Expression analysis of fecundity related genes in FecB carrier and non-carrier ewes by semi-quantitative PCR

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ABSTRACT

In the present study, an attempt has been made to analyze the expression of genes associated with fecundity in FecB carrier and non-carrier ewes using semi-quantitative PCR. Ovary tissues were collected from one Garole (homozygous for FecB), one non-carrier Deccani and one heterozygous crossbred (Garole x Deccani) ewe with known litter sizes. Total RNA was extracted and reverse transcribed to generate cDNA. Expression of eight genes, likely to be associated with the regulation of ovulation rate, viz., BMPRIB, BMP15, STAT5, SMAD5, SMAD9, FSHR, LHCGR and GDF9 was analyzed by semi quantitative RT-PCR. Six genes, viz., BMPRIB, BMP15 STAT5, SMAD5, LHCGR and GDF9 showed differential level of expression across ewes of different breeds and FecB genotypes having different litter sizes. The expression of BMPRIB, SMAD5 and LHCGR was found to be higher in ewes homozygous for FecB mutation while the mRNA level of SMAD9 and FSHR was found to be similar across all the three genotypes. This preliminary study thus shows differential expression of investigated genes in FecB carrier and non-carrier ewes, which would further guide to analyze specific gene(s) quantitatively across specific physiological stages and in genotypes to elucidate their probable role in litter size.

Key Words: Fecundity, ovulation rate, litter size, RT-PCR

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INTRODUCTION

India possesses a wide variety of sheep populations represented by 40 descript breeds and several non-descript populations adapted to varied climatic conditions and different geographical regions. Sheep play a major role in Indian agricultural economy by contributing in terms of meat, by far the major product of economic importance from sheep, manure and wool as well. Economics of sheep production in India is therefore largely governed by the litter size. Most of the Indian sheep produce single offspring in a lambing except Garole and a few other breeds viz. Kendrapara sheep breed of Odisha (Patro et al., 2006). Garole is a breed of Sunderbans, West Bengal, in India and is well known for its high fecundity (Ghalsasi & Nimbkar, 1993).

Prolificacy in sheep is determined mainly by the ovulation rate that is the number of ova shed in each estrous cycle. Folliculogenesis is a highly ordered genetically controlled process involving the expression of several genes under precise temporal and spatial regulation. Mutations causing increased ovulation rate have been discovered in the BMPRIB, GDF9, BMP15 and other genes, discovered from the expressed inheritance patterns (Moore et al., 2004; Chu et al., 2005; Chu et al, 2006). The fecundity mutations have been identified as point mutations commonly known as FecB, FecXI, FecX2w (Davis et al., 2008). The identification of these mutated genes point to the TGFß superfamily; which act as autocrine/paracrine regulators of ovarian follicular development and ovulation through the BMP/SMAD signaling pathway (Galloway et al., 2000)

Most of the TGFß superfamily members exert their influence on target cells by binding to and forming hetero-tetrameric complexes with two types of Ser/Thr kinase receptors on the cell surface designated as type-I and type-II. Other genes implicated in the process include various SMADs and transcription factors like STAT5 and the role of these in increased ovulation rate is yet to be ascertained (Massague and Wotton 2000, Miyazawa et al., 2002, Chang et al., 2002). The FecB mutation was initially identified in Booroola Merino sheep originating from Australia with its origins traced back to Garole sheep from Bengal (Davies et al., 2002). The spontaneous mutations leading to alterations in ovulation rate in Inverdale (FecXI) and (FecXH) ewes were shown to involve ligands rather than receptors of the TGFß superfamily due to separate point mutations identified in the bone morphogenetic protein (BMP15) gene on X chromosome. Similarly, in Cambridge and Beclare ewes, mutations in both BMP15 and GDF9 lead to a marked increase in ovulation rate. (Souza et al., 2004)

FecB or the Booroola is a dominant autosomal gene mutation with an additive effect on ovulation rate. The effect of Booroola gene has been shown to be due to a single point mutation (Q249R) in the intracellular kinase-signaling domain of the BMPRIB gene, which is expressed in oocytes and granulosa cells (Mulsant et al., 2001; Souza et al., 2001 and Wilson et al., 2001). Garole sheep breed of India is known to be a carrier of Booroola (FecB) gene mutation (Davies et al., 2002). Also more recently, Kendrapara sheep of Orissa has been shown to carry the FecB mutation (Kumar et al., 2008). By introgression of the FecB mutation in sheep of low prolificacy, it is possible to increase the litter size and hence increase the productivity in sheep (Banerjee et al., 2010). The relationship between FecB genotype and litter size or the ovulation rate needs to be investigated and understood for improving sheep production. In this direction the present study attempts to analyze the expression

profile of fecundity related genes in whole ovarian tissue of FecB carrier and non-carrier ewes by semi-quantitative PCR.

MATERIALS AND METHODS

Collection of ovaries: Ewes of different breeds and FecB genotypes were selected from the flocks at the Nimbkar Agricultural Research Institute (NARI) at Phaltan in Maharashtra State for collection of ovaries. The sampling was done at NARI, following standard laparoscopy surgical procedure. The samples were collected in RNAlater solution (Ambion, USA) and stored at -80°C after transportation to the laboratory. Ovary tissues were collected from ewes of known FecB genotypes and litter size records of at least three lambings viz. Garole BB homozygous for FecB (Av. litter size: 2.67), Crossbred B+, heterozygous for FecB (Av. litter size: 1.20) and Deccani ++, wild type non-carrier (Av. litter size: 1.00) ewes. The Garole and crossbred ewes were in the luteal phase of oestrus while the Deccani ewe was not in oestrus. It was in the sexually quiescent phase. The ovary tissues were from right ovaries of Garole and Deccani and left ovary of Crossbred.

Selection of genes and primer designing: Eight genes viz. BMPRIB, BMP15, STAT5, SMAD5, SMAD9, FSHR, GDF9 and LHCGR, likely to be involved in folliculogenesis, were selected for expression analysis. Specific primer pairs for each of the genes associated with ovulation rate were designed from the sheep or cattle sequences available in the GenBank database of NCBI for semi-quantitative as well as quantitative real time PCR analysis (Table 1). Heterologous primer pairs were designed for LHCGR and SMAD4 gene transcripts using bovine sequence while for the rest of the genes including GAPDH, homologous primer pairs were designed using sheep sequences by PrimerSelect program of Lasergene software (DNASTAR, USA).

Table 1. Details of the designed primers used for gene expression analysis

Gene	Primer Sequence	Amplicon size	Annealing temp. (°C)	NCBI Accession no.
BMPRIB	F 5'AACCGGTGGAGCAGTGACGAG 3'	125bp	55	AF312016
	R 5'TCTGACATTTTGGCAAGGGTTTTC 3'			
BMP15	F 5' GAGCATGATGGGCCTGAAGTAA 3'	106bp	55	NM_001114767
	R 5' GGGTATAGAGATGGGGAGCAATGA 3'			
STAT5	F 5' GCCCCCAGCCCTACAGC 3'	121bp	55	NM_001009402
	R 5' GAGAAATCCCGGTGGTGAATG 3'			
SMAD5	F 5' TCACAGACCCTTCGAATAACAAA 3'	129bp	50	NM_001077107
	R 5' CTCCACCAACGTAGTACAGATGAA 3'			
SMAD9	F 5' TCGCCAGGATGTCACAAG 3'	95bp	54	NM_001076928
	R 5' GGGAGCCCATCTGAGTCA 3'			
FSHR	F 5' CTGTGATGCTGCGGGGCTTTTTC 3'	102bp	58	NM_001009289
	R 5' ATGGCGTGGGTGATGGTATGC 3'			
GDF9	F 5' GGCATTCCCTCCACCCTAAAAG 3'	105bp	55	NM_001142888
	R 5' AGCTGGCACTCTCCTGGTCTCTG 3'			
LHCGR	F 5' TGGGCTCTACCTGCTACTCATT 3'	109bp	53	L36329
	R 5' AAGCCAGCAACACTACACCCATTC 3'			
GAPDH	F 5' GCC CAA AAC ATC CCT GCT TCT 3'	86 bp	50-60	AF272837
	R 5' ATG CCA GTG AGC TTC CCG TTG AG 3'			

RNA extraction: Total RNA was extracted from the ovary tissue samples collected from ewes of different genotypes/litter sizes using TRIzol method. Briefly, the tissue samples were homogenized by adding 1 ml of TRIzol (Invitrogen, USA) per 100 mg of tissue using GlasCol homogenizer (GlasCol, USA). The tissue suspension in TRIzol was centrifuged at 12000xg for 10 min. To the supernatant, 200µl of chloroform was added and shaken vigorously. The samples were kept at room temperature for 15 min. The mixture was then centrifuged at 12000xg for 15 min and supernatant was collected into fresh tube. RNA was precipitated by adding 0.5 ml isopropanol and incubating at room temperature for 10 min. The RNA was pelleted by centrifugation at 12000xg for 15 min and supernatant was discarded. The pellet was washed with 75% ethanol and re-suspended in 30-50µl RNA storage solution and stored at -80º C till used. RNA quantity and quality was assessed by Nanodrop spectrophotometer and also using Experion Bioanalyzer (BioRad, USA).

Reverse Transcription-PCR: Equal amount of good quality total RNA (1 µg) from all samples was reverse transcribed adding 0.5 µg of oligo (dT) primers, 4 µl of 25mM MgCl2, 1 µl of 10 mM dNTP mix, 2 µl of 0.1 M DTT, 40 U of RNase inhibitor and 200 U of MMLV- Reverse transcriptase using Superscript first strand cDNA synthesis kit (Invitrogen, USA). RNA was incubated at 65ºC for 5 min. followed by 50ºC for 60 min and 85ºC for 5 min.

After standardization of PCR for all the eight genes and the housekeeping gene GAPDH, cDNA synthesized from equal amount of total RNA extracted from ovary tissues was subjected to semi-quantitative PCR analysis. Primers for the housekeeping gene GAPDH were added to the same tube and it was co-amplified with each gene as an internal control. The standard amplification mixture contained 200 µM of dNTPs, 10 pmole of each primer, 10x buffer and 1 unit of Taq DNA polymerase (Bangalore Genei, India). PCR conditions optimized were 95ºC for 2.5 min, 28 cycles of 94ºC for 30 sec., respective annealing temperatures for 30 sec. and 72ºC for 30 sec., without any final extension step. Samples were taken out at 28th and 30th cycles to optimize the amplification for differential expression.

The amplified product of each gene and the DNA molecular marker having known concentration (Fermentas, USA) of each band were analyzed on 3% agarose gel.

RESULTS AND DISCUSSION

Since its first detection in 1982, major advances have been made in discovering the mechanism of action of the FecB mutation. FecB is currently known to be present in at least 48 sheep breeds across 19 countries (Davis, 2008). In the present study, total RNA extracted from the ovary tissue samples of carrier and non-carrier sheep was first evaluated for its integrity and quantity. The concentration of all the samples as measured by Nanodrop varied from 300-900 ng/µl. The O.D ratio of 260 and 280 nm for most of the RNA samples was in the range of 1.9 - 2.0 indicating purity of the respective samples. The integrity of the RNA samples was further assessed by using Experion Bioanalyser (BioRad). The results indicated good quality of RNA samples with RIN values ranging from 8.2-9.6 and ratios of 18s and 28s peak areas also indicating good quality RNA (Fig.1).

First strand cDNA was synthesized and was successfully employed for the amplification of all the target gene transcripts across breeds and FecB genotypes in Garole BB, Crossbred (Garole X Deccani) B+ and Deccani ++ ewes. After standardization of PCR amplification, 5µl of the amplified product was checked in 3% agarose gel, along with a suitable size molecular DNA marker. Specific amplified products as per expected size were observed in agarose gel (Fig. 2).

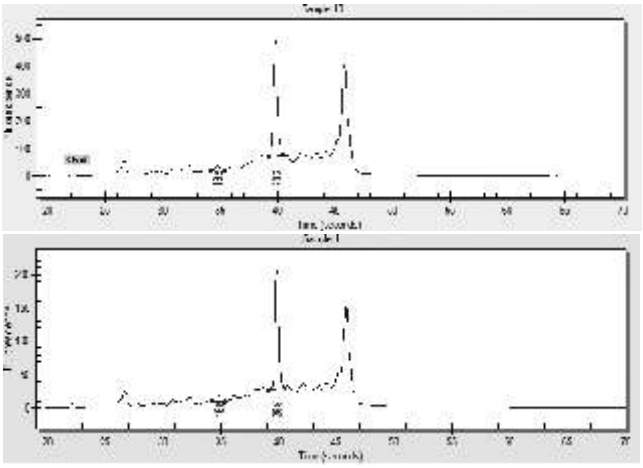

Fig 1. Experion Bioanalyser based analysis of total RNA extracted from sheep ovaries showing 18S and 28S rRNA peaks.

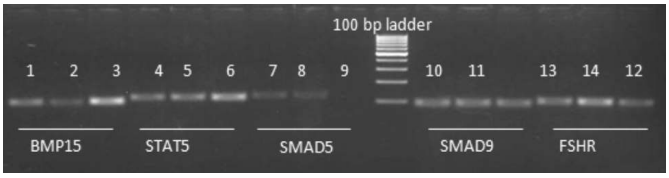

Fig 2. Ehidium bromide stained 3% agarose gel showing the amplified gene transcripts across ++ Deccani (1,4,7,10 and 13), BB Garole (2,5,8,11 and 14) and B+ Crossbred (3,6,9,12 and 15) ewes respectively along with 100 bp DNA ladder.

After successful amplification of all the gene transcripts across different breeds and FecB genotypes, semi-quantitative RT-PCR analysis was performed to check whether a specific transcript is up or down regulated across ewes of different breeds and FecB genotypes and consequently of different litter sizes. The annealing temperatures varied with primer sets and were determined to avoid non-specific amplification. Different concentrations of primers and Taq DNA polymerase were then standardized for co-amplification of target and GAPDH internal control to get the specific amplifications of the target and the standard genes. The amplified transcripts were analysed on 3% agarose gel. Specific PCR amplification for GAPDH control was observed across all the 8 target genes in the cDNA synthesized from sheep of different breeds/genotypes (Fig. 3). Co-amplification of house-keeping gene by putting reaction in single tube was carried out in this study as compared to amplification of house-keeping and target genes done in separate tubes by earlier workers (Cun-ling et al., 2007), which is always a better choice, since it avoids variations due to pipetting errors.

As indicated, after normalizing with internal control, 28th cycle was optimized for respective genes across different breeds/FecB genotypes. Semi-quantitative RT-PCR showed varied levels of gene transcripts across animals of different breeds, FecB genotypes and litter sizes (Fig. 3). Differential expression of mRNAs encoding BMP/SMAD pathway molecules in antral follicles of high and low fecundity Hu sheep has been reported by Xu et al. (2010). Their study showed that there was significant difference in the levels of GDF9 and TGFßR1 mRNAs in the antral follicles of the high and low fecundity groups which possibly suggests that differences in expression levels of GDF9 and its receptor genes could influence ovulation rate. Barzegari et al. (2010) have also found the polymorphism in BMP15 and GDF9 genes to be associated with fertility and ovulation rate in sheep in Iran. Differences in the BMP15 mRNA expression are known to exist in the pre-antral and antral follicles of ewes heterozygous for the Woodlands mutation and their wild type genotypes (Feary et al., 2007). The data in the present study showed that the expression of BMP15 mRNA was lower in crossbred ewes heterozygous for FecB mutation than both wild type and homozygous BB genotype.

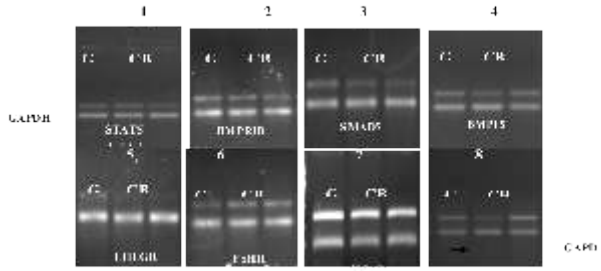

Fig. 3. Semi-quantitative RT-PCR analysis of 8 genes across FecB carrier and non-carrier ewes. GAPDH was used as an internal control for normalizing the band intensities after 28th cycle. (G: Garole, CB: Crossbred, D: Deccani).

Expression of BMPRIB was found to be up regulated in the Garole BB ewe as also reported earlier in sheep ovaries using immunohistochemistry showing strong expression in granulosa cell layer of follicles from primary to late antral stages of development (Montgomery et al., 2001). The expression of FSHR and SMAD9 was found to be nearly similar across all the genotypes. SMAD5, possibly implicated in signaling pathway, showed higher expression in the Garole ewe (Fig. 3). The LHCGR mRNA level was higher in Garole (BB) ewe as compared to other genotypes which was also demonstrated by Cun-ling et al. (2007) by semi-quantitative RT-PCR. Their results showed significantly higher mRNA levels of FSHR and LHCGR for homozygous (BB) genotype in right ovary but there was no difference in the left ovary, which might be due to the anatomical and cyclical physiological differences existing between right and left ovaries. The results in this study showed that GDF9 gene expression was higher in the Deccani non-carrier ewe as compared to BB Garole and the B+ Crossbred ewes suggesting that difference in the expression level of this gene might influence the rate of ovulation in sheep. Expression of STAT5 was higher in crossbred as compared to Garole and wild type Deccani sheep. STAT5 is a signaling molecule involved in regulation of expression of several genes through JAK/STAT pathway including those regulating the growth and differentiation of mammalian cells (Nakasato et al., 2006).

This is a preliminary report on expression analysis of genes presumably involved in regulating ovulation rate in FecB carrier and non-carrier sheep of Indian origin. The results thus necessitate carrying out further investigations in more animals after synchronization of estrus to establish the implication of variation in expression levels of these target genes on ovulation rate and litter size in sheep.

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