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Genetic polymorphism of FecB gene in NARI Suwarna flock

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Abstract
Aim: This work was conducted to study the polymorphism and also the inheritance pattern of the FecB gene in the NARI Suwarna sheep which were procured from Nimbkar Agricultural Research Institute, Phaltan, Maharashtra and are being maintained at Instructional Livestock Farm Complex, College of Veterinary Science, Rajendranagar, Hyderabad.
Materials and Methods: DNA isolation and gene-specific amplification of FecB gene was performed from blood samples of 25 NARI Suwarna sheep. The amplicons were then subjected to PCR - RFLP and Nucleotide sequencing to identify different genotypes with respect to the target gene.
Results: Out of 25 samples screened for FecB gene, four samples were homozygote (BB) with 108bp and 32bp, 15 samples were heterozygote (Bb) with 140bp, 108bp and 32bp and the remaining six samples were wild type (bb) with 140bp. The *AvaII* digestion of 140bp exon 8 yielded three genotypic patterns indicating only two allelic variants whereas the *AvaII* digestion of PCR product 198bp for exon 9 and 153bp for exon 10 did not produce any restricted fragments, indicating the absence of restriction site. The exon 8 showed three genotypes (BB, Bb and bb) in NARI Suwarna flock with genotypic frequencies as 0.16, 0.60 and 0.24 and the allelic frequencies were 0.46 and 0.54 for the alleles B and b, respectively. The results suggested that the polymorphism showed only in exon 8 region of FecB gene.
Conclusion: The FecB gene was present in some of the animals studied and absent in others. Out of three segments of FecB gene (exon 8, 9 and exon 10) only exon 8 showed polymorphism and the remaining segments are thought to be non-polymorphic and conserved among the breeds. Further, study with larger sample size is needed to elucidate the existing polymorphism in this gene.

Keywords: NARI Suwarna, FecB gene, *AvaII*, Molecular genetic characterization, Polymorphism

Introduction
Sheep is an important livestock species in India. It contributes greatly to the agrarian economy, especially in the arid/semi-arid and hilly areas where crop and /or dairy farming are not economical. They play an important role in the livelihood of a large percentage of small and marginal farmers and landless labour engaged in sheep rearing.
While several studies have been carried out with mucin from various sources, this study was designed to compare the properties of mucin extracted from the African giant snail and the intestines of cow and pig that could influence the performance of dosage forms.
The sheep population in India is 65.07 million and contributes to around 12.71% of the total livestock population as per 19th Livestock Census-2012 (Food and Agriculture Organization). The productivity of Indian sheep is lower than those of agriculturally advanced countries. The major reasons for the low productivity are inadequate grazing resources, incidence of diseases leading to high mortality, morbidity consequent reduction in productivity and lack of organized efforts for genetic improvement. Improvement in fertility traits is an important task for sheep producers, which have a major impact on efficiency and profitability in lamb meat production. Hence, much of the emphasis is to be focused to study the genes associated with fertility for overall development of the productivity. Hence, the present study is accentuated on the FecB gene which is the fecundity gene.
The expression of the FecB gene varies in different breeds, resulting in different levels of prolificacy. The difference observed in prolificacy is due to various factors, like environmental variation, ewe parity, background breed, selection and maternal nutrition. The negative effects of FecB gene is less body mass, embryonic mortality, born weak lambs and lower survival rate of lambs, besides high ovulation rate and litter size [1]
The Garole is micro sheep native of West Bengal, which is having FecB gene mutation. It is the latest sensation in the World of domestic species by virtue of its prolificacy, lambing frequency, disease resistance and other extraordinary merits rarely or not even observed in

other sheep breeds of the world. Mostly Indian sheep breeds have lambing with one lamb except for Garole sheep, which is renowned for its high reproductive efficiency and prolificacy due to presence of Booroola fecundity (FecB) gene.

NARI Suwarna, the sheep under study is a synthetic breed, developed at Nimbkar Agricultural Research Institute (NARI) at Phaltan, Maharashtra State [2]. It is a cross between Lonad Deccani (≥75%) and Garole (≤25%) sheep and the results showed that one copy of FecB led to an increase in litter size per ewe conceived by 0.37 lambs [3].

Materials and Methods

Ethical approval

All the animal experiments were conducted after approval of committee for the purpose of control and supervision of experiments on animals.

Experimental Animals

About 15 NARI Suwarna sheep (females and males) were procured from NARI, Phaltan, Maharashtra and are maintained at Instructional Livestock Farm Complex, College of Veterinary Science, Rajendranagar, Hyderabad. These animals along with their progeny were used to study the inheritance of FecB gene and to explore the DNA polymorphism of FecB gene by using PCR-RFLP technique. A total of 25 NARI Suwarna sheep were eventually covered under the present investigation.

Blood Collection and Genomic DNA extraction

About 8-10 ml of blood was collected from each animal aseptically for the DNA isolation. Genomic DNA was isolated by Phenol-Chloroform method, as described by Sambrook *et al*. [4] with few modifications. The quality of DNA was checked by gel electrophoresis and purity by Nano drop taking ratio of optical density (OD) value at 260 and 280 nm. Good quality DNA having OD ratio be-tween 1.7 and 1.9 was used for further work.

PCR-RFLP of exon 8, 9 and 10 regions of FecB gene

The amplification of the exon 8, 9 and 10 portions of FecB gene by using specific primers i.e., exon 8 forward (5′-GTCGCTATGGGGAAGTTTGGATG-3′), reverse (5′-CAAGATGTTTTCATGCCTCATCAACACGGTC-3′) [5], exon9 forward(5′-TATCAAAGGGACGGGGTCCTGG-3′), reverse (5′-TCGATGGGCAATTGCTGGTTGC- 3′) [6] and exon10 forward (5′-TGACATACCACCCAACACTC-3′), reverse (5′-ACACATCTCCTAGCGACCTC-3′) [7].

PCR reaction was carried out containing 1x Taq Buffer, 0.1 mM dNTPs, 30 pM Primers-Forward and reverse, 1.5 mM $MgCl_2$, 0.3 unit Taq Polymerase and autoclaved milli Q water by using (GeneAmp®, PCR system 9700, Applied Biosystem) with the following conditions: initial denaturation of 3 min at 95°C followed by 35 cycles of denaturation 30sec at 94°C, annealing temperature ranged from 57ºC to 61ºC depending on the primer used and extension at 72°C each of 30 seconds and lastly the final extension of 5-10min at 72°C. The amplified PCR products were checked by 8% polyacrilamide gel electrophoresis (PAGE). A 50bp DNA ladder (Fermentas) was also run alongside the samples to ascertain the size of the amplified products. After electrophoresis the amplified products were visualized under UV light in a UV transilluminator. An aliquot of 10µl of PCR product was digested with 0.50µl of *AvaII* restriction enzyme and 1x RE buffer 1.0 µl in a water bath for 3-4hr. The restriction enzyme digested PCR products were separated by 3% of agarose gel.

Statistical Analysis

The genotypic and allelic frequencies of FecB gene

The genotypic and allelic frequencies of FecB gene in NARI Suwarna sheep were estimated as per Hardy–Weinberg formulae [8].

Genotypic frequencies

$$Homozygote\ mutant\ genotype\ (BB) = P = \frac{Number\ of\ BB\ type\ animals}{Total\ number\ of\ animals}$$

$$Heterozygote\ genotype\ (Bb) = H = \frac{Number\ of\ Bb\ type\ animals}{Total\ number\ of\ animals}$$

$$Homozygote\ wild\ genotype\ (bb) = Q = \frac{Number\ of\ bb\ type\ animals}{Total\ number\ of\ animals}$$

Allelic frequencies

$Frequency\ of\ B\ allele\ = P + ½\ H$

$Frequency\ of\ b\ allele\ = Q + ½\ H$

Sequencing of PCR product of 140bp exon8 of FecB gene

Representative samples of purified PCR product were sequenced by automated sequencer (ABI Genetic Analyzer 3700) using Sanger's dideoxy chain termination method at Centre for DNA Fingerprinting and Diagnostics, Hyderabad. Amplified PCR products were subjected to custom DNA sequencing from both ends (5' and 3' ends) using the forward and reverse primers. The forward and reverse sequences for each PCR fragment were assembled to form contig of respective region. Nucleotide sequences were visualized by using Chromas software. (Ver.1.45, http:// www. technelysium.com.au/chromas.html). The obtained sequence was first blasted (www.ncbi.nlm.nih.gov/BLAST) to ascertain that sequences were of FecB gene. The reference NARI Suwarna sequence was subjected to multiple alignment with reported sequences of other breeds of sheep by using clustalW software programme.

Results

PCR-RFLP of exon 8, 9 and 10 of FecB gene

The *AvaII* digestion of 140bp PCR product of exon 8 yielded three genotypic patterns with two allelic variants. Out of 25 samples screened for FecB gene, four samples were homozygote (BB) with 108bp and 32bp, 15 samples were heterozygote (Bb) with 140bp, 108bp and 32bp and the remaining six samples were wild type (bb) with 140bp (Figure-1). The genotypic frequency for this fragment were 0.16, 0.60 and 0.24 for BB, Bb and bb respectively. Allelic

frequencies 0.46 and 0.54 for B and b respectively (Table-1). The *AvaII* digestion of 198bp product of exon 9 (Figure-2) and 153bp of exon 10 (Figure-3) did not produce any restricted fragments.

Comparative Nucleotide sequence analysis of exon 8 portion of FecB gene with other sheep breeds

The exon 8 (140bp) of FecB gene in NARI Suwarna revealed the restriction site for *AvaII* enzyme at 108 position (Figure-4). So the banding pattern was 108bp and 32bp which is in homozygous condition.

A 140 nucleotide sequence of exon 8 of the FecB gene was compared with the NARI Composite (Acc.No.HM853667.1), Garole Fecundity (Acc.No.GQ863576.1), Nilagiri (Acc.No.LC152970.1) and Marwari (Acc.No.HM853663.1). The exon 8 of FecB gene sequence of NARI Suwarna is deviating from other breeds of sheep. The comparison of the nucleotides at coding region is shown in Figure-5.

The nucleotide sequence of exon 8 of FecB gene of NARI Suwarna was differing at 19 bases when compared with other sheep breeds (NARI Composite, Garole Fecundity, Nilagiri and Marwari). The 19 different nucleotide positions at which NARI Suwarna differing with other breeds of sheep breeds is presented in Table -2.

The NARI Suwarna FecB homozygous (BB) showed homology of 99.8, 99.8, 99.8 and 99.7 percent with NARI Composite, Garole Fecundity, Nilagiri, and Marwari by BLAST analysis.

Table 1: Genotypic and allelic frequencies of FecB gene

Sample Size	Genotypic Frequency			Allelic Frequency	
25	BB	Bb	bb	B	b
Estimated Value	0.16	0.60	0.24	0.46	0.54

Table 2: Nucleotide changes at different positions in NARI Suwarna differing with other sheep breed

Position	24	25	26	27	29	30	31	32	34	36
NARI Suwarna	T	A	G	G	T	G	A	A	A	T
Other sheep breeds	G	G	A	A	G	T	G	G	G	G

Position	39	40	41	42	44	46	47	54	55
NARI Suwarna	C	G	T	T	A	A	C	T	T
Other sheep breeds	G	A	A	A	G	T	A	A	A

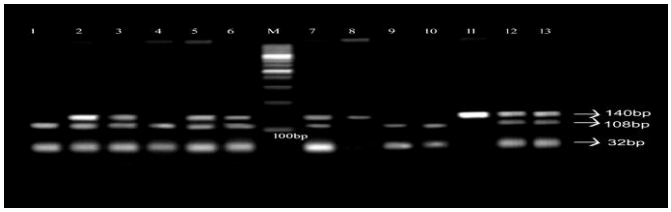

Fig 1: *AvaII* digested product of 140bp exon 8 in NARI Suwarna (13 animals). Lane 1-13 – *Ava II* digested product; M - 100bp marker.

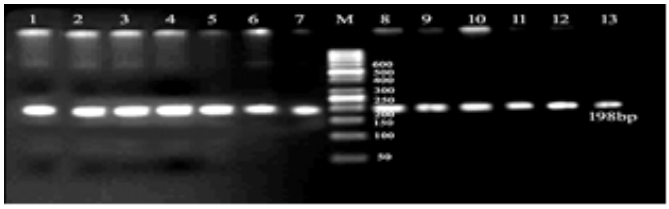

Fig 2: *AvaII* digested product of 198bp exon 9 in NARI Suwarna Lane 1-13 - *Ava II* digested product; M - 50bp marker

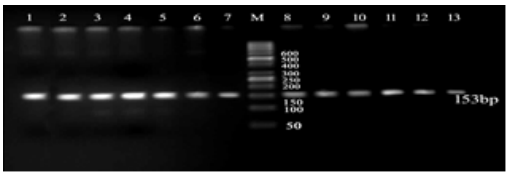

Fig 3: *AvaII* digested product of 153bp exon 10 in NARI Suwarna Lane 1-13 - *Ava II* digested product; M - 50bp marker.

5'GTCGCTATGGGGAAGTTTGGATGTAGGTGAACATTGCCGTTAAGACGCTGTGTTAG
TGTTCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCG↓GACC
GTGTTGATGAGGCATGAAAACATCTTGA 3'

Fig 4: *AvaII* restriction site in nucleotide sequence of 140bp exon 8 of FecB gene in NARI Suwarna.

```
1                                                          60
NS      GTCGCTATGGGGAAGTTTGGATGTAGGATGAACATTGCCGTTAAGACGCTGTGTTAGTGT
NC      GTCGCTATGGGGAAGTTTGGATGGGAAAGTGGCGTGGCGAAAAGGTAGCTGTGAAAGTGT
GF      GTCGCTATGGGGAAGTTTGGATGGGAAAGTGGCGTGGCGAAAAGGTAGCTGTGAAAGTGT
NIL     GTCGCTATGGGGAAGTTTGGATGGGAAAGTGGCGTGGCGAAAAGGTAGCTGTGAAAGTGT
MAR     GTCGCTATGGGGAAGTTTGGATGGGAAAGTGGCGTGGCGAAAAGGTAGCTGTGAAAGTGT

          61                                                      120
NS      TCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCGGACCGTGTTGA
NC      TCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCGGACCGTGTTGA
GF      TCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCGGACCGTGTTGA
NIL     TCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCGGACCGTGTTGA
MAR     TCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCAGACCGTGTTGA

          121                 140
NS      TGAGGCATGAAAACATCTTGA-
NC      TGAGGCATGAAAACATCTTGA-
GF      TGAGGCATGAAAACATCTTGCA
NIL     TGAGGCATGAAAACATCTT---
MAR     TGAGGCATGAAAACATCT----
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Note: NS (NARI Suwarna), NC (NARI composite), GF (Garole Fecundity), NIL (Nilagiri), MAR (Marwari)
Variation in nucleotide sequence in NARI Suwarna are highlighted in yellow color.

Fig 5: Comparative Nucleotide Sequences of Exon8 FecB gene in NARI Suwarna compare with other sheep breeds.

Discussion

Exon 8 yielded three genotypic patterns with two allelic variants. Out of 25 samples screened for FecB gene, four samples were homozygote (BB) with 108bp and 32bp, 15 samples were heterozygote (Bb) with 140bp, 108bp and 32bp and the remaining six samples were wild type (bb) with 140bp. However, the results obtained in the present study were in contrary with few of the publicized literature. Pardeshi *et al.* [9], reported 110bp in Garole sheep and 140bp undigested fragment in Deccani, Madras red and Bannur sheep; Prasad *et al.* [6] reported 110bp and 140bp fragments of FecB gene in Garole sheep and 140bp fragment in Munjal, Nali, Nali crosses; Debnath *et al.* [10] reported 110 and 140bp bands in Shahabadi sheep; Dutta *et al.* [11] reported 110bp fragment for the 140bp amplified product of FecB gene in Garole and Maskur *et al* [12] reported 110,110 and 140bp and single uncut 140bp fragments for the 140bp amplified product of FecB gene in Indonesian fat tailed sheep. The literature where the results were not in agreement with the present findings were; Asadpour *et al.* [13] reported 160bp size fragment for the 190bp amplified product of FecB gene in Zel sheep, Shafieyan *et al.* [14] found 190bp undigested fragment in Lory sheep and Tanesh e*t al.* [15] done SSCP in 190bp fragment of FecB gene in chhotanagpuri sheep. These differences might be due to inclusion of some portion of introns along with exon 8.

From the available literature, it was found that, there were three genotypes (BB, Bb and bb) recorded by Davis *et al.* [16] in Han ewes, Kumar *et al.* [17] and Kolte *et al.* [18] in Garole sheep, Asadpour *et al.* [13] in Zel sheep, Debnath and Singh [10]

in Shahabadi sheep and Tanesh e*t al*. [15] in chhotanagpuri sheep. However, Pardeshi *et al*. [9] reported only two genotypes in Garole and only one genotype bb was recorded by Guan *et al*. [19] in Chinese Hu sheep, Ghaffari *et al.* [20] in Shal sheep and Dutta *et al.* [11] in Garole.

The genotypic frequencies for homozygote FecB, heterozygote FecB and the wild type were 0, 0.15 and 0.85 as reported by Asadpour *et al*. [13] in Zel sheep; 0, 0.76 and 0.24 by Debnath and Singh [10] in Shahabadi sheep; 0.060, 0.268 and 0.672 by Maskur *et al* [12] in Indonesian fat tailed sheep and 0.250, 0.324 and 0.424 by Tanesh e*t al*. [15] in chhotanagpuri sheep respectively. However, the frequency of 1.0 for the single genotype bb was reported by Guan *et al*. [19] in Chinese Hu sheep and Dutta *et al.* [11] in Garole the allelic frequencies obtained in the present study were 0.46 and 0.54 for the alleles B and b, respectively. The values for the same alleles reported by Debnath and Singh [10] were 0.37 and 0.63 in Shahabadi sheep; 0.99 and 0.01 by Asadpour *et al.* [13] in Zel sheep; 0.61 and 0.39 by Polley *et al*. [21] in Garole; 1.0 and 0 by Dutta *et al.* [11] in Garole; 0.193 and 0.807 by Maskur *et al* [12] in Indonesian fat tailed sheep and 0.413 and 0.587 by Tanesh e*t al*. [15] in chhotanagpuri sheep for B and b alleles, respectively.

There were no reports of PCR-RFLP with *AvaII* restriction enzyme in exon 9 portions of FecB gene. However Prasad *et al*. [6] reported that there was no polymorphism with *SspI* restriction enzyme in exon 9 of FecB gene in Munjal, Nali and Nali crosses. However Kumar *et al.* [7] reported two fragments of 18bp and 135bp with *XhoI* restriction enzyme in Garole and Muzaffarnagari sheep. These findings suggested that the exon 10 possessed the site for *XhoI* enzyme but not for the *AvaII*.

Debnath and Singh [10] compared the Shahabadi FecB heterozygous (Bb) carrier with that of Garole (GQ863576), Muzaffarnagari (GQ863577), Balangir and Bonpala FecB non-carriers. The Shahabadi FecB heterozygous carrier showed similarity of 97.9, 99.3, 98.6 and 98.6 percent with Balangir, Bonpala, Garole and Muzaffarnagari sheep, respectively. Probably these nucleotides might be specific to this breed in this region.

Conclusion

The FecB gene was present in some of the animals studied and absent in in others. Out of three segments of FecB gene (exon 8, 9 and exon 10) only exon 8 showed polymorphism and the remaining segments are thought to be non-polymorphic and conserved among the breeds. Further, study with larger sample size is needed to elucidate the existing polymorphism in this gene.

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