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Detection of polymorphism of FecG gene in indigenous sheep of Arunachal Pradesh

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Abstract
Various physiological processes having direct impact on litter bearing ability namely folliculogenesis, oogenesis and ovulation are modulated by FecG or growth differentiation factor 9 (GDF9) on chromosome 5. Therefore, a study on molecular analysis of FecG gene was done for a better understanding of the reproductive ability of the indigenous sheep of Arunachal Pradesh. The aim of the study was to identify allelic variants of ovine FecG. Blood samples were collected from 50 randomly selected ewes. Genomic DNA extracted and PCR amplified for FecG gene with specific primer set. PCR fragment with length 139 bp was obtained. Genotypes were determined by restriction fragment length polymorphism (RFLP) method with DdeI restriction enzyme. Upon sequencing, a single restriction site was found. Thus, producing a single genotype AB, made up of two segments of 100 and 39bp. The locus of FecG gene was found to be monomorphic. According to the results the investigated animals were non-carriers of the mutation.

Keywords: Sheep, PCR, RFLP, FecG, polymorphism

Introduction
The worth of rearing small ruminants is never unknown for the poor farmers in comparison to the other livestock species, who cannot meet the expense of maintaining a cow or a buffalo find goat/sheep as an alternative source of livelihood. Sheep rearing plays a major role in rural economy particularly in the arid, semi-arid and mountainous areas of the country. As the farmers can acquire maximum benefits in terms of wool, meat, milk, skin and manure, most of them are engaged in sheep rearing. India is bestowed with a rich source of ovine germplasm, consisting of 43 numbers of registered breeds of sheep with a wide range of genetic variation. The total sheep population of the country has shown a considerable increase of 14.1% over previous census accounting 74.26 million (Twentieth Livestock Census).

Arunachal Pradesh, a state located in the north eastern part of India, dominated by the tribal population, is mainly dependent on agriculture and animal husbandry as their source of livelihood. The total indigenous sheep population of the state is 7085 (Twentieth Livestock Census) which are mainly concentrated in Tawang and West Kameng districts of the sates. The Indigenous sheep of this region play role to generate income to the family besides providing meat and wool, and are quite adaptive to the local climatic conditions and have shown better performance even in poor feeding and managemental practices.

Studies have shown that the reproductive processes like ovulation rate, litter size, fertility, normal follicular growth etc. are controlled by a set of genes collectively known as the fecundity (Fec) genes (Hanrahan *et al*., 2004) [11]. Various genes that have been reported to affect prolificacy in sheep includes the FecB which is bone morphogenetic protein receptor type 1B (BMPR1B) on chromosome 6, FecG which is growth differentiation factor 9 (GDF9) on chromosome 5 and FecX which is bone morphogenetic protein 15 (BMP15) on chromosome X (El-Fiky *et al*., 2017) [9]. GDF9 or the FecG is essential for the overall process of folliculogenesis, oogenesis, and ovulation and thus plays a major role in female fertility (Castro *et al*., 2015) [4]. Thus it is required to study the presence of fecundity gene (FecG) in the indigenous sheep population of Arunachal Pradesh for better understanding and improvement of reproduction. The detection of polymorphism of FecG and consequently the genetic information obtained from the study can be applied to improve the breeding, selection and ultimately conservation programmes. This study has been an attempt to detect the polymorphism of the FecG gene across the indigenous sheep of Arunachal Pradesh.

Materials and Methods

The indigenous sheep from West Kameng district of Arunachal Pradesh (which have derived its name from a tributary of Brahmaputra, i.e. Kameng river) was taken into consideration. The district/area lies between 27.3 ° N latitude to 92.3 ° E longitude. Approximately 3 ml of whole blood samples were collected from the jugular vein into vacutainers containing EDTA (2.7%) from 50 randomly selected ewes. The blood samples were properly mixed to prevent any clotting, than they were labelled and were taken into the laboratory in ice boxes right after collection and stored at -20º Celsius. Isolation of genomic DNA was done using Phenol Chloroform extraction Procedure (Sambrook and Russell, 2001) [18] with slight modification. The quality of Genomic DNA was checked by 0.8 % horizontal submarine agarose gel electrophoresis. The purity of the genomic DNA was assessed by UV spectrophotometer (Nanodrop Spectrophotometer, by checking the optical density (OD) value at 260 and 280 nm. The samples having OD ratio (269 nm/280nm) 1.7 to 1.9 were used for the experiment. The concentration of genomic DNA was estimated in spectrophotometer and the samples showing concentration between 50-90µg/ml of DNA were taken. PCR amplification of DNA was carried out in total volume of 50 ml. The reaction mixture contained PCR master mix (26 µl), nuclease free water (20 µl), forward and reverse primers (1 µl each) and DNA template (2 µl). Primer set suggested by Hanrahan *et al*. (2004) [11] was used to amplify FecG gene. The forward and reverse primers were: Forward: 5' CTT TAC TCA GCT GAA GTG GGA CAAC 3' and Reverse: 5'ATG GAT GAT GTT CTG CAC CAT GGT GTG AAC CTGA 3'.PCR cycling conditions were, an initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 30seconds, annealing at 62°C for 30 seconds and extension at 72°C for 30 seconds and the final extension at 72°C for 4 min. The confirmation of PCR amplification was done by running 1.5% agarose gel electrophoresis with 100bp ladder. For PCR-RFLP, PCR products were then digested in 20 µl reaction volume using 15µl of PCR products, 1 µl nuclease free water, 2 µl of buffer and 2 µl of restriction enzyme (DdeI) and kept for an incubation period of 16 hours at 37°C. 4% agarose gel electrophoresis was run to check the enzyme digested products along with 50 bp ladder for 2 hour at 85 V. The bands were visualized in the gel documentation system. Samples were outsourced for sequencing at Molbiogen (Apical Scientific Sequencing) after purification of respective PCR product. The analysis of molecular data was carried out by software Bioedit.

Results and Discussions

PCR amplification of FecG gene resulted in 139 bp amplicon (Fig. 1) for the indigenous sheep of Arunachal Pradesh. Ghaffari *et al*. (2009) [10] in Shal sheep; Shi *et al*. (2010) [19] in Xinjiang Cele black sheep; Debnath and Singh (2016) [7] in Balangir, Shahabadi and Bonpala sheep breeds; Mustafa *et al.* (2018) [14] in Lohi sheep and Mohamed *et al*. (2020) [13] in Watish Sudanese desert sheep also reported similar product size for FecG gene. The PCR-RFLP digestion of the amplified product upon DdeI (restriction enzyme) digestion produced single type of banding pattern yielding two fragments of 100 and 39 bp (Fig. 2). The population studied exhibited monomorphism for FecG gene. Arbitrarily, this could be distinguished as AB genotype in indigenous sheep of Arunachal Pradesh. Ghaffari *et al*. (2009) [10] in case of Shal sheep; Polley *et al*. (2010) [16] in Garole sheep; Kolosov *et al*. (2015) [12] in Russian sheep; Debnath and Singh (2016) [7] in Balangir, Shahabadi and Bonpala; Mustafa *et al*. (2018) [14] in Lohi sheep and Pineda *et al*. (2018) [15] in Colombian Creole hair sheep reported similar observation of single genotype of FecG gene. Contrary to the present finding more than one type of genotype were reported by different researchers such as Chang *et al*. (2009) [5] in Small Tail Han, White Suffolk, Texel and Tibetan sheep, Roy *et al*. (2011) in Bonpala sheep, Bahrami *et al*. (2014) [2] in Hisari sheep, Kolosov *et al.* (2014) in Russian sheep breeds, Barakat *et al*. (2017) [3] in Egyptian sheep breed; Dash *et al*. (2017) [6] in Kendrapara sheep; Al-Khuzai *et al*. (2019) [1] in Awassi sheep of Egypt and El Araby *et al*. (2019) [8] in Barki and Rahmani Sheep of Egypt

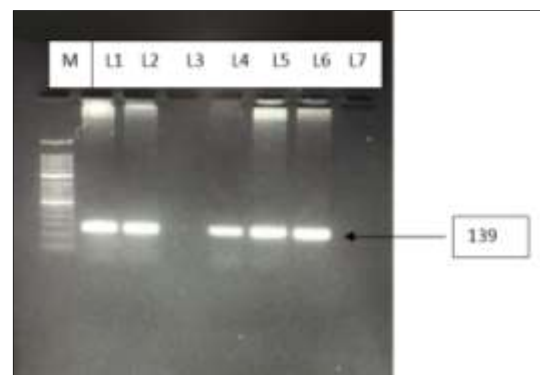

Fig 1: PCR amplification of FecG gene. (M = 50 bp Ladder, L1, L2 L4, L5 and L6 = Amplified product, L3 and L7 = Blank)

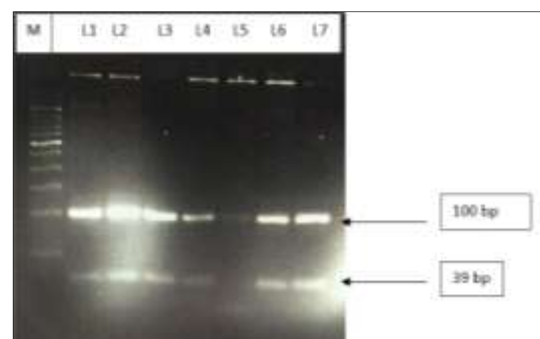

Fig 2: PCR-RFLP OF FecG Gene Using DdeI (M = 50 bp and L1 – L7: Lanes)

Few samples representing AB genotype in FecG gene were sequenced (in both forward and reverse directions) by automated DNA sequencer (Applied Biosystem, USA) which was done by out sourcing as shown in Fig. 3 and Fig. 4. Based on the scores obtained by BLAST, other nucleotide sequences of FecG gene of *Ovisaries* were obtained from NCBI for comparative analysis. Multiple alignments of the sequences were performed using Clustal W software as shown in Fig. 5. DdeI restriction site was detected at nucleotide 100th position of the 139 bp fragment of FecG gene (Fig. 6).

GTTTGGCCNACAATACAACCCTCGATACTGTAAAGGGGACTGTCCCAGGGCGGTCGGACATCGGTAT
GGCTCTCAGGTTCACACCATGGTGCAGAACATCATCCATAAT

Fig 3: Partial sequence of *FecG* Gene and ITS *DdeI* Restriction Site

GTTTGGCCNACAATACAACCCTCGATACTGTAAAGGGGACTGTCCCAGGGCGGTCGGACATCGGTATG
GCTCTCAGGTTCACACCATGGTGCAGAACATCATCCATAAT

Fig 4: Partial sequence of *Fec G* gene sequence of AB genotype

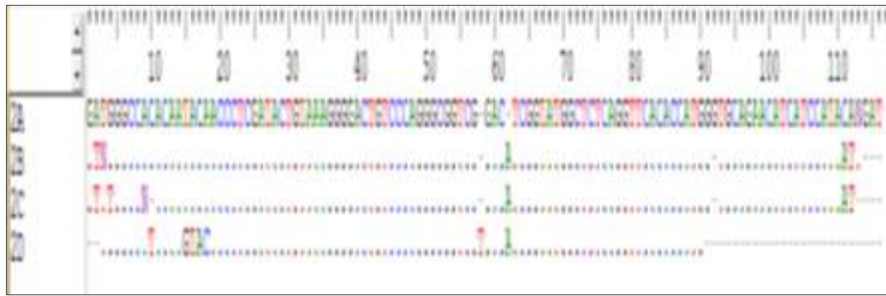

Fig 5: Clustal W sequence alignment of *FecG* gene

Fig 6: *DdeI* restriction site of *FecG* gene

One restriction site was found for the restriction enzyme DdeI when the sequence obtained after PCR-RFLP was analysed in BLAST upon submission to NCBI.

Conclusion

From the present study it can be concluded that the FecG gene is monomorphic in all 50 investigated indigenous sheep of Arunachal Pradesh. Only one genotype arbitrarily designated as AB was detected. However, further study may be undertaken to confirm it in a substantially large population.

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