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Characterization of the Luteinizing Hormone Beta (LH-β) Subunit Gene in the Goat

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ABSTRACT

Background: Luteinizing hormone (LH) plays a critical role in ovulation and maintenance of pregnancy in female and gamete production in male during fertile phase of life. Physiological disturbance of this hormone leads to conditions like delayed ovulation, anovulation, cystic ovarian disease and lack of sexual desire in male. Since, there had been no report of molecular characterization of β-subunit of luteinizing hormone (LH) of Indian goat, the present study aimed to clone and characterize genomic DNA encoding LHβ subunit.

Methods: Genomic DNA was extracted from goat blood and amplified using specific LHβ gene primers. After cloning and transformation, plasmids were isolated from randomly selected white colonies. Presence of insert was confirmed by restriction enzyme digestion of plasmids. After confirmation by PCR, plasmids were sent for DNA sequencing.

Result: Analysis of sequence revealed an insert of 1006 bp size as expected. Comparison of nucleotide sequence revealed the cloned gene to be is LHβ encoding 141 amino acids. It showed 97.3 and 91.7% similarity with sheep and cattle respectively. Inferred amino acid sequence showed absolute similarity (100%) with sheep and buffalo. The common and essential features such as twelve cysteine molecules, a single potential N-glycosylation site, the CAGY region and another tetrapeptide CGPC are all found in the goat sequence too.

Key words: Cloning, DNA, Genomic, Goat, Luteinizing hormone beta (LHβ), Sequencing.

INTRODUCTION

Goat is one of the oldest domesticated animals. Goat have been popularly known as mortgage lifters of India along with sheep. India occupies first position in terms of goat population and goat milk production. Since ages Goats have been poor people's most reliable livelihood resource. India has 34 registered breeds of goats. Goats are resistant to many diseases and they have ability to survive in harsher conditions compared to other ruminants. A lot of genetic diversity exists in Indian breeds with respect to production potential, adaptability and other parameters, thus it is necessary to study the Indian goat at genomic level.

Luteinizing hormone (LH) is a heterodimeric glycoprotein consisting of a common alpha subunit coupled to a unique beta subunit (Pierce and Parsons, 1981), each encoded by different genes and linked by non-covalent bonds. Within the same species, the α-subunits of these hormones have identical amino acid sequence except for heterogeneity at their amino termini and is encoded by a single gene in mammalian genome, while their β-subunits are unique and confer hormonal specificity (Naylor *et al*., 1983) and is encoded by multigene family. The beta subunit of LHβ confers its specific biologic action and is responsible for the specificity of the interaction with the LH receptor. LH is produced by gonadotropic cells in the anterior lobe of the pituitary gland.

Both α-and β-subunits of the LH evolved from a single common ancestor through gene duplications. The first duplication produced an α-and a β-subunit followed by a second duplication of ancestral β-subunit to yield LHβ subunit genes and ancestor of TSHβ and FSHβ.

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The genes encoding for alpha and beta subunits are localized on different segments in the genome. After proper post translational modification/processing they are associated by non-covalent bonds to form biologically active hormones.

LH acts synergistically with the follicle stimulating hormone (FSH) to promote the growth and development of gonads, to control gametogenesis and to regulate gonadal endocrine functions (Moyle *et al.,* 1994). LH regulates follicular maturation, ovulation, and the maintenance of luteal activity in female and stimulates Leydig cells to secrete androgen along with other gonadotropin in males. LH is being used clinically in the conditions like anovulation, delayed ovulation, polycystic ovarian disease and repeat breeding in females and cryptorchidism and general hypoplasia in young animals in males.

The nucleotide sequences and amino acids of LHβ of different species like mammals, birds, amphibians, reptiles and fish has been studied. The nucleotides and deduced amino acids sequences of the LHβ subunit molecule available for 7 mammalian species, namely, Buffalo [DQ489560], Cattle [M11506], Sheep [S64695], Horse [S41704], Mouse [U25145], Pig [D00579] and Human [X00264] were used for the analysis. Isolation and analysis of these genes in different species may help in understanding and production of recombinant hormones (Clark *et al.*, 2005). Analysis of the nucleotide and deduced amino acid sequences of LHβ may further provides insight into the evolution of this family of hormones (Wallis, 2001).

In this study, we determined the unknown sequence of DNA coding for β-subunit of LH in Indian non-descript local goat (*Capra hircus*) and characterized the sequence by cross-species comparison with known sequences from other species.

MATERIALS AND METHODS

Animals and genomic DNA

Blood was collected from the jugular vein of the goat undergoing clinical trial under the Division of Pharmacology, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, India. Standard proteinase-K digestion followed by phenol:chloroform:isomayl alcohol (25:24:1) extraction was performed to isolate genomic DNA from peripheral blood leucocytes (Sambrook and Russell, 2001).

Quality, purity and concentration of DNA

The genomic DNA isolated from goat blood was checked for its quality, purity and concentration. The quality of the genomic DNA was checked by 0.6% submarine gel electrophoresis. Purity of isolated genomic DNA was checked spectrophotometrically, samples having OD ratio (A_{260}/A_{280}nm) between 1.7-1.9 was considered pure and used for further analysis. The concentration of genomic DNA was estimated by recording the OD (1 OD value at 260nm is equivalent to 50µg/ml of double stranded DNA). Concentration of DNA was calculated using the following formula:

$$\text{DNA concentration (µg/ml)} = OD_{260} \times 50 \times \text{Dilution factor}$$

Primers

Specific primers were designed from conserved region of heterologous sequences of β-subunit gene available on NCBI GenBank. The sequence used are as follows:
Forward primer: 5′-GCAGGGGAGGCACCAAGG-3′
Reverse primer: 5′-ATGGGCATGGGAGGTTGAAGT-3′

Polymerase chain reaction (PCR)

The goat genomic DNA isolated from the blood cells was used as a template for amplification of β-subunit gene. The reaction was performed in a 25 µl volume. The PCR was performed using Taq DNA polymerase (Promega corporation, USA) under the following thermal conditions: one cycle at 94°C for 5 mins, then 35 cycles each at 90°C for 1.5 min, 59°C for 1.5 min and 74°C for 1.5 min. and finally, an extension at 74°C for 1 min in a thermal cycler (Eppendorf, Germany). The amplified PCR product was analysed on 1.5% agarose gel electrophoresis with a 5000 bp DNA ladder. Elution of DNA fragment from the gel was carried out using GenElute extraction kit (Sigma).

Cloning of LHβ gene

The eluted product was cloned in pGEMT Easy TA cloning vector (Promega Corporation, USA) following the manufacturer's protocol followed by transformation into DH5α strain of *E. coli* and plated on LB agar plate containing ampicillin (50 µg/ml), IPTG (25 µl/ml) and X-gal (20 µg/ml). The plates were incubated overnight at 37°C and screened for the blue and white colonies. The white colonies were picked up and inoculated into 5 ml of LB broth containing ampicillin. Plasmids were extracted from bacterial culture derived from identified single colonies by miniprep method (Sambrook and Russell, 2001). The plasmids were screened by digesting with *EcoRI* and *NotI* in order to release the insert.

Sequencing

The recombinant plasmids encoding the complete LHβ gene was sequenced at DNA sequencing facility, Delhi University (South Campus). Double orientation, single strand sequencing was done using SP6 and T7 promoter primers.

Sequence analysis

Seven mammalian LHβ sequences namely cattle (M11506), buffalo [DQ489560], sheep (S64695), goat (AM258985), horse (S41704), pig (D00579), mouse (U25145) and human (X00264) were retrieved from GenBank and used for analysis. These sequences were analyzed using the BLAST and Clustal W programs. A phylogenetic tree was constructed from the data provided by the multiple sequence alignment patterns following the Neighbour-Joining method by the MEGA X program (Kumar *et al.*, 2018).

RESULTS AND DISCUSSION

Isolation of genomic DNA

The genomic DNA isolated from the blood shown a single band on 0.6% agarose gel electrophoresis. The concentration of DNA was found to be 500 µg/ml. The isolated DNA had an A_{260}/A_{280} of 1.8 indicating a highly purified preparation. The size of the amplified PCR product carried out at an annealing temperature of 59°C revealed a 1000 bp product as expected (Fig 1).

Cloning of PCR amplified LHβ gene

The T/A cloning vector pGEMT-Easy and purified 1006 bp product was ligated in the ratio of 1:3 using T_4 DNA ligase. The resulting construct was used for transformation of DH5α competent cells. After plating the transformed. *E. coli* cells over LB agar plates containing ampicillin, IPTG, X-gal, the plates were incubated at 37°C overnight which resulted in the formation of white and blue colonies. The white colonies with the recombinant plasmids were grown overnight in LB containing penicillin and subsequently the plasmids were isolated.

Characterization of recombinant pGEMT-Easy plasmids

Characterization by PCR

The isolated plasmids analyzed for the insert by PCR using gene specific primers. The product run on 1.5% agarose gel gave a gene amplicon of size 1006 bp (Fig 2; Lane 6 and 7). No amplification was observed in plasmid isolated from blue colonies (Fig 2; Lane 1) as negative control.

Characterization by restriction endonucleases

The plasmids that gave positive results with the PCR was further characterized by restriction endonucleases digestion.

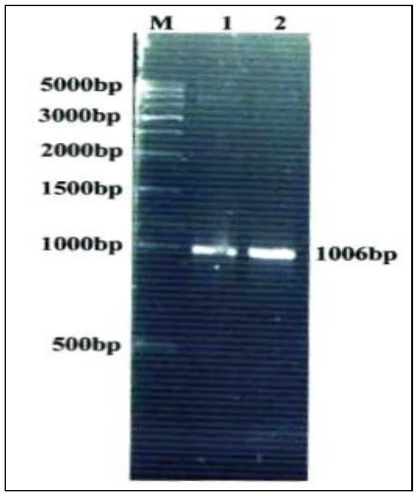

Fig 1: Characterization of LHβ gene PCR product.
Lane M: 5000 bp DNA ladder.
Lane 1 and 2: PCR amplicon of LHβ gene.

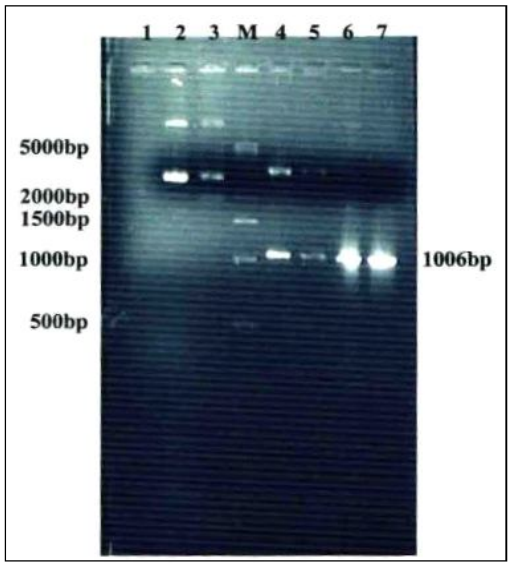

Fig 2: Screening of recombinant pGEMT-Easy clones by *EcoR*I digestion and colony PCR.
Lane M: 5000 bp DNA ladder.
Lane 1: No amplicon from blue colony plasmid.
Lane 2 and 3: Plasmid from blue colony (No insert).
Lane 4 and 5: Positive recombinant clones (Digestion *by Eco R*I).
Lane 6 and 7: Positive colony PCR product.

Insert of LHβ gene was released form pGEMT-Easy vector using *EcoRI* (Fig 2; Lane 4 and 5). Digestion of *NotI* produced two distinct bands of 399 and 607 bp (Fig 3; Lane 3 and 4).

Sequencing

The recombinant clone was sequenced and found to be 1006 bp and the sequence was submitted to GenBank (accession number AM258985). The sequence shows start codon ATG at position 19-21 and the stop codon TAA at 974-976, thus it represents the complete coding region (CDS) of the gene. It codes for 141 amino acids and contain two introns and three exons. A BLAST search for this sequence in NCBI confirmed its similarity with other mammalian LHβ sequences. The sequencing results revealed a high similarity with other mammalian species. The nucleotide sequence and the deduced amino acid sequence is shown in (Fig 4). The ORF of the goat LHβ subunit encodes 141 amino acids with a predicted molecular mass of 15.17 kDa. Goat LHβ subunit consists of a signal peptide 20 amino acids long and a mature subunit 121 amino acids long. A total of 12 cysteines and one putative N-glycosylation sites exist in this subunit (Fig 4).

Sequence analysis

Align report

Some of the available nucleotide and amino acid sequence of LHβ gene from various mammalian species were aligned and compared with respect to the goat LHβ sequence. The alignment report of amino acid is depicted in (Fig 5).

Homology

The nucleotide and deduced amino acid sequences were compared with other mammalian sequences available in GenBank. Amino acid and nucleotide sequence alignment was performed using the Clustal method (Higgins and Sharp, 1988). Clustal W analysis of nucleotide sequences revealed a high level of similarity with the nucleotide sequences of other mammalian species. The goat LHβ sequence had the highest homology with buffalo (99.6%) and sheep (97.3%) at the nucleotide level. At the amino acid sequence level, the goat LHβ sequence had 100% homology with sheep and buffalo (Basavarajappa et al., 2008). The unique amino acid residues, CAGY (34th to 37th amino acid residues), were found conserved across different species. These conserved residues are responsible for binding to the common alpha subunit (Gharib *et al*., 1990).

Phylogenetic analysis of the LH-β hormone family

Based on the multiple sequence alignment of 141 amino acid residues, a phylogenetic tree was constructed (Fig 6).

The present work was undertaken to clone and characterize the gene encoding LHβ of Indian Goat. The action of lutropin (LH), follitropin (FSH) and in some species, chorionic gonadotropin (CG) on gonadal receptors are required for fertility. These gonadotropins and other member of the glycoprotein hormone family, thyrotropin (TSH) are composed of a conserved α-subunit and a hormone specific

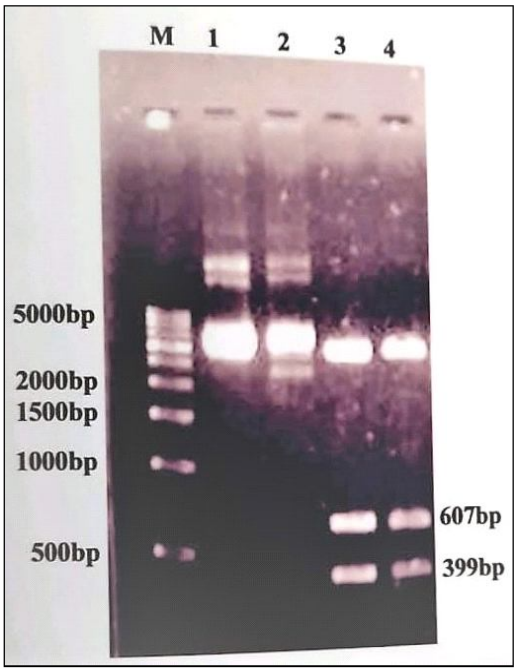

Fig 3: Characterization of RE digestion of recombinant plasmids by *Not* I enzyme.

Lane M: 5000 bp DNA ladder.

Lane 1 and 2: Negative recombinants.

Lane 3 and 4: Positive recombinants with insert having internal cut of *Not* I enzyme.

β-subunit that enables them to distinguish LH, FSH and TSH receptors. Both hormone subunits have similar architectures and are of 15 kDa and 23 kDa respectively. The hormone specificity, species specificity and relative biological activity are determined almost exclusively by the β-subunit (Ward *et al*., 1991).

The genomic DNA isolated from goat blood was amplified using specific LH gene primers. At an annealing temperature of 59°C, PCR gave distinct band of 1006 bp as expected. The cloned LHβ-subunit of goat is 1006 bp fragment long and it extends immediately from 5' of the translational start codon (ATG) to 3' of translational stop codon (TAA) and thus spanned the entire coding region. The gene consists of two introns and three exons. The sequence of junction splice sites is identical with consensus sequences at exon intron boundaries. Introns length were 297 and 235 bp. Intron 1 is inserted between the amino acid codons -16/-15 in the apoprotein signal sequence. Intron 2 is inserted between codons +41 and +42. While the lengths of these introns are slightly shorter than those of other mammalian species, their relative location within the transcriptional unit are identical indicating strong conservation of gene structure within the glycoprotein hormone family as well as between mammalian species.

The nucleotide base at position 330 was 'A' in the studied goat LHβ sequence while it was 'G' in all other mammalian species used to compare this sequence. Since, this positioned at intron-exon junction, the sequence should

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                                  19-21
1      GCAGGGGAGGCACCAAGGATGGAGATGCTCCAGGTAAGTCTGTAGGGCCCCTTGTGACTC   60
1                                M   E   M   L   Q ~~~~~~~~~~~~~~~~~~~~~~~~~~~~
61     CATCCAGGCCACAGCTGGCAGGAAGTGGGAGAGTCCGGGGACCTGGTAAAGGAGGCCTCT  120
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
121    TTAGAAGAGTGTGGGGAGAAGAGTAGGCCTGACGGTGGGAGGAGGGCAGCAGGTGGGGCC  180
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
181    TGAGGTGTTGGGGTGTCTGGGGTCCCTGGGGATGGAAAATCCTTGAATGGAAGGTGGCAG  240
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
241    GCACAGGAGCTGGGTCCCTGAACGTGTGCATGCAGGGCTTGGGGGTGGGGTGAGGATCTG  300
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
301    GCTGGCCCTGAGGCACTGGCCTTGTCCCAAGGACTGCTGCTGTGGCTGCTGCTGGGCGTG  360
6      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ G   L   L   L   W   L   L   L   G   V
361    GCCGGGGTGTGGGCTTCCAGGGGGCCACTGCGGCCGCTGTGCCAGCCCATCAACGCCACC  420
16      A   G   V   W   A   S   R   G   P   L   R   P   L   C   Q   P   I   N   A   T
421    CTGGCGGCTGAGAAGGAGGCCTGCCCTGTCTGTATCACTTTCACCACCAGCATCTGCGCC  480
36      L   A   A   E   K   E   A   C   P   V   C   I   T   F   T   T   S   I   C   A
481    GGCTACTGCCCCAGCATGGTGAGCTGCCGAGGGCGGGCGGGTGCCACCAACCCAGCTCCA  540
56      G   Y   C   P   S   M ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
541    GGCAATTACTCGGGGCTAGGCCCACAGAAGACCCGCAGTGGCAGTGGGGGTGGAAGGGTG  600
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
601    GCCTGCTGCCTGGGGAAGGGGCCGGGCAGGTGGGAAGGAGAGCACAGAGGGTCCCTGGGA  660
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
661    TCTGTGGGCTGCAGTGGGGGAGCTCGGGGGAGAGCTCAGCCCCATGGAGACACTCAAGCT  720
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
721    CCCTGCCCTCCAGAAGCGGGTGCTGCCTGTCATCCTGCCGCCCATGCCCCAGCGGGTGTG  780
62     ~~~~~~~~~~~ K   R   V   L   P   V   I   L   P   P   M   P   Q   R   V   C
781    CACCTACCACGAGCTGCGCTTTGCCTCCGTTCGGCTCCCCGGCTGCCCACCTGGCGTGGA  840
78      T   Y   H   E   L   R   F   A   S   V   R   L   P   G   C   P   P   G   V   D
841    CCCAATGGTCTCTTTCCCCGTGGCCCTCAGCTGTCACTGTGGGCCCTGCCGCCTCAGCAG  900
98      P   M   V   S   F   P   V   A   L   S   C   H   C   G   P   C   R   L   S   S
901    CACTGACTGCGGGGGTCCCAGAACCCAACCCTTGGCCTGTGACCACCCCCCGCTCCCAGA  960
118     T   D   C   G   G   P   R   T   Q   P   L   A   C   D   H   P   P   L   P   D
                        974-976
961    CATCCTCTTCCTCTAAGGATGCCCCACTTCAACCTCCCATGCCCAT   1006
138     I   L   F   L
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Fig 4: Nucleotide and deduced amino acid sequences of the Indian non-descript goat LHβ gene (Accession number: AM258985), the first nucleotide of the ATG translational start codon as +1.

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           1
Goat       MEMLQGLLLW LLLGVAGVWA SRGPLRPLCQ PINATLAAEK EACPVCI [ 47]
Sheep      .......... .......... .......... .......... ....... [ 47]
Buffalo    .......... .......... .......... .......... ....... [ 47]
Cattle     ...F...... .......... .......... .......... ....... [ 47]
Pig        .......... ...S...... .........R .........N ....... [ 47]
Mouse      ..R....... ...SPSV... .........R .V.......N .F..... [ 47]
Human      .........L ...SMG.A.. ..E....W.H ....I..V.. .G..... [ 47]

Goat       TFTTSICAGY CPSMKRVLPV ILPPMPQRVC TYHELRFASV RLPGCPP [ 94]
Sheep      .......... .......... .......... .......... ....... [ 94]
Buffalo    .......... .......... .......... .......... ....... [ 94]
Cattle     .......... .......... .......... .......... ....... [ 94]
Pig        .......... ....V....A A...V..P.. ..R..S...I ....... [ 94]
Mouse      .......... ....V....A A...V..P.. ..R..A.... ....... [ 94]
Human      .VN.T..... ..T.M...QA V...L..V.. ..RDV..E.I ......R [ 94]

Goat       GVDPMVSFPV ALSCHCGPCR LSSTDCGGPR TQPLACDHPP LPDILFL [141]
Sheep      .......... .......... .......... .......... ....... [141]
Buffalo    .......... .......... .......... .......... ....... [141]
Cattle     .......... .......... .......... .......... ....... [141]
Pig        ....T..... .......... ...S...... A......R.L ..GL... [141]
Mouse      ....I..... ....R..... ...S...... ...M...L.H ..GL.L. [141]
Human      ....V..... ....R..... R.TS.....K DH..T....Q .SGL... [141]
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Fig 5: Alignment of amino acid sequences of the LH beta subunit. The sequences for alignment were from six species (Goat [AM258985], Sheep [S64695], Buffalo [AY765376], Bovine [M11506], Pig [D00579], Mouse [U25145], Human [X00264]. Amino acids similar with goat sequence are indicated by a dot (.).

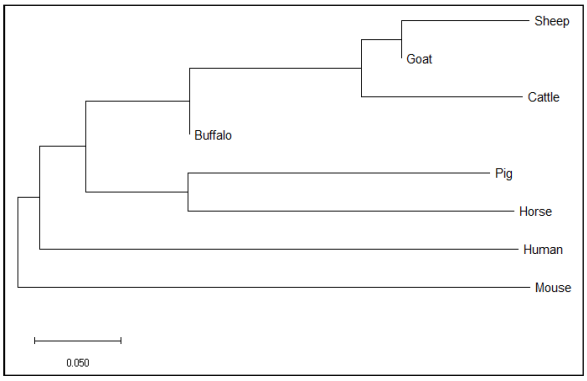

Fig 6: An un-rooted phylogenetic tree of LHβ subunits from different species using Molecular Evolutionary Genetics Analysis Version X (MEGA X) software. The tree was constructed by the Neighbour-Joining method from similar sized (141 amino acid residues) LH beta sequences shown in Fig 5.

be 'G'. The goat LHβ subunit was amplified by PCR using Taq polymerase which is known to introduce error in the amplified fragment. It is therefore every possibility that the 'A' at position 330 is an artefact introduced by Taq polymerase. This error can be checked by resequencing the other clones containing LHβ gene.

The LHβ subunit is 97.7% and 92.3% similar with 9 and 15 bp shorter than the ovine (Accession number S64695) and bovine (Accession number M11506) LHβ subunit respectively. The additional bases present in intron doesn't affect the reading frame. Almost all differed and inserted nucleotides were found in the intron region only irrespective of species which is 100% in case with that of sheep and cattle.

Nucleic acid sequence comparison allows determination of not only the number of base changes which results in amino acid changes (replacement changes) but also the number of base changes which have no effect on amino acid sequence (silient changes). Determination of both silient and replacement substitution thereby provides an index of the rate of neutral genetic drift at silient or intron sites as well as on index of divergence at replacement sites. For a given set of related genes, the rate of sequence divergence at replacement sites is nearly linear when analyzed over recent evolution.

A comparison of the amino acid sequence of polypeptide deduced from coding sequence as presented in (Fig 4) indicate that the LHβ is 141 amino acids long with a leader (signal) sequence of 20 amino acids and a mature subunit of 121 amino acids. The molecular weight calculated is 15.2kDa. The signal sequence is rich in hydrophobic residues and remain same in sheep, cattle and buffalo but the difference is observed with other mammalian species. The difference observed with other mammalian species may not affect the biological activity of the hormone, since this sequence is cleaved from the nascent peptide and it plays an important role in maturation and secretion of hormone (von Heijne, 1985).

In apoprotein molecule the position of 12 cysteine residues is conserved among all species. These twelve cysteine residues form six disulfide bridges helping in the formation of three large loop, which in turn play a critical role in heterodimer formation and/or secretion (Furuhashi *et al*., 1995; Sato *et al*., 1997). Formation of additional loop, known as seat belt, is largely responsible for influence of

β-subunit on receptor binding specificity, probably through an influence on hormone conformation. It is noted that a single potential glycosylation site (Asn-X-Thr) at position 13 to 15 is aligned with that of vertebrate species except human. This conserved N-glycosylation site is required for signal transduction, intercellular stability and subunit folding (Matzuk and Boime, 1988; Sairam and Bhargavi, 1985). The region important for biological activity, the "CAGY" region (Hayashizaki *et al*., 1985; Ledley *et al*., 1976; Pierce and Parsons, 1981) is also conserved among all these species and this region is found in the beta subunit of other glycoprotein hormones also.

The common tetrapeptide sequence (Cys-Gly-Pro-Cys), which is found in the active site of thioredoxin and LHβ subunit of various mammals, is also found in the goat LHβ sequence. LH has an intrinsic thioredoxin activity and this activity is important in receptor activation and in post receptor signal transduction (Boniface and Reichert, 1990). This catalytic tetrapeptide sequence does not exist in any non-mammalian LHβ-subunit so far examined, so this hypothesis is restricted to mammals.

A recombinant single chain beta alpha equine chorionic gonadotropin/luteinizing hormone (eCG/LH) with active biological potential was produced for applications in assisted reproductive technologies or diagnostic assays through expression of the eCG/LH gene construct (Bhardwaj *et al*., 2018). Based on the sequence of gonadotropin subunit of goat, recombinant lutropin can be produced in an in-vitro system for long term treatment in assisted reproduction of goat without possible risk of antibody production.

CONCLUSION

Introduction of this genomic fraction of LH into eukaryotic cell lines and transgenic animals will facilitate analysis of the cellular and molecular mechanisms which regulate expression of the LH-gene, alongwith exploitation of recombinant protein (LH) in pharmaceutical of human and animal therapy.

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