding pattern in 2.5 percent agarose gel indicating same genotype of animals are presented in University Sheep and Goat Breeding Farm. The details of the comparison data and specific nucleotide replacement were represented in the alignment Fig. 3. The sequences from the BMP-15 gene corresponded to the GenBank accession number AF236078S2. Nucleotide sequence of PCR product revealed that 141 nt were present in the target region of BMP-15 gene. Sequencing result reveled that no mutation occurred where the C nucleotide has not changed to T at this locus (C-T) for exon-2 of BMP-15 gene in all experimental animals, indicating that wild type allele was present in this herd of Garole sheep.

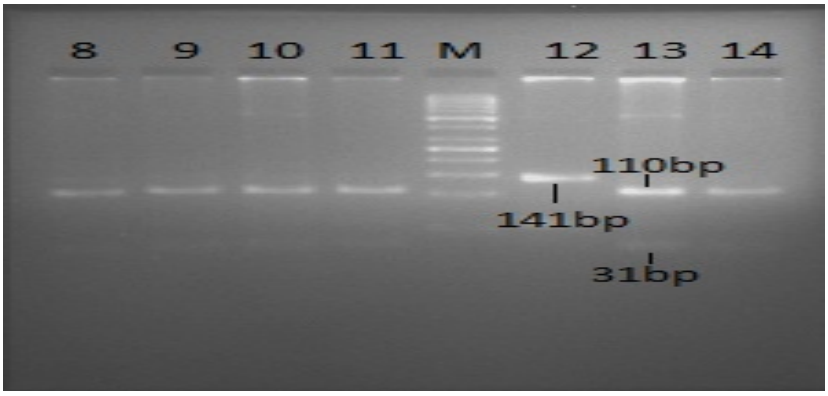
Nucleotide sequence analysis: Multiple alignment analysis revealed different nucleotide variations present in different positions of the nucleotide sequence of Garole sheep in comparison to the other species. The nucleotide alignment report was presented in Fig. 3. A close look at ClustalW Multiple sequence alignment result showed that there were no differences of nucleotide sequence of exon-2 of BMP-15 gene in studied Garole sheep with that of NCBI available exotic *Ovis aries* nucleotide sequence.

Result of multiple sequence alignment showed that nucleotide "A" was observed at position 407 in the sequence of Black Bengal goat and 6045 in of Ganjam goat whereas it was nucleotide "G" in the sequence of Garole sheep. Similarly at position 814 of the fragment nucleotide "G" was observed in the present study whereas in buffalo the nucleotide at this position is "C".

BLAST analysis of deduced nucleotide sequence revealed homology of 100 percent with Balochi, Lohi and Hu exotic sheep Breed. 100% of homology was also observed with Beetal goat breed of Punjab, and exotic goat breed of Boer, Teddy, Markhoz and Guizhou. However, the similarities both with Black Bengal goat of West Bengal and Ganjam goat of Odisha were 99%. Nucleotide sequence of Garole sheep showed cent percent similarity with *Bos taurus*. However, it was 99 percent identical to the sequence of *Bubalus bubalis*. BLAST analysis of exon-2 of BMP-15 gene of Garole sheep revealed homology of 100 percent with exotic sheep



Lane 1-7 : PCR product(141bp)
Lane M : 50bp molecular marker
Fig. 1. Ethidum Bromide-Stained gel (2.5%) of PCR products of exon 2 of BMP-15 gene in Garole sheep

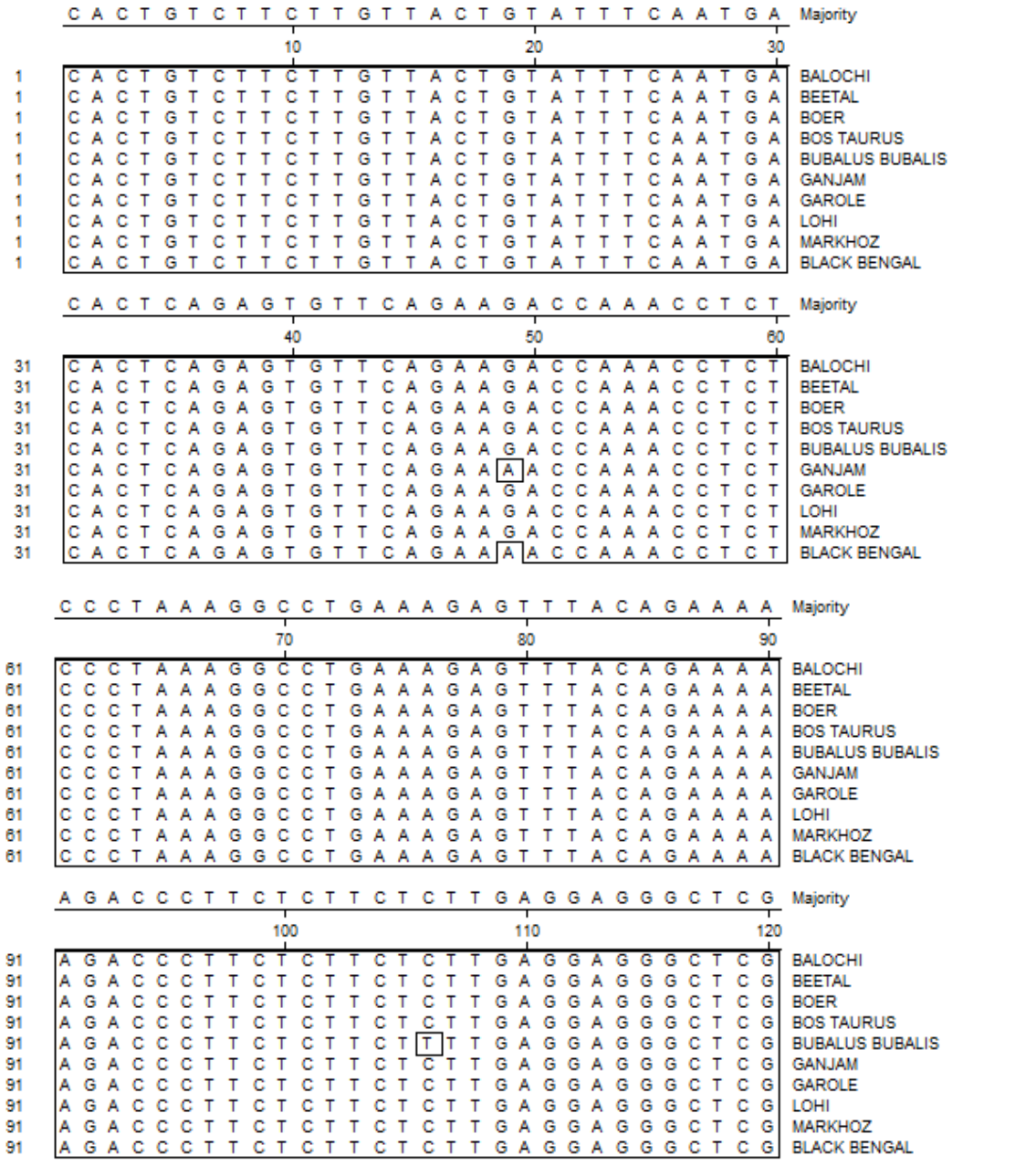


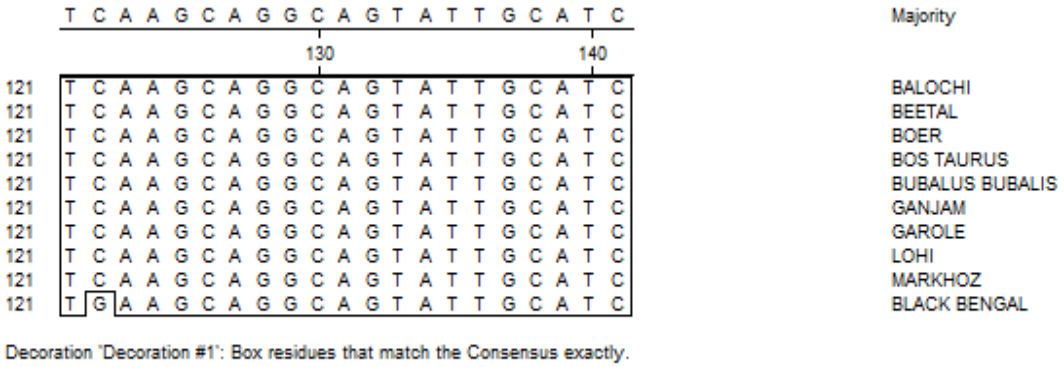
Lane 8-11 and13-14: Wild type Genomic(++) having 110 and 31bp.
Lane 12: PCR products of Exon-2 of BMP-15 Gene in Garole breed.
Lane M: 50bp molecular marcer
Fig. 2. RFLP pattern of PCR amplification exon-2 of BMP-15 gene digested with *Hinf1* RE in Garole sheep breed

breed (*Ovis aries*), 99 percent with Indian Goat (*Capra hircus*) and *Bubalus bubalis*. Percent identity/divergence among nucleotide sequences of exon-2 of BMP-15 gene (141 bp) of Garole sheep and other species were also depicted in Fig. 4.

The phylogenetic tree based on the basis of nucleotide sequences show the relative closeness among different species in the evolutionary time scale. The phylogenetic tree was constructed on the basis of nucleotide for exon-2 of BMP-15 gene of Garole sheep and the result was depicted in Fig. 5. The phylogenetic tree constructed on the basis of nucleotide sequences of different species revealed that Garole sheep

form a cluster with Lohi and Bolochi sheep breed, Beetal, Boer and Markhoz goat breed indicating their closeness in the evolutionary timescale. The distance of origin of this group gradually increased from Ganjan goat breed. The Ganjam goat breed came next in the hierarchy of closeness of origin.





Ovis aries: Balochi Breed, Pakistan (Accession no.JN655672.1), Lohi Breed, Pakistan (Accession no. JN655671.1), Hu Breed, China (Accession no. EU402923.1)
Capra hircus: Beetal Breed, India (Accession no.JQ350891.1), Teddy Breed, Pakistan (Accession no. JN655669.1), Markhoz Breed, Iran (Accession no. GU732196.1), Guizhou White Breed (Accession no. FJ429281.1), Boer Breed, Africa (Accession no. EU847289.1), Black Bengal Breed, India (Accession no. FJ665809.1), Ganjam Breed, India (Accession no. JQ350892.1)
Bos Taurus: (Accession no. EU712722.1)
Bubalus bubalis: (Accession no. HM854729.1)

Fig. 3. Multiple sequence alignment report of exon-2 of BMP-15 gene (141 bp) of Garole sheep with other

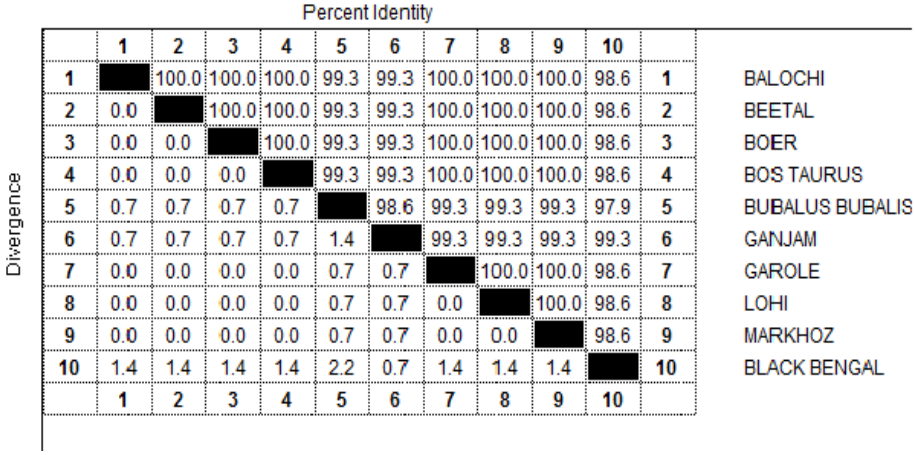


Fig. 4. Percent identity/divergence among nucleotide sequences of exon-2 of BMP-15 gene (141 bp) of Garole sheep and other species

DISCUSSION

The basic finding of the present study was the absence of polymorphism at the exon-2 of BMP-15 gene in Garole sheep, indicating that all Garole sheep were monomorph for exon-2 of BMP-15 gene. None of animal in this sheep under study carried the exon-2 mutation in the BMP-15 gene. The Kendrapara sheep was also genotyped for the presence of exon-2 mutations by Kumar *et al*. (2008). None of

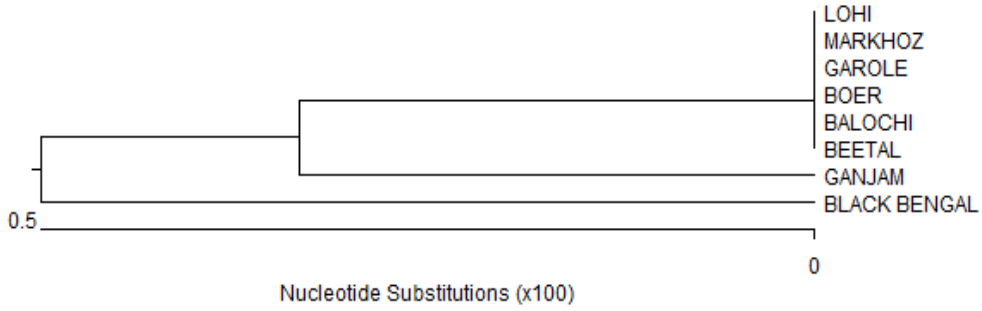


Fig. 5. Phylogenetic analysis based on nucleotide sequences of exon 2 of BMP15 gene (141 bp) of Garole sheep and other species

this breed were also found carrier for Galway mutation. The findings of the present study are also in line with those of Hu *et al*. (2007) in Chinese goats who reported that none of the polymorphism of the ovine fecundity major gene FecX was tested in 550 goats from six breeds (or flocks). Also, none of the polymorphisms were found in the coding region of BMP-15 mature peptide of Markhoz goats of Iran by using of PCR-RFLP with DdeI digestion (Arefnezhad, 2007). The absent of mutation in BMP-15 of six breeds of Chinese goats was reported by He *et al*. (2006). Zare *et al*. (2007) also detected no mutations in point of BMP-15 gene from 240 blood samples of Shal ewes by using of PCR-RFLP techniques. However, the Lacaune sheep (Bodin *et al*., 2002), the Belclare/ Cambridge sheep (Hanrahan *et al*., 2004) and Small Tailed Han sheep (Chu *et al*., 2007) are the sheep breed where mutations in this gene has been detected. As the population under study did not reveal any polymorphism for exon-2 of BMP-15 gene, the prolificacy could not be correlated with polymorphism in Garole sheep. The result indicated that the twinning/multiple lambing in this breed of Garole sheep is not linked with the Galway mutation. So far, no mutation in exon-2 of BMP-15 gene affecting prolificacy has been reported in Garole sheep.

Sequencing result reveled that no mutation occurred where the C nucleotide has not changed to T at this locus (C-T) for exon-2 of BMP-15 gene in all experimental animals, indicating that wild type allele was present in this herd of Garole sheep. However, Hanrahan *et al*. (2004) reported the C to T change at nucleotide 718 introduced a premature stop codon in the place of glutamic acid at amino acid residue 239 of the unprocessed protein, which presumably resulted in complete loss of BMP-15 function in Belclare and Cambridge sheep breed. Forward and reverse sequencing identified the *Hin*f I RFLP polymorphism as a C/T single nucleotide change at position 718 of the BMP-15 gene in Small Tailed Han sheep in China as reported by Chu *et al*. (2007). Nucleotide C-T base change in exon-2 of BMP-15 gene leading to the amino acid change of arginine to histidine and a premature stop codon in the place of glutamic acid as reported by Javanmard *et*

al. (2011) in fat-tailed sheep and Kasiriyan *et al*. (2011) in Iranian Sanghsari sheep.

Multiple alignment analysis revealed different nucleotide variations present in different positions of the nucleotide sequence of Garole sheep in comparison to the other species. Several polymorphic sites other than those reported in sheep have been published for exon-2 of BMP-15 gene in goats in different studies. Jiao *et al*. (2007) reported that exon-2 of BMP-15 has A963G (S300G) and C1050G (L329V) mutations. Further, Feng *et al*. (2009) observed that the A963G mutant of BMP-15 gene (exon-2) in Jining Grey goats has three genotypes (AA, AG and GG) while only AA genotype was found in Liaoning cashmere, Angora and Inner Mongolia Cashmere goats. Ran *et al*. (2009) found seven substitutions in mature peptide sequence of BMP-15 in white goat population of China. Nucleotide sequencing of exon-2 of BMP-15 gene also revealed absent mutation in the studied Garole sheep. Hence, further extensive study based on a large number of observations may be taken up in future for validity of this present finding.

The BLAST analysis of present study showed 99-100 % homology between species for the obtained sequence of exon-2 of BMP-15 gene of Garole sheep. The multiple alignment and homology analysis of Garole sheep sequence with those of other species revealed different degrees of nucleotide variations present among them. The information generated can be useful for further DNA level studies of this gene in Garole sheep. From the phylogenetic tree based on nucleotide sequence, it was evident that Black Bengal goat was most distant from this Garole sheep breed.

In conclusion, all the ewes of Garole sheep breed under study carried wild type homozygous genotype for exon-2 of BMP-15 gene. However, in present study, a small sample of animals was found that were presenting this feature. Therefore, further investigation on large population is required to confirm the present finding and also to the link of this mutation with increased prolificacy in Garole sheep.

ACKNOWLEDGEMENT

The authors wish to acknowledge the help and support rendered by Vice-Chancellor of West Bengal University of Animal and Fishery Sciences, Kolkata-700 037, West Bengal, India for providing necessary facilities to carry out this research endeavor.

REFERENCES

Arefnezhad B, 2007. Molecular analysis of BMP-15 (Bone morphogenetic protein-15) gene in Markhoz goats. M.Sc. Thesis, Department of Animal Science, Shiraz University, Shiraz, Iran

Bodin L, Di Pasquale E, Fabre S, Bontoux M and Monget P *et al.*, 2007. A novel mutation in the Bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulation rate and sterility in Lacaune sheep. Endocrinology, 148(1): 393–400

Bodin L, M San Cristobal FL, Mulsant P, Bibe B and Lajous D *et al*., 2002. Segregation of a major gene influencing ovulation in progeny of Lacaune meat sheep. Genet, 34: 447-464

Chu MX, Liu ZH, Jiao CL, He YQ and Fang L *et al*., 2007. Mutations in BMPR-IB and BMP-15 genes are associated with litter size in Small Tailed Han sheep (*Ovis aries). J Anim Sci*, 85: 598–603

Feng FJ, Li XL, Zhou RY, Zheng GR and Li LH *et al*., 2009. Characterization and SNP identification of part of the goat melanophilin gene. J Biochem.Gene, 47(3-4): 198-206

Galloway SM, McNatty KP, Cambridge LM, Laitinen MPE and Juengel JL *et al*., 2004. Mutations in an oocyte derived growth factor gene (BMP-15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet, 25: 279–283

Galloway SM, 2000. Mutations in the genes for oocyte derived growth factors GDF 9 and BMP-15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). Biol Reprod, 70: 900-909

Hanrahan JP, Gregan SM, Mulsant P, Mullen M and Davis GH *et al.*, 2004. Mutations in the genes for Oocyte-derived growth factors GDF9 and BMP-15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare Sheep (*Ovis aries*). Biology Reprod, 70(4): 900–909

He YQ, Chu MX, Wang JY and Fang L, 2006. Polymorphism on BMP-15 as a candidate gene for prolificacy in six goat breeds Chinese. J Anhui Agric Univ, 33: 61-64

Hua GH, Chen SL, Ai JT and Yang LG, 2007. None of polymorphism of ovine fecundity major genes FecB and FecX was tested in goat. Anim Reprod Sci, doi:10.1016/j.anireprosci. 2007. 08.013

Javanmard A, Azadzadeh N and Esmailizadeh KA, 2011. Mutations in bone morphogenetic protein 15 and growth differentiation factor 9 genes are associated with increased litter size in fat-tailed sheep breeds. Vet Res Commun, 35: 157–167

Jiao Cl, Ming-Xing C, Jin-Yu W, Li F and Sucheng Y, 2007. Cloning and sequence analysis of exon-2 of BMP-15 gene in Jining Grey Goats. Chinese Anim Vet Sci, 34(12): 52-55

Kasiriyan MM, Hafezian SH and Hassani1 N, 2011. Genetic polymorphism BMP-15 and GDF9 genes in Sangsari sheep of Iran. International J Gen and Mol Bio, 3(1): 31-34

Kumar SS, Mishra AK, Kolte AP, Dash SK and Karim SA, 2008. Screening for Booroola (FecB) and Galway (FecXG) mutations in Indian sheep. Small Ruminant Res, 80: 57–61

Monteagudo LV, Ponz R, Tejedor MT, Lavina A and Sierra I, 2009. A 17 bp deletion in the bone morphogenetic protein 15(BMP-15) gene is associated to increased prolificacy in the Rasa Aragonesa sheep breed. Anim Reprod Sci, 110: 139-146

Miller SA, Dykes DD and Polesky HF, 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res,16:1215

Ran XQ, Lin JB, Zhi-Yong D, Cheng Q and Wang JF, 2009. Diversity of BMP-15 and Gdf9 genes in white goat of Guizhou province and evolution of the encoded proteins. Zoological Res, 30(6): 593-602

Zare Y, Nejati-Javaremi A and Rahimi G, 2007. Detection of polymorphisms in two point of gene associated with twinning (BMP-15) in Shal sheep. The 5thNational Biotechnology Congress of Iran. Tehran, Iran, pp 483

Article received on 02.01.2016 and accepted for publication on 01.05.2016