STUDIES ON GROWTH HORMONE GENE POLYMORPHISM IN NELLORE SHEEP THROUGH PCR-SSCP

By

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B.V.Sc & A.H
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CERTIFICATE

I certify that Mr. **B.RAJITH REDDY** has satisfactorily prosecuted the course of research and that the thesis entitled **"STUDIES ON GROWTH HORMONE GENE POLYMORPHISM IN NELLORE SHEEP THROUGH PCR-SSCP"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.

Date: 14-12-2018

Place: Hyderabad

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DECLARATION

I, Mr. **B.RAJITH REDDY** hereby declare that the thesis entitled "**STUDIES ON GROWTH HORMONE GENE POLYMORPHISM IN NELLORE SHEEP THROUGH PCR-SSCP**" submitted to **P.V. NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY** for the degree of **MASTER OF VETERINARY SCIENCE** is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

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Place: Hyderabad

CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON GROWTH HORMONE GENE POLYMORPHISM IN NELLORE SHEEP THROUGH PCR-SSCP"** submitted in partial fulfilment of the requirements for the degree of the **"MASTER OF VETERINARY SCIENCE"** of **P.V. NARSIMHA RAO TELANGANA VETERINARY UNIVERSITY** is a record of the bonafide research work carried out by **B.RAJITH REDDY,** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All the assistance and help received during the course of the investigation has been duly acknowledged by the author of the thesis.

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B.Rajith

(Dr.B.Rajith Reddy)

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LIST OF ABBREVIATIONS

=	:	Equals
%	:	Per cent
±	:	Plus or Minus
>	:	Greater than
<	:	Less than
≤	:	Less than or equal to
°C	:	Degree Celsius
µg	:	Microgram
µl	:	Microlitre
µm	:	Micrometre
APS	:	Ammonium persulfate
BL	:	Body length
bp	:	Base Pair
BW	:	Body weight
DAHD	:	Department of Animal Husbandry, dairying & fisheries
DMRT	:	Duncan's Multiple Range Test
DNA	:	Deoxyribo Nucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
DW	:	Distilled water
EDTA	:	Ethylene Diamino Tetra acetic Acid
g	:	Gram
GH	:	Growth Hormone
GHIH	:	Growth Hormone Inhibiting Releasing
GHRH	:	Growth Hormone Releasing Hormone
GLM	:	Generalized Linear Model
h	:	Hour

HAW : Height at withers
HG : Heart girth
ILFC : Instructional Livestock Farm Complex
IU : International unit
kbp : Kilo-base pair
kg : Kilogram
lbs : Pounds
LRS : Livestock Research Station
mA : milli Ampere
MAS : Marker Assisted Selection
mg : Milligram
Mgcl2 : Magnesium chloride
min : Minute
ml : Millilitre
mM : Milli Molar
mm : Millimetre
NDDB : National Dairy Development Board
NFW : Nuclease Free Water
ng : Nano gram
nm : Nanometre
OD : Optical density
oGH : Ovine Growth Hormone
P : Probability
PAGE : Poly Acrylamide Gel Electrophoresis
PCR : Polymerase Chain Reaction
pm : Pico moles
RBC : Red Blood Cells
rpm : Revolutions per minute
SDS : Sodium Dodecyl Sulphate
sec : Seconds

SNP	:	Single Nucleotide Polymorphism
SSCP	:	Single Strand Conformation Polymorphism
TBE	:	Tris Boric acid EDTA
TEMED	:	N, N,N',N'-Tetra Methyl Ethylene Diamine
UTR	:	untranslated region
UV	:	Ultra Violet
V	:	Volts
w/v	:	Weight by Volume
WBC	:	White Blood Cells

ABSTRACT

Name of the author : B.RAJITH REDDY

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ABSTRACT

A total of about 50 unrelated animals of Nellore brown sheep belonging to different flocks including, Livestock Research Station (LRS), Mamnoor, Instructional Livestock Farm Complex (ILFC), Rajendranagar of P.V.Narsimha Rao Telangana Veterinary University and farmers flocks from Shamshabad region of Ranga Reddy district were screened to explore the polymorphism of different regions of growth hormone (GH) gene using PCR-SSCP technique.

The PCR-SSCP of 5' regulatory region (210 bp) yielded three SSCP patterns **11, 12** and **22** corresponding to two allelic variants **1** and **2.** The allelic frequency of **1** and **2** were 0.45 and 0.55, genotypic frequencies were 0.24, 0.42 and 0.34 in **11, 12,** and **22**, respectively. The exon-4 (191 bp) yielded two genotypic patterns **11** and **12** corresponding to two allelic variants **1** and **2**. The allelic frequency of **1** and **2** were 0.80 and 0.20, genotypic frequencies were 0.60 and 0.40 in **11** and **12**, respectively**.** The PCR-SSCP yielded two conformational patterns **11** and **12** in exon-5 (480 bp) indicating polymorphism in Nellore sheep with two alleles **1** and **2.** The allelic frequency of **1** and **2** were 0.85 and 0.15, respectively, genotype frequencies of **11** and **12** were 0.70 and 0.30, respectively. The PCR-SSCP of 3' UTR (320 bp) yielded two different conformational patterns **12** and **13** corresponding to three allelic variants **1, 2** and **3**. The allelic frequencies of **1, 2** and **3** were 0.50, 0.31 and 0.19, respectively and genotype frequencies were 0.62, 0.38 for **12** and **13**, respectively.

The least square means obtained for body weight, body length, height at withers and heart girth were 25.93 ± 0.20 kg, 71.46 ± 0.43 cm, 74.17 ± 0.17 cm, 75.70 ± 0.53 cm, respectively at 12 months of age in Nellore sheep.

PCR-SSCP patterns of different portions of GH gene were correlated with body weights and morphometric traits at 12 months of age. It was observed that the overall mean BW, BL, HAW and HG values were 26.03 ± 0.42 kg, 72.00 ± 0.77 cm, 74.54 ± 0.30 cm and 75.97 ± 1.16 cm in animals with pattern **11**, while with pattern **12** the values were 25.88 ± 0.35 kg, 70.81 ±0.71 cm, 74.21 ± 0.29 cm and 75.76 ± 0.81 cm and with

pattern **22** were 25.91 ± 0.32 kg, 71.18 ± 0.75 cm, 73.87 ± 0.29 cm and 75.44 ± 0.94 cm, respectively in 5' regulatory region (210 bp) of GH gene. The present findings showed slight variation in body weights and morphometric traits. In exon-4 (191 bp), mean BW, BL, HAW and HG in pattern **11** were 26.08 ± 0.25 kg, 71.93 ± 0.55 cm, 74.35 ± 0.25 cm and 75.96 ± 0.68 cm and in pattern **12** were 25.71 ± 0.35 kg, 70.75 ± 0.67 cm, 73.91 ± 0.21 cm and 75.32 ± 0.88 cm, respectively. The present study clearly shows slight variation in body weights and morphometric traits. The overall mean BW, BL, HAW and HG were 26.11 ± 0.27 kg, 71.68 ± 0.53 cm, 74.24 ± 0.21 cm, 75.52 ± 0.67 cm in animals with pattern **11** of SSCP portion of exon-5 (480 bp),whereas for pattern **12**, the corresponding values were 25.50 ± 0.25 kg, 70.94 ± 0.74 cm, 74.00 ± 0.30 cm, 76.13 ± 0.89 cm, respectively. The present study indicated slight variation in BW and other morphometric traits between patterns obtained. BW, BL, HAW and HG were 25.75 ± 0.23 kg, 71.16 ± 0.61 cm, 74.24 ± 0.23 cm, 75.10 ± 0.63 cm, for those showing pattern **12** of 3' UTR (320 bp) region, and in those with pattern **13** the values for BW, BL, HAW and HG were 26.22 ± 0.38 kg, 71.94 ± 0.53 cm, 74.06 ± 0.27 cm, 76.69 ± 0.93 cm, respectively. The animals with pattern **13** had slightly higher values for BW and HG.

Identification of different SSCP patterns confirmed the polymorphism within 5' regulatory region, exon-4, exon-5 and 3'UTR of GH gene in Nellore sheep. This investigation indicated that SSCP analysis is a valuable tool for the identification of genetic polymorphism. The SSCP patterns (various genotypes) obtained in the present study for various segments of growth hormone gene and their relationship with body weights and other morphometric traits at 12 months age did not show significant differences among genotypes. To establish the real connection between genotypes and various traits if any requires large population of wide genetic base with accurate data.

CHAPTER-I

INTRODUCTION

India endowed with a vast resource of livestock population including 299.6 million bovines, 65.1 million sheep, 135.2 million goats, 10.3 million pigs and 729.2 million poultry (Last update June 20, 2017, NDDB).

Currently in India, livestock is one of the fastest growing subsectors of agriculture and its share of GDP is quickly increasing. This growth is driven by the rapidly increasing demand for livestock products, this demand being driven by population growth, urbanization and increasing incomes in developing countries. Livestock plays a vital role in the upliftment of farmers in unfavourable conditions, as India has many fluctuations in environment it is difficult to have a satisfiable and dependable income from crops.

Sheep is one of the important species of livestock contributing to the livelihood of resource poor farmers of rural areas in arid and semi-arid regions especially where crop and dairy framings are not economical. It contributes to the farm households not only by acting as a source of livelihood and nutritional security, but also as a moving asset, which can be liquidated during crisis periods within short time. They play an important role in the livelihood of a large percentage of small and marginal farmers and landless labourers engaged in sheep rearing.

India has 42 registered breeds of Sheep. Out of these, the high genetic merit (more meat / milk / wool yield per animal) indigenous registered breeds are only 14.The reasons for low productivity of sheep in India are poor exploitation of genetic potential of native stock, inadequate feed resources, heat stress and poor health monitoring (DAHD,2012,overview of sheep farming sector in India).

Nellore sheep is a popular and tall mutton breed found in the northern Andhra Pradesh. Of late, Nellore sheep were introduced in almost all districts of Telangana and replaced local Deccani breed which is smaller in body size with coarse fleece not in demand. Nellore breed is good contributor to mutton production in Telangana and Andhra Pradesh. It can withstand harsh climate conditions and is known for disease resistance and heat tolerance. Based on coat colour pattern Nellore sheep is classified in to three varities viz. Palla, Jodipi, and Brown or Dora.

As the demand for sheep meat increased tremendously, it is necessary to increase the meat production to reach the mark. Nellore is the best breed and a right choice for improving meat production in Telangana and Andhra Pradesh.

Growth is influenced by growth hormone (GH), which is a 191-amino acid, single polypeptide chain that is synthesized, stored and secreted by somatotropic cells (eosinophil cells) within the lateral wings of the anterior pituitary in vertebrates. Growth hormone (GH) is regulated by the neurosecretory nuclei of the hypothalamus. These cells release growth hormone-releasing hormone (GHRH or somatocrinin) and growth hormone-inhibiting hormone (GHIH or somatostatin) into the hypophyseal portal venous blood surrounding the pituitary gland. Growth hormone being an anabolic hormone, promotes body growth by enhancing growth of muscles, bones and organs in the body. The GH gene has a direct effect on the synthesis and secretion of growth hormone and plays an important role in animal growth (Gadelha *et al.*, 2013).

Improvement of meat production depends on growth candidate hormones and genes. An understanding of variation in these genes causing variation in the quantitative traits could be useful for the genetic improvement of these traits through marker assisted selection (MAS). Highly polymorphic genetic markers may dissect the variation existing in quantitative traits and quantify the effect of the genes that contribute to the variation.

Single Strand Conformation Polymorphism (SSCP) was first reported by Orita *et.al* (1989) in a study of human DNA. It is a simple and most sensitive and appropriate method for mutation detection (Bastos *et al.,* 2001) in the amplified DNA fragments. PCR-SSCP analysis is a valuble tool for establishment of allelic variants in the genes (Vyas *et al.,* 2008). This technique is used for future selection programs to improve the production in animals based upon the patterns and analytical studies. PCR-SSCP forms the base for marker assisted selection, which is used for rapid improvement in production traits.

The ovine GH (oGH) gene has been mapped to chromosome 11 (11q25) (Ofir and Gootwine, 1997). The structure of this gene is similar to that found in other species and very homologous to the bovine GH gene (Gordon *et al.,* 1983). The GH gene spans 2.6 to 3.0 kbp in most mammals and comprises of a 5' regulatory region, five exons, four introns and a 3' untranslated region (UTR). The studies on polymorphism in sheep GH gene enable to understand the existing genetic variability in the population and its usefulness for improvement of economic traits. This polymorphism if associated with economic traits can form a base to improve the animal production through selection.

The literature reviewed indicated that the studies on Single Strand Conformation polymorphism of GH gene in sheep and its association with growth traits were scanty especially in Indian breeds.

Hence the present study was carried out in Nellore sheep with the following objectives:

1. To study the Single Strand Conformation Polymorphism (SSCP) in 5' regulatory region, exon-4, exon-5 and 3' UTR of GH gene in Nellore sheep.
2. To study the morphometry and body weight of Nellore sheep and their association with GH gene SSCP patterns.

CHAPTER-II

REVIEW OF LITERATURE

2.1. Growth hormone gene polymorphism

Bastos *et al.* (2001) studied growth hormone gene polymorphism in Portuguese indigenous sheep breed “Churra da Terra Quente” through SSCP and reported two SSCP patterns in exon-4 and five in exon-5. The pattern I in exon-4 and exon-5 are more frequent (72.5% and 47.5%, respectively) when compared to other patterns.

Malveiro *et al.* (2001) studied growth hormone gene polymorphism through PCR-SSCP in exon-4 and exon-5 of growth hormone gene in Algarvia goat. The study revealed six (AA, BB, CC, DD, EE and FF) different conformational patterns in exon-4 and five (AA, BB, AB, AC and BC) in exon-5. Within these patterns, CC pattern in exon-4 and BC pattern in exon-5 are more frequent (35.2% and 44.4%, respectively).

Marques *et al.* (2003) studied the polymorphism in growth hormone gene in two ecotypes (Jarmelista and Ribatejano) of Serrana goat through PCR-SSCP. The study revealed high degree of genetic polymorphisms showing different conformational patterns (AA, AB, AC, CF, ABC, ABE, ABF, ACF, ABCF and ABDE) in exon-4. ABDE pattern was absent in Jarmelista ecotype whereas Ribatejano ecotype showed only five patterns (AB, AC, ABC, ABDE and ABCF). In exon-5, five conformational patterns (AB, ACD, ABCD, ABCE and ABFG) were identified.

Min *et al*. (2005) studied the genetic variability in 5' promoter region of growth hormone gene in LuBei white goat, Boer goat and their F1 hybrid and first backcross generation by PCR-SSCP using two sets of primers. The results showed that there were five substitution mutations in the two fragments. The result showed that in 26-239 bp most of the Boer goats and hybrid generation presented as AA genotype, while most LuBei white goats presented as BB genotype. In 225-429 bp all breeds presented more CC genotype.

Marques *et.al (*2006) screened growth hormone gene (Five exons and the 5' UTR and 3' UTR) for mutations using PCR-SSCP procedure in "Serra da Estrela" sheep. The study revealed growth hormone gene is highly polymorphic. The study revealed four SSCP patterns in 5' UTR, two in exon-4, five in exon-5 and seven in 3' UTR.

Shiri *et al.* (2006) studied genetic polymorphisms at the GH gene by SSCP technique in the Iranian indigenous sheep breed (Kordian sheep) and reported that the fragment exon-4 of growth hormone gene showed three conformational patterns with the frequencies of 75%, 22% and 3% for pattern 1, pattern 2 and pattern 3, respectively.

Gupta *et al.* (2007) observed the polymorphism in exon-4 and exon-5 of growth hormone gene by PCR- SSCP in Black Bengal goat. There were seven haplotypes (A,B, C, D, E, F and G) in exon-4 and five haplotypes (A, B, C, D and E) in exon-5. Within these haplotypes the B haplotype of exon-4 and exon-5 were more frequent (38% and 40%, respectively).

Lan *et al.(*2007) studied growth hormone gene polymorphism in 5' UTR region, exon-5 and flanking 3' UTR region based on PCR-SSCP technique in 5 indigenous goat breeds (Xinong Sannen, Laoshan, Guanzhong, Shaannan White, Guizhou White) and 2 immigrant breeds (Boer and Angora) in china. Three pairs of primers i.e. GH1 and GH2 were used for amplification of 5' UTR region, and GH5 for exon-5 and flanking 3' UTR. The 5' flanking region showed EE and EF patterns with GH1 primer, CC and CD patterns with GH2 primer. The exon-5 and its flanking 3' UTR region amplified with GH5 primer also showed polymorphism (PQ, PR, QQ and QR patterns).

Kumar *et al.* (2008) studied genetic variability in promoter region, exon-4 and exon-5 of growth hormone gene in Sirohi goat based on PCR- SSCP technique. The study revealed eight conformational patterns (AA, AB, AC, AD, AE, AF, AG and AH) in the promoter region and six patterns in exon-4 (EA, EB, EC, ED, EE and EF) and also in exon-5 (FA, FB, FC, FD, FE and FF).

Vyas *et al*. (2008) amplified several fragments of growth hormone gene in Jamunapari goat to study polymorphism through SSCP technique and concluded that the SSCP analysis is a valuable tool for the establishment of allelic variants in the genes. They reported that 7 SSCP patterns for the fragment 1011 to 1344 bp, 5 patterns for 1401 to 1604 bp and 4 patterns for 1705 to 2093 bp fragments respectively.

Gupta *et al.* (2009) studied Single Nucleotide Polymorphism (SNP) in exon-4 and GH-E5 region (covering exon-5 and part of 3' UTR) of GH gene based on SSCP technique in Jakhrana goat and reported six SSCP patterns (AA, AB, AC, BB, BC and CC) in exon-4 corresponding to three alleles (A, B and C) and six SSCP patterns (AA, AB, AC, BB, BC and CC) corresponding to three alleles (A, B and C) in GH-E5 region .

Mousavizadeh *et al*. (2009) studied the growth hormone gene polymorphism in Talli goat based on PCR-SSCP and reported nine conformational patterns in exon-4 with frequencies of 27.7% for the homozygous pattern (AA) and 72.2% for all of other heterozygous patterns (A/B, A/C, A/B/C, A/B/D/E, A/B/C/F, A/C/F, A/B/E, A/B/F). The results suggested that the exon 4 of the GH gene in Talli goats was highly polymorphic.

An *et al.* (2010) studied PCR-SSCP polymorphism in exon-4 and 5 of growth hormone gene in four goat breeds (Xinong Saanen, Boer, F1 and F2 generations of Boer X Guanzhong) .The study revealed three haplotypes (E, F and G) and three conformational patterns (EE, EF and GG) in exon-5 region. Within these haplotypes, E haplotype was found to be more frequent. The exon-4 region of GH gene did not reveal any polymorphism.

Wickramaratne *et al*. (2010) studied the polymorphism through PCR-SSCP in GH1 (5' promoter), GH5 (intron-3, exon-4 and intron-4) and GH7 (exon-5 and 3'-downstream region) of GH gene in Osmanabadi and Sangamneri goats. The study revealed five, three and six distinct band patterns, respectively in corresponding regions of gene in Osmanabadi whereas three, four and four patterns were observed in Sangamneri.

Azari *et al* (2011) studied PCR-SSCP polymorphism of 365 bp region of exon-5 of growth hormone gene in Dalagh sheep and noticed three conformational patterns with frequencies like G1=0.84, G2=0.13 and G3=0.03 respectively.

Tahmoorespur *et al.* (2011) studied genetic polymorphism on growth hormone gene at exon-5 based on PCR-SSCP technique in Iranian purebred Baluchi sheep. The study revealed three conformational patterns (G1, G2, and G3) in exon-5.

Yousefi and Azari (2012) studied growth hormone gene polymorphism based on PCR-SSCP in Zel sheep and reported three different conformational patterns viz., G1, G2 and G3 in exon-5 with the frequencies of 19%, 51% and 30% for G1, G2 and G3 respectively.

Dettori *et al.* (2013) studied growth hormone polymorphism in Sarda breed goat based on PCR-SSCP. The study revealed exon-4 was most polymorphic with nine different conformational patterns, while exon-5 revealed five different polymorphic patterns

Hajihosseinlo *et al*. (2013) studied PCR-SSCP analysis of (a part of intron 3, exon-4, and a part of intron 4) ovine growth hormone gene in Makooei sheep. The study revealed five banding patterns (AA, AB, BB, CC, and CD) with frequencies (31.3, 64.6, 1, 1, and 2.1) respectively. Four alleles A, B, C, and D with frequencies of (0.63, 0.327, 0.028, and 0.015) were detected.

Moradian *et al*. (2013) studied polymorphism in 200bp fragment of exon-4 segment of growth hormone gene based on PCR-SSCP technique. The study revealed five SSCP patterns, representing five different genotypes (AA, AB, BB, CC and CD) with four (A, B, C and D) allelic variants. Allele A and AB genotype is common among them.

Jia *et al.(*2014) studied growth hormone gene polymorphism in 5' regulatory region, exon-4 and 3' untranslated region (UTR) based on PCR-SSCP technique in four sheep breeds(Tibetan, Small Tail Han, German Merino and Poll Dorset sheep).The study revealed two alleles in both 5' regulatory region and exon-4 (A and B) and 3 genotypes (AA, AB, and BB). However 3 alleles (A, B, and C) with 4 genotypes (AA, AB, BB, and AC) were found in 3' UTR.

Nejhad *et al. (*2014) studied genetic polymorphism in 365 bp region of exon-5 of growth hormone (GH) gene in Alpine and Saanen goats with PCR-SSCP technique. The study revealed that there were four (G1, G2, G3 and G4) conformational patterns with frequencies (0.38, 0.21, 0.23 and 0.18) for Alpine goat and (0.48, 0.21, 0.17 and 0.14) for Saanen goat and concluded that GH gene was polymorphic and showed that PCR-SSCP is an appropriate tool for detecting polymorphism and evaluating genetic variability.

Bahrami *et al.* (2015) studied genetic variability on growth hormone in the Mehraban sheep and observed polymorphism in exon-5 with five different genotype patterns (AA, AB, BB, AC and CC) with three allelic variants(A,B and C) having 0.19, 0.27, 0.24, 0.19 and 0.10 frequencies respectively.

In a study on Single Nucleotide Polymorphism (SNP) in five exons and four introns of GH gene in Nilagiri sheep Cauveri *et al.* (2016) reported absence of polymorphism in all exons but the introns were polymorphic with two variants (SNP-G1 and SNP-G2) in intron-I and intron-II regions respectively.

Dayal *et al*. (2016) studied PCR-SSCP polymorphism in 245 bp fragment (partial intron 1, exon-2 and partial intron 2) and 472 bp fragment (partial intron 2, exon-3 and intron 3 and partial exon-4) of growth hormone gene in Black bengal goat. The study revealed 4 genotypes AA, AB, AC and CC with three alleles A, B and C at locus in 245 bp , whereas 472 bp fragment revealed 5 genotypes AA, AB, AC, BB and CC with only 3 alleles A, B and C. AB genotype and A allele was predominant in both the fragments of Black Bengal goat.

Farag et *al*. (2016) studied PCR-SSCP polymorphism in exon-5 of growth hormone gene in Barki, Rahmani and one crossbred (Rahmani x Awase) sheep breeds of Egypt. The exon-5 showed two conformational patterns. The pattern I was recorded to be more frequent (83.3, 92.86 and 90%) than pattern II (16.7, 7.14 and 10%) in Barki, Rahmani and crossbred, respectively.

Moradi *et al.* (2016) explored genetic polymorphism in exon-4 of the GH gene in the Lori-Bakhtiari sheep using PCR-SSCP and found two different conformational patterns.

2.2 Body weights and morphometry in sheep

2.2.1 Body weight

Rao *et al*. (2002) analysed the data on body weights recorded at quarterly interval on 156 Nellore jodipi and 44 Nellore palla maintained at Livestock Research

Station, Palmaner and reported that the overall least squares means of body weights at 12 months age in Nellore jodipi is 25.61 ± 0.53 kg, whereas the corresponding mean body weight in Nellore palla is 27.61 ± 0.89 kg.

Nehra and Singh (2006) reported that the overall least squares means for 12 months body weight in Marwari sheep were 20.70 ± 0.31 kg and observed that the sex ,year and season of birth significantly affected the body weights at all the ages. However the season of birth had no influence on body weights at 3 and 6 months age.

Kandalkar (2007) analysed the growth traits of Deccani sheep at Network Project of Sheep Improvement, Mahatma Phule Krishi Vidyapeeth, Rahuri (M.S.) and observed that the overall least-squares means of body weight at 12 months of age was 23.41 ± 0.28 kg. There was a significant effect of sire, sex, and season on body weight at all ages.

Dass (2008) conducted the research on the body weights at various ages of Pugal sheep of north-western arid and semi-arid region in Bikaner district of Rajasthan and reported that the mean body weight at 12 months of age was 29.74 ± 0.28 Kg.

Ravimurugan and Devendran (2009) studied the mean body weights in 84 Ramnad white sheep maintained at district Livestock farm, Padukottai and reported that the least-squares means for adult body weight was 31.05 ± 0.57 kg in male and 23.50 ± 0.27 kg in females. There was a significant influence of sex on bodyweights.

Reddy *et al.* (2009) reported that the least squares means for body weights at 12 months age in Nellore sheep is 24.67 ± 0.56 kg. Non-genetic factors significantly influenced the body weightat birth, 3, 6, 9 and 12 months of age, except for effect of season of birth on body weight at 9 months of age.

Balasubramanyam *et al.* (2010) studied the body biometry traits of (5647 animals)Madras Red sheep during the period of 2003-04 from 11 villages in and around

Kancheepuram districts in Tamilnadu and reported the overall mean body weight was 21.20 ± 0.08 kg at 12 months age under field conditions. Sex had significant influence on body weight at all stages of growth.

Ravimurugan *et al.* (2010) while studying the growth traits of Kilakarsal (Keezhakaraisal) sheep reported that the least-squares means for body weight at 12 months of age in males and females was 24.75 ± 1.04 kg and 21.94 ± 0.44 kg, respectively. Age and sex of the animal were significant source of variation and the body weight increases as the age advances. They also reported that the body weights recorded for males are higher than females.

Thiruvenkadan *et al*. (2011) analyzed the data of body weights on 2,365 Mecheri sheep (1,201 males and 1,164 females), maintained at the Mecheri Sheep Research Station, Pottaneri, Tamilnadu. The study revealed that the overall mean body weight at 12 months age was 21.10 ± 0.10 kg .Males were heavier than females at almost all stages of growth and the differences tended to increase with age.

Chikurdekar *et al*. (2012) reported that the least square means of 12 months body weight of Sangamneri strain of Deccani sheep was 23.94 ± 0.18 kg.

Pragati *et al.* (2012) studied the growth performance in Edka sheep of Odisha on 1360 sheep belong to five localities and reported the overall least-squares mean body weight at 12 months of age as 18.57 ± 0.06 kg.The age had a significant effect on body weight.

Devendran *et al*. (2014) carried out detailed studies on body weights at different ages in Madras red sheep and reported that the overall average body weight at 12 months of age ranged from 19.88 ± 1.14 kg to 22.48 ± 0.13 kg. The growth rate decreased as the age advanced and was the lowest during 9-12 months. Growth rates were higher in males during 3-6 months while it was higher in females during birth, 3 and 9-12 months.

Joshi *et al*. (2014) evaluated the growth parameters in Magra sheep and reported the least-squares mean body weight at 12 months of age was 28.55 ± 0.20 kg.

Bangar *et al.* (2017) while studying the body biometry traits of Deccani sheep at ICAR-Network Project on Sheep Improvement, MPKV, Rahuri, and Maharashtra reported the least-squares mean body weight at 12 months of age was 25.00 ± 0.10 kg.

Jalajakshi *et al.* (2017) collected data on body weights of Nellore Brown sheep during the period of 2010-15 maintained under farm and also in field condition. The observed Least square means for body weights in semi-intensive system at 12 months of age was 24.67 ± 0.20 kg whereas in extensive system is 22.45 ± 0.64 kg. System of rearing and sex had highly significant influence on body weights at all ages studied.

Kumar *et al.* (2017) studied the body weights at different ages of Nellore jodipi sheep for three years viz., 2013-14, 2014-2015 and 2015-16 and reported that the overall least squares means for body weight at 12 months of age was 27.41 ± 0.18 kg, respectively. Year of birth and sex of animal had significant effect on body weights at all the stages.

Vani *et al.* (2017) carried out for prediction of least square means of body weights in Nellore brown sheep by using statistical methods. Data on body measurements were collected from 897 Nellore brown sheep from five divisions of Kadapa district in Andhra Pradesh. The Least square means for birth weights at 12 months of age in 97 males is 38.35 ± 0.83 kg and in 800 females is 29.96 ± 0.16 kg, overall least square means were 30.43±0.21 kg. The Sex had significant effect on body measurements at all ages and male animals showed higher values than females at all stages of growth.

Reddy *et al.* (2017) studied the data on body weights at 12 months of age recorded on 519 Nellore Brown sheep maintained at Livestock Research Station, Mamnoor. The overall least squares means for body weight at 12 months of age were 27.06 ± 0.03 kg. The Males were significantly heavier than females at all ages. The season of birth significantly influenced the body weight at 12 months of age.

2.2.2 Morphometric traits

2.2.2.1 Body length

Gopal and Hari (2007) carried out biometrical studies in Muzaffarnagari sheep and reported that the overall means for body length at 12 months of age was found to be 80–83 cm. Sex significantly influenced the ($P \leq 0.01$) body length at 12 months age.

Dass (2008) reported that the least-squares means of body length in Pugal sheep as 63.20 ± 0.43 cm at 12 months age.

Mandal *et al.* (2008) analysed the biometrical measurements (heart girth, body length and height at withers) in Muzaffarnagari sheep and found that the overall means for body length at 12 months of age was 72.10 ± 0.20 cm.

Ravimurugan and Devendran (2009) studied the biometry of body length at various ages in 84 Ramnad white sheep maintained at Livestock farm, Padukottai, Tamilnadu and reported that overall mean for at 12 months age body length was found to be 63.00 ± 0.48 cm. while the least-squares means for the same age were 66.82 ± 0.87 cm and 59.19 ± 0.41 cm, respectively in males and females. There was a significant difference in body length among sexes, males showed higher body lengths.

Ravimurugan *et al.* (2010) studied body biometry traits of 181 Kilakarsal (Keezhakaraisal) sheep at 12 months of age and reported that the least-squares means for body length in males was 56.48 ± 1.02 cm and the corresponding value recorded in females was 55.41 ± 0.43 cm respectively. Age showed a significant effect on body length. The study revealed that body lengths in females were higher than males.

Thiruvenkadan *et al*. (2011) carried out a detailed study on the growth traits of Mecheri sheep and reported the mean body length pooled over sexes was found to be 66.00 ± 0.40 cm in adult Mecheri sheep at 12 months age.

Panda *et al*. (2014) in his characterization study on mutton type in indigenous sheep of Puri district in Odisha reported that the average body length at 12 months age to be 55.52 ± 0.18 cm in 69 males and 51.86 ± 0.16 cm in 377 females. It was also observed that there was a medium to high correlation between body length and other morphometric traits.

Rani *et al*. (2014) investigated correlations among body weights and several body measurements in Nellore sheep and reported the overall least-squares means for body length was 71.14 ± 0.50 cm. They reported body weight, chest girth and paunch length are positively correlated with body length and are significant at all age groups.

Yadav *et al.* (2014) studied morphometric traits of five ecotypes of Deccani sheep (Sangamneri, Kolhapuri, Lonand, Solapuri and Madgyal) and reported that the adult body length ranged from 71.6 to 78.5 cm.

Gowane *et al.* (2015) carried out studies on various morphometric measurements of Malpura sheep and found that the least-squares means of body length was 80.37 cm in males and 68.21 cm in females, respectively at 12 months age. The study indicated that the age was a very important factor for determining the size of the morphometric parameter, as there was linear increase in all the parameters as age advanced.

Vani *et al.* (2017) carried out biometrical studies in Nellore brown sheep belonging (897 animals) to five divisions of Kadapa district in Andhra Pradesh. The Least square means for Body length at 12 months of age in 97 males was 76.85 ± 1.01 cm and in 800 females was 66.00 ± 0.26 cm, overall least squares means were 66.64 ± 0.33 cm. They reported that the males showed higher values than females in all ages.

2.2.2.2 Height at withers

Gopal and Hari (2007) studied biometrical measurements in 431 Muzaffarnagari sheep and found that the overall means for height at withers at 12 months of age was 73–79 cm.The influence of sex is found to be highly significant in 9-12 month age. Body measurements in males had significantly higher values than females.

Dass (2008) reported that the least-squares means of height at withers in Pugal sheep was 62.88 ± 0.31 cm at 12 months age.

Mandal *et al*. (2008) studied biometrical measurements in Muzaffarnagari sheep and found that the overall means for height at withers at 12 months of age was 72.61 ± 0.20 cm.

Ravimurugan and Devendran (2009) studied morphometric measurements in 84 Ramnad White sheep breed and reported the least-squares means for height at withers was 74.22 ± 0.71 cm in males and 69.88 ± 0.33 cm in females, respectively at 12 months age. The study revealed there was a significant effect of sex on body measurements. The least square means clearly shows Males had higher values than females studied.

Ravimurugan *et al.* (2010) studied body biometry traits of Kilakarsal (Keezhakaraisal) sheep at 12 months of age and reported that the least-squares means for height at withers as 69.79 ± 1.33 cm and 68.37 ± 0.56 cm, respectively in males and females

Thiruvenkadan *et al*. (2011) studied the growth traits of Mecheri sheep and reported the mean height at withers pooled over sexes to be 67.00 ± 0.40 cm at 12 months age.

Panda *et al.* (2014) studied the body height parameters in indigenous sheep of Puri district and reported that the average height at withers at 12 months of age was 60.22 ± 0.29 cm in males and 56.60 ± 0.24 cm in females in mutton type indigenous sheep of Puri district.

Rani *et al*. (2014) studied body biometry traits in Nellore sheep and reported that the overall least-squares means for height at withers as 76.91 ± 0.40 cm.

Yadav *et al*. (2014) studied morphometric traits of five ecotypes of Deccani sheep (Sangamneri, Kolhapuri, Lonand, Solapuri and Madgyal) and reported that the overall mean for height at withers ranged from 68.7 to 76.5 cm.

Gowane *et al*. (2015) recorded the morphometric measurements of Malpura sheep and found that the least-squares means of height at withers were 78.27 and 74.91 cm, respectively in males and females at 12 months age.

Vani *et al.* (2017) carried out analysis on body measurements data of 897 Nellore brown sheep from five divisions of Kadapa district in Andhra Pradesh and reported that the least square means for height at withers at 12 months of age in 97 males is 81.31 ± 1.02 cm and in 800 females is 71.51 ± 0.23 cm, overall least means were 72.03 ± 0.29 cm. Males showed higher values than females at all ages.

2.2.2.3 Heart girth

Gopal and Hari (2007) recorded biometrical measurements on 431 Muzaffarnagari sheep, and found that the overall means for heart girth at 12 months of age was 79–81 cm. The effect of sex is found to be highly significant for heart girth.

Comparison of body measurements in two sexes indicates that males have significantly higher values than females.

Dass (2008) reported that the least-squares mean of heart girth in Pugal sheep was 73.63 ± 0.63 cm at 12 months age.

Mandal *et al.* (2008) studied biometrical measurements in Muzaffarnagari sheep and found that the overall means for heart girth at 12 months of age was 73.43 ± 0.22 cm.

Ravimurugan and Devendran (2009) studied morphometric measurements in 84 Ramnad White sheep and reported the least-squares means at 12 months age for heart girth was 81.65 ± 0.82 cm and 73.44 ± 0.39 cm in males and females, respectively. Whilst, the overall mean for corresponding trait was reported as 77.54 ± 0.45 cm. Age and sex were highly significant source of variation in measurements.

Ravimurugan *et al. (*2010) studied distribution and characterization of Kilakarsal (Keezhakaraisal) sheep and recorded biometry traits of 181 sheep and reported least-squares means for heart girth at 12 months age in males was 75.28 ± 1.43 cm and the corresponding value recorded in females was 69.54 ± 0.60 cm respectively.

Thiruvenkadan *et al*. (2011) studied the growth traits of Mecheri sheep and reported the mean heart girth pooled over sexes to be 74.00 ± 0.40 cm at 12 months age.

Panda *et al.* (2014) reported the average heart girth at 12 months age in mutton type indigenous sheep of Puri district to be 68.19 ± 0.27 cm in males and 63.67 ± 0.30 cm in females.

Rani *et al.* (2014) studied body biometry traits in Nellore sheep and reported the overall least-squares means for heart girth as 78.04 ± 0.49 cm. Heart girth was found to be positively and significantly correlated with body length and body weight at all ages studied in both the sexes.

Yadav *et al.* (2014) studied morphometric traits of five ecotypes of Deccani sheep (Sangamneri, Kolhapuri, Lonand, Solapuri and Madgyal) and reported that the overall mean for heart girth ranged from 74.5 to 78.1 cm.

Gowane *et al*. (2015) analyzed the morphometric measurements of Malpura sheep and found that the least-squares means of heart girth at 12 months age were 86.18 cm and 78.27 cm, respectively in males and females.

Vani *et al.* (2017) carried out for prediction of least square means of heart girth in Nellore brown sheep by using statistical methods. Data on body measurements were collected from 897 Nellore brown sheep from five divisions of Kadapa district in Andhra Pradesh. The Least square means for heart girth at 12 months of age in 97 males is 82.96 ± 1.15 cm and in 800 females is 73.39 ± 0.26 cm, overall least square means were 73.00 ± 0.30. The Sex had significant effect on body measurements at all ages and male animals showed higher values than females at all stages of growth.

2.3 Association of SSCP patterns with body weights and morphometric traits

Min *et al.* (2005) studied the association between (5' promoter region) growth hormone gene polymorphism and growth traits in Boer and Boer x LuBei white goats. Animals with AA genotype had higher body weight at one year and those with BB genotype in LuBei white goat showed lower birth weight and weaning weights.

Lan *et al.* (2007) studied the association of SSCP patterns in 5' UTR region, exon-5 and flanking 3' UTR regions of growth hormone with growth traits in 5 indigenous sheep breeds (xinong sannen, laoshan, Guanzhong, Shaannan White, Guizhou White) and reported that there is a little influence of different patterns of growth hormone gene on weight in one-year-old animals.

Kumar *et al*. (2008) studied the correlation between polymorphism in the promoter region, exon-4 and exon-5 of the growth hormone gene and growth traits in Sirohi goats. The animals having AH variant in the promoter, EF variant in exon-4 and FF variant in exon-5 regions had significantly higher body weights at birth, nine months and six months of age, respectively.

An *et al.* (2010) studied variability in growth hormone exon-5 region in three Chinese goat populations (Boer goat, F1 and F2 generations of Boer X Guanzhong dairy goats). In Boer goats, the females with EE and EF genotypes had greater body weight, body length and chest girth than those with EG genotype. In F1 goats, the females with EE and EF genotypes had greater body weight and females with EF genotype had greater height at withers and body length. In F2 goats, the females with EF genotype had greater body weight and the females with EG genotype had greater chest girth.

Wickramaratne *et al*. (2010) studied the association between polymorphism at 5' promoter region of growth hormone gene and growth traits in Osmanabadi and Sangamneri goat breeds of India. The study revealed GT genotype in both breeds was associated with heavy body weight and GG with low body weight. In Sangamneri breed GT genotypes were 6.5% taller than GG.

Tahmoorespur *et al*. (2011) studied growth hormone gene polymorphism in Exon-5 and its association with estimated breeding values of growth traits in Iranian purebred Baluchi sheep. The study revealed a significant effect of polymorphism on 6 months body weight.

Yousefi and Azari (2012) investigated the association between SSCP genotypes of exon-5 of GH gene and body weights in Zel sheep. The statistical analysis revealed no significant differences between the genotypes studied.

Hajihosseinlo *et al*. (2013) studied polymorphism in Exon-4 of growth hormone gene and its association with body length, height at withers and chest girth in Makooei Sheep.The study reported that, only heart girth had significant association with SSCP patterns of exon-4 in GH gene.

Moradian *et al*. (2013) studied association between polymorphism in the Exon-4 of growth hormone gene and growth traits in Makooei sheep. The study revealed significant differences in weights at weaning, six months and nine months of age among the genotypes.

Jia *et al*.(2014) investigated the correlation between polymorphisms in the 5' regulatory region, exon-4 and 3' untranslated region (UTR) of growth hormone gene and growth traits in various sheep breeds .The study revealed that only two alleles (A and B) and 3 genotypes (AA, AB, and BB) for both 5' regulatory region and exon-4 whereas 3 alleles (A, B, and C) and 4 genotypes (AA, AB, BB, and AC) were found for the 3' UTR.In Tibetan sheep, the association analysis indicated statistically significant differences in the scores of weight, length, and heart girth for the 5' regulatory region and polymorphism exon-4 is associated with differences in weight, length, wither height, and heart girth. Different genotypes differed in weight, length, wither height, and heart girth at 3' UTR. For exon-4, Poll Dorset sheep with genotype AA showed a lower score than those of genotypes BB and AB. With regard to the 3' UTR, Poll Dorset sheep with genotype AC showed higher scores than those of genotypes AA and AB.

Dayal *et al.* (2016) studied the correlation between polymorphisms in the growth hormone gene i.e in exon-3 and exon-4 (partial) and growth traits in Black Bengal goat. Animals having AC genotype had the highest birth weight, whereas animals having CC genotype had lowest birth weight.

CHAPTER-III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Experimental Animals

The present study was carried out on fifty unrelated stock of Nellore Brown sheep **(Fig.1, 2, 3)** maintained at Livestock Research station Mamnoor, Institutional Livestock farm complex (ILFC) Rajendranagar unit of P.V. Narsimha Rao Telangana Veterinary University, and few animals from farmers flocks belonging to areas near to Shamshabad of RangaReddy district.

3.1.2 General Laboratory Preparation

Molecular grade reagents were used for the preparation of all solutions and buffers. The reagents and labware were availed from many suppliers including GeNei (Bangalore), Himedia (Mumbai), Merck (Mumbai) and Sigma (USA). All the aqueous solutions were prepared using double distilled water and wherever necessary, solutions were autoclaved at 121ºC and 15 lbs pressure for 15 minutes. Neutral glassware of borosil were used throughout the study.

Glassware were soaked in neutral detergent (Labolene) overnight and then scrubbed and washed thoroughly under running tap water. Then they were washed with de-ionized water, then with single distilled water and finally rinsed with double distilled water, air dried, packed and sterilized in hot air oven at 160ºC for 2 hours. Filter assemblies, caps wrapped in double paper, micropipette tips, deep well plates, troughs, microfuge tubes etc. were sterilized by autoclaving at 121°C/15lbs/15 minutes.

3.1.3. GH Gene Primers for PCR

Growth hormone (GH) gene different regions were amplified by using four sets of species specific primers corresponding to 5' regulatory region, exon-4, exon-5 and 3' UTR obtained from available literature (Jia *et al.,* 2014) and the annealing temperature of these primers was optimized by gradient PCR. The details of the primers used are presented in Table. 1.

3.2 METHODS

3.2.1 Sample Collection

About 10 ml of blood was collected from each animal aseptically from the external jugular vein into vacutainer tubes containing EDTA (2.7%). The tube was 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 degree centigrade until further processing.

3.2.2 Isolation of Genomic DNA

Genomic DNA was isolated from the whole blood by phenol-chloroform extraction method by standard protocol (Sambrook *et al.,* 1989) with slight modifications. All the chemicals and reagents used for the DNA isolation are enlisted in Appendix I. The steps followed in the isolation of DNA from blood samples are detailed below.

Step 1: The collected blood samples (10 ml) were transferred from vacutainer tubes into 15 ml capacity centrifuge tubes.

Table 1. Details of primers used for amplification of different regions of GH gene

S.No	Region	Primer sequence	Amplified product	Authors
1	5' Regulatory region	F: 5' AACAGGATGAGTGAGAGGAGGT 3' R: 5' CCATCATAGCTGGTGAGCTG 3'	210 bp	Jia *et al*.(2014)
2	Exon-4	F: 5' GGACTTGGAGCTGCTTCGCAT 3' R: 5' GGAAGGGACCCAACAATGCCA 3'	191 bp	Jia *et al.(*2014)
3	Exon-5	F: 5' AGCAGAGTCTTCACCAACAGC 3' R: 5' TAGTTCTTGAGCAGCGCATC 3'	480 bp	Jia *et al.(*2014)
4	3' UTR	F: 5' TGGCAGGAGCTGGAAGATGT 3' R: 5' CCTACTCAGACAATGTGATGCAA 3'	320 bp	Jia *et al*.(2014)
F:Forward, **R:**Reversed				

Fig.1: Nellore ram

Fig.2: Nellore ewe

Fig.3: Nellore flocks

(a)

(b)

Step 2: Chilled RBC lysis buffer (double the quantity of blood) was added and mixed end to end. The tubes were incubated on ice for about 10 min and centrifuged at 4000 rpm for 10 min and discard Supernatant containing plasma and lysed RBC by pipetting.

Step 3: Step 2 was repeated for 3-4 times till the WBC pellet became free from red tinge.

Step 4: Next DNA extraction buffer was added 3 ml/10 ml of blood. The WBC pellet was dispersed and incubated at 37 degree Celsius for 30 min in water bath.

Step 5: Next 200 µl 10% SDS was added and mixed by inversion.

Step 6: 40 µl proteinase K (25 µg/µl) was added and incubated at 50 degree celsius in water bath overnight.

Step 7: Next morning, equal volume of Tris-saturated Phenol (pH>8) was added and mixed by inversion for about 10 min.Centrifuged at 4000 rpm for 10 min. The upper aqueous phase containing DNA was collected into 15 ml capacity centrifuge tubes.

Step 8: Step 7 was repeated with Phenol: Chloroform: Isoamyl alcohol (25:24:1) and with Chloroform: Isoamyl alcohol (24:1) and then the upper aqueous phase was collected into fresh 15 ml capacity centrifuge tubes.

Step 9: 3 M sodium acetate (1/10th volume) was added to aqueous phase and mixed gently.

Step 10: Two volumes of isopropanol was added and mixed by gentle inversion and kept at room temperature for 5 min for precipitation of DNA.

Step 11: The precipitated DNA along with 500 µl of isopropanol was taken into 1 ml eppendorf tubes. Centrifuged at 10,000 rpm for 10 min. The supernatant was discarded by inversion.

Step 12: The DNA pellet was washed in 70% ethanol twice. Centrifuged at 10,000 rpm for 10 min. The supernatant was discarded by inversion.

Step 13: Finally, the DNA pellet was air dried by inverting the tube on to blotting paper and then the pellet was dissolved in 200 µl of nuclease free water (NFW).

3.2.3 Evaluation of Purity, Quality and Concentration

3.2.3.1 Purity of DNA

The purity of the genomic DNA was assessed by checking the optical density (OD) at 260 nm and 280 nm which indicates the amount of DNA and amount of protein respectively in a given sample. The ratio of optical densities at 260 nm and 280 nm was used as a criterion for purity. The samples having OD ratio (260 nm/280 nm) 1.7 to 1.9 were used for experiment.

3.2.3.2 Concentration of DNA:

The concentration of genomic DNA was estimated by taking OD value at 260 nm. The genomic DNA concentration was measured by using the formula given by Sambrook and Russel (2001).

$$\text{DNA concentration } (\mu g/ml) = \text{OD} \times \text{Dilution factor} \times 50$$

3.2.3.3 Quality of DNA

Genomic DNA quality was checked to ensure intact DNA without any shearing. Horizontal submarine agarose gel electrophoresis was performed to check the quality of DNA. Agarose of 0.8% w/v was dissolved in 1x TBE buffer by heating. The agarose solution was cooled and poured into the gel casting tray with comb after adding ethidium bromide (0.5 µg/ml). After solidification of gel, comb is removed and submerged in a gel tank having 1x TBE buffer.

The isolated DNA samples were mixed with 1/6th volume of 6x gel loading buffer and loaded into the wells using a micropipette. The electrophoresis was carried out at 70 volts for about one hour. Then the gel was visualized under UV light and photographed using gel documentation system (Syngene). After checking the quality, the isolated DNA was diluted to 50-100 ng/µl with nuclease free water (NFW) for further analysis.

Note: Agarose Gel Electrophoresis reagents are presented in Appendix II

3.2.4 Polymerase Chain Reaction (PCR)

The amplification of the 5’ regulatory region, exon 4, exon-5 and 3' UTR portions of GH gene with specific primers (Bioserve) available in literature (Table 1) was carried out in 0.2 ml capacity PCR tubes, using thermal cycler (Eppendorf). A master mix for PCR amplification was prepared as presented in the Table 2.

Table 2. Composition of PCR reaction mix for amplification

S.No	Components	Volume	Final concentration
1	10x Taq buffer	1.25 µl	1 X
2	dNTPs (10Mm)	0.5 µl	0.4 mM
3	Primer-Forward (50 pm)	1 µl	4 pm
4	Primer-Reverse (50 pm)	1 µl	4 pm
5	MgCl2(25 mM)	0.5 µl	1 mM
6	Taq Polymerase(1 unit/µl)	0.5 µl	0.5 unit
7	Autoclaved Mille Q water	6.75 µl	

An aliquot of 11.5 µl of master mix per sample was drawn into thin-walled PCR tubes and 1 µl (50-100 ng) of template DNA was added for making 12.5 µl. The PCR tubes were marked for the identification, then spinned briefly for proper mixing and mounted in PCR machine.

3.2.4.1 PCR reaction conditions of different GH gene segments

The PCR protocol with specific annealing temperatures for each of the primers for 5' Regulatory region, Exon-4, Exon-5 and 3' UTR are detailed in Table 3, 4, 5 and 6.

Table 3. PCR reaction conditions for 5' regulatory region

Step	Process	Temperature (°C)	Time
1	Initial denaturation	94	7 min
2	Cyclic denaturation	94	30 sec
3	Primer annealing	63.7	30 sec
4	Cyclic extension	72	30 sec
5	Steps 2 to 4 were repeated for 36 cycles		
6	Final extension	72	5 min
7	Hold	4	Forever

Table 4. PCR reaction conditions for exon-4

Step	Process	Temperature (°C)	Time
1	Initial denaturation	94	7 min
2	Cyclic denaturation	94	30 sec
3	Primer annealing	63.2	30 sec
4	Cyclic extension	72	45 sec
5	Steps 2 to 4 were repeated for 36 cycles		
6	Final extension	72	5 min
7	Hold	4	Forever

Table 5. PCR reaction conditions for exon-5

Step	Process	Temperature (°C)	Time
1	Initial denaturation	94	7 min
2	Cyclic denaturation	94	30 sec
3	Primer annealing	62.9	30 sec
4	Cyclic extension	72	45 sec
5	Steps 2 to 4 were repeated for 36 cycles		
6	Final extension	72	5 min
7	Hold	4	Forever

Table 6. PCR reaction conditions for 3' UTR

Step	Process	Temperature (°C)	Time
1	Initial denaturation	94	7 min
2	Cyclic denaturation	94	30 sec
3	Primer annealing	61.5	30 sec
4	Cyclic extension	72	45 sec
5	Steps 2 to 4 were repeated for 36 cycles		
6	Final extension	72	5 min
7	Hold	4	Forever

The annealing temperatures ranged from 61.5 °C to 63.7 °C depending on the primers used. The PCR tubes were kept in thermal cycler and the programme was executed. Each PCR amplification programme took about 2 hours. At the end of the PCR, the tubes were taken out and stored at -20°C until further use. The PCR products were confirmed by agarose gel electrophoresis and visualized in gel documentation system.

3.2.4.2 Checking of the amplified product

Horizontal submarine agarose gel electrophoresis (2% w/v) was carried out to check the amplified product. A 5 µl PCR product mixed with 1 µl of 6x gel loading dye was loaded along with 100 bp ladder as a marker in a separate lane. The electrophoresis was done at 80V for 2h. The amplified product in the gel was observed under UV transilluminator and documented by gel documentation system (Syngene).

3.2.5. Single Strand Conformational Polymorphism (SSCP)

Single strand conformational polymorphism was used to screen samples for polymorphism in GH gene. Formamide dye and polyacrylamide gel (12% concentration) were made for SSCP study. The composition of the formamide dye and polyacrylamide gel mix were given in appendix- III.

3.2.5.1 Procedure for SSCP

Glass plates, spacers and combs were washed with detergent, rinsed initially under running tap water till no remains of detergent were left and finally in double distilled water before drying. Before use, glass plates, spacers and comb were cleaned with methanol and dried. The procedure for casting of gel was as follows:

1. Glass plates were fitted by putting the 1 mm spacer between the two plates at its position and then clamps were applied. Flat end of glass plates was sealed by rubber gasket. Then the freshly prepared polyacrylamide gel mix was poured into the space between plates and comb was inserted immediately.

2. The above gel was allowed to polymerize at room temperature for 1h.

3. After polymerization, rubber gasket was removed and gel was put in electrophoresis tank with notched plate facing towards the buffer reservoir.

4. The reservoir of the electrophoresis tank was filled with 1x TBE (Same as agarose gel), the comb was removed carefully and the gel was given a pre-run at 160 V for 30 min, in a vertical gel electrophoresis system.

5. About 5 µl of PCR product was taken in a 0.2 ml PCR tube and 12 µl formamide dye was added in it and mixed properly. Then, PCR tubes were sealed with the parafilm.

6. PCR product and formamide dye mix were denatured at 95°C for 5 min (By keeping on 95°C hot water on heater) and snap cooled on ice for 15-20 min.

7. The product was loaded in the gel carefully. The electrophoresis was performed at 4°C for 10-12 h at 110 V. The current, voltage and running time were standardized as per the size and composition of PCR product.

8. After running was over, gel was kept for silver staining to visualize the banding pattern.

3.2.5.2 Silver Staining

The gel was stained by silver nitrate as described by Bassam *et al*. (1991) with minor modifications in appendix- III.

The following were the steps involved in the silver-staining:

1. The gel along with plate was placed on a clean surface by keeping the notched plate facing upwards.

2. The upper plate was separated gently by taking care to avoid breakage of the gel.

3. The first row loaded was marked by cutting the upper portion of the gel from that side.

4. The gel along with the plate was placed in a suitable sized tray. The gel was removed from glass plate by flooding with distilled water so as to transfer the gel into the tray. Then distilled water was removed and the gel was fixed.

5. About 300 ml of freshly prepared 10% ethanol was added in the tray, shaken gently and the plate was removed carefully after the gel got detached. The gel was agitated slowly for 5 min until the tracking dye was no longer visible.

6. Ethanol was discarded and 300 ml of 1% of the nitric acid was added and gel was kept on rocker for 3 min.

7. The gel was rinsed 3 times for 3 min each with double distilled water.

8. A volume of 300 ml of 0.1% silver nitrate was added to the tray and agitated slowly for 20 min.

9. The gel was rinsed 2 times for 30 sec each with double distilled water so as to remove all the traces of silver nitrate and other chemicals.

10. For developing the gel 300 ml of 3% sodium carbonate with 450 µl of 37% formaldehyde (added at the last minute) freshly prepared was poured in the tray and the gel was gently shaken till the development of distinct bands.

11. The reaction was stopped by adding 200 ml of triple distilled water

12. The gel was visualized under white light and documented by the gel documentation system and scanned by scanner for better quality of gel photo.

13. The different patterns observed were recorded for further analysis.

3.2.5.3 Identification of unique band patterns or Genotyping

The most common band pattern identified was named as 1. If there are more bands, in addition to the common bands, they were marked as 2, 3, 4 etc., depending on the band pattern.

3.2.5.4 Allelic and genotypic frequency

The allelic and genotypic frequencies for each fragment were calculated by using POPGENE software (Yeh *et al.*, 1997).

3.2.5.5 Phenotypic data recording on body weight and other morphometric traits

The data on body weight (BW) and other morphometric traits viz., body length, height at withers and heart girth were recorded at 12 months of age. The body weight (in kg) was recorded using a hanging spring balance. The other morphometric traits (in cm) were recorded using measuring tape as per Banait *et al.* (2002) and Narsimham *et al.* (2003) as mentioned below:

Body length (BL): Distance from point of shoulder to the point of tuber ischii.

Height at withers (HAW): Distance from the base of the hoof to the highest point of withers.

Heart Girth (HG): Body circumference around the chest just behind the elbow joint.

3.2.6 Statistical analysis

Statistical analysis was performed to study the association of each genotype on body weight and different morphometric traits of Nellore sheep. The univariate GLM model of SPSS 17 was used to perform the analysis according to the following statistical model:

$$\mathbf{Yij = \mu + Gi + Sj + eij}$$

Where, Yij = dependent variable

μ = overall population mean,

Gi = fixed effect of genotype

Sj = fixed effect of sex

eij = random error with NID (0,σ2).

Significant differences between least square means of different genotypes were tested by Duncan's method (DMRT). Values were considered significant at ($P \leq 0.05$) and presented as least square means ± standard errors.

CHAPTER-IV

RESULTS

The present investigation was carried out in fifty unrelated Nellore brown sheep to study the polymorphism in 5' regulatory region, exon-4, exon-5 and 3'UTR of growth hormone gene through PCR-SSCP technique with four sets of primers and also association of SSCP patterns with body weights and morphometry at 12 months of age.

4.1 Isolation of genomic DNA and determination of its quantity and quality

The blood samples (approximately 10 ml) were collected from jugular vein using EDTA (2.7%) vacutainer tubes aseptically. The good quality genomic DNA was isolated through phenol-chloroform extraction method of Sambrook *et al.* (1989).The Purity and concentration of isolated DNA was evaluated based on OD values (260:280) through Nano drop ((JENWAY Genova Nano).The quality of the DNA was checked for any shearing by agarose gel electrophoresis (0.8%) and appeared as a single band. The DNA was diluted with nuclease free water to adjust the concentration between 50-100 ng/µl and used as a template in PCR reaction.

4.2 PCR reaction

The PCR reactions were set with four sets of species specific primers available in literature for the amplification of different segments of growth hormone gene corresponding to 5' regulatory region, exon-4, exon-5 and 3' UTR in 0.2 ml capacity flat PCR tubes, using thermal cycler (Eppendorf). The details of oligonucleotide primers used in this study are presented in Table 1.

4.2.1 PCR amplification of 5' regulatory region (210 bp) of GH gene

210 bp of 5' regulatory region (**Fig. 4)** of growth hormone gene was amplified using genomic DNA as template(50-100 ng/µl) with a set of primers(forward:5'

AACAGGATGAGTGAGAGGAGGT3', reverse:5'CCATCATAGCTGGTGAGCTG 3') and visualized through 2% agarose gel electrophoresis in gel documentation system along with 100 bp ladder. The PCR reaction was optimized using different concentrations of $MgCl_2$, dNTPs and the primers. The optimized conditions that gave the best yield were 1 mM, 0.4 mM and 4 pm concentrations of $MgCl_2$, dNTPs and the primers, respectively. 36 PCR cycles of initial denaturation, denaturation, extension, final extension and hold were standardized and annealing temperature was fixed at 63.7°C for 5' regulatory region of growth hormone gene.

4.2.2 PCR amplification of exon-4 (191 bp) of GH gene

191 bp of exon-4 (**Fig. 5**) of growth hormone gene was amplified using genomic DNA as template (50-100 ng/µl) with a set of primers (Forward:5' GGACTTGGAGCTGCTTCGCAT 3', reverse: 5'GGAAGGGACCCAACAATGCCA 3') and visualized through 2% agarose gel electrophoresis in gel documentation system with 100 bp ladder. The PCR reaction was optimized using different concentrations of $MgCl_2$, dNTPs and the primers. The optimized conditions that gave the best yield were 1 mM, 0.4 mM and 4 pm concentrations of $MgCl_2$, dNTPs and the primers, respectively. 36 PCR cycles of initial denaturation, denaturation, extension, final extension and hold were standardized and annealing temperature was fixed at 63.2 °C for exon-4 of growth hormone gene.

4.2.3 PCR amplification of exon-5 (480 bp) of GH gene

The exon-5 (480 bp) of growth hormone gene **(Fig.6)** was amplified using genomic DNA as template (50-100 ng/µl) with a set of primers, (forward:5' AGCAGAGTCTTCACCAACAGC 3', reverse: 5' TAGTTCTTGAGCAGCGCATC 3') and confirmed on 2% agarose gel electrophoresis in gel documentation system along with 100 bp ladder. The PCR reaction was optimized using different concentrations of $MgCl_2$, dNTPs and the primers. The optimized conditions that gave the best yield were 1 mM, 0.4 mM and 4 pm concentrations of $MgCl_2$, dNTPs and the primers, respectively. 36 PCR cycles of initial denaturation, denaturation, extension, final extension and hold were standardized and annealing temperature was fixed at 62.9 °C for exon-5 of growth hormone gene.

4.2.4 PCR amplification of 3' UTR (320 bp) of GH gene

The 3' UTR (320 bp) of growth hormone gene was amplified (**Fig. 7**) using genomic DNA as template (50-100 ng/µl) with a set of primers, (forward:5' TGGCAGGAGCTGGAAGATGT3', reverse: 5' CCTACTCAGACAATGTGATGCAA 3' primers) and verified through 2% agarose gel electrophoresis in gel documentation system with 100 bp ladder. The PCR reaction was optimized using different concentrations of $MgCl_2$, dNTPs and the primers. The optimized conditions that gave the best yield were 1 mM, 0.4 mM and 4 pm concentrations of $MgCl_2$, dNTPs and the primers, respectively. 36 PCR cycles of initial denaturation, denaturation, extension, final extension and hold were standardized and annealing temperature was fixed at 61.5°C for 3' UTR of growth hormone gene.

4.3 PCR-SSCP of different regions of GH gene

The PCR products of 5' regulatory region, exon-4, and exon-5 and 3' UTR of Growth hormone gene were used for SSCP screening to detect the polymorphism.

4.3.1 PCR-SSCP of 5' regulatory region of GH gene

About 5 µl of PCR amplified product of 5' regulatory region (210 bp) of GH gene was denatured by mixing 12 µl of formamide dye at 95°C for 5 min (by keeping in hot water at 95°C) and snap cooled on ice for 15-20 min. The denatured fragments were then resolved on 12% polyacrylamide gel for 10-12 hours at 110 V-120 V and further subjected to silver staining. The results observed three different SSCP patterns **11, 12** and **22 (Fig. 8)** corresponding to two allelic variants **1** and **2**. Out of 50 samples, twelve samples showed pattern **11**, twenty one samples showed pattern **12** and seventeen samples showed pattern **22**.

4.3.2 PCR-SSCP of exon-4 of GH gene

About 5 µl of PCR amplified product of exon-4 (191 bp) of GH gene was denatured by mixing 12 µl of formamide dye at 95°C for 5 min (by keeping in hot water at 95°C) and snap cooled on ice for 15-20 min. The denatured fragments were then resolved on 12% polyacrylamide gel for 10-12 hours at 110 V-120 V and further subjected to silver staining. The results observed two different SSCP patterns **11** and **12** (**Fig. 9)** corresponding to two allelic variants **1** and **2**. Out of 50 samples, thirty samples showed Pattern **11** and twenty individuals showed pattern **12**.

4.3.3 PCR-SSCP of exon-5 of GH gene

About 5 µl of PCR amplified product of exon-5 (480 bp) of GH gene was denatured by mixing with 12 µl of formamide dye at 95°C for 5 min (by keeping in hot water at 95°C) and snap cooled on ice for 15-20 min. The denatured fragments were then resolved on 12% polyacrylamide gel for 10-12 hours at 110 V-120 V and further subjected to silver staining. The results observed two different SSCP patterns **11** and **12** (**Fig. 10**) corresponding to two allelic variants **1** and **2**. Among 50 samples, thirty five samples showed pattern **11** and fifteen samples showed pattern **12**.

4.3.4 PCR-SSCP of 3' UTR of GH gene

About 5 µl of PCR amplified product of 3' UTR (320 bp) of GH gene was denatured by mixing with 12 µl of formamide dye at 95°C for 5 min (by keeping in hot water at 95°C) and snap cooled on ice for 15-20 min. The denatured fragments were resolved on 12% polyacrylamide gel for 10-12 hours at 110 V-120 V and further subjected to silver staining. The results observed two different SSCP patterns **12** and **13** (**Fig. 11**) corresponding to three allelic variants **1, 2** and **3**. Among 50 samples, thirty one samples showed pattern **12** and nineteen samples showed pattern **13**.

4.3.5 Identification of unique band patterns or Genotypes

In the current study, the PCR-SSCP of different segments of GH gene revealed polymorphism in 5' regulatory region, exon-4 and 3' UTR and in exon-5.The number of alleles and genotypes (Table. 7) observed in each fragment of GH gene studied are presented.

4.4 Allelic and genotypic frequency

The allelic and genotypic frequencies for each fragment (namely, 5' regulatory region, exon-4, exon-5 and 3' UTR) of the growth hormone gene calculated are presented in Table 8.

Table 7. Allelic variants and genotypes (SSCP patterns) observed in different portions of GH gene

Gene segment	Alleles observed			Genotypes observed			
5' Regulatory region	1	2	-	**11** (N=12)	**12** (N=21)	**22** (N=17)	-
Exon 4	1	2	-	**11** (N=30)	**12** (N=20)	-	-
Exon 5	1	2	-	**11** (N=35)	**12** (N=15)	-	-
3' UTR	1	2	3	**12** (N=31)	**13** (N=19)	-	-

The number in parenthesis indicate number of individuals showing that specific pattern

Table 8. Frequencies of different alleles and SSCP genotypes in different regions of GH gene

Gene segment	**Allele frequency**			**Genotype frequency**			
	1	**2**	**3**	**11**	**12**	**22**	**13**
5' regulatory region	0.45	0.55	-	0.24	0.42	0.34	-
Exon-4,	0.80	0.20	-	0.60	0.40	-	-
Exon-5	0.85	0.15	-	0.70	0.30	-	-
3' UTR	0.50	0.31	0.19	-	0.62	-	0.38

4.5. Body weight and morphometry

The data on body weights, body length, height at withers and heart girth were recorded at 12 months of age in 50 unrelated sheep belonging to different institutional farms and from farmers in Shamshabad area of RangaReddy district. The observed over all mean for body weight (BW), body length (BL), height at withers (HAW) and heart girth (HG) in Nellore sheep are presented in Table 9.

Table 9. Least squares means of body weight and morphometric traits over all the genotypes.

	Body weight(kg)	Body length(cm)	Height at withers(cm)	Heart girth(cm)
Over all mean	25.93 ± 0.20	71.46 ± 0.43	74.17 ± 0.17	75.70 ± 0.53

Fig. 4: Agarose gel electrophoresis showing PCR amplified product of 5' regulatory (210bp) region of GH gene

Lane 1-4: 210 bp, Lane M: 100 bp ladder

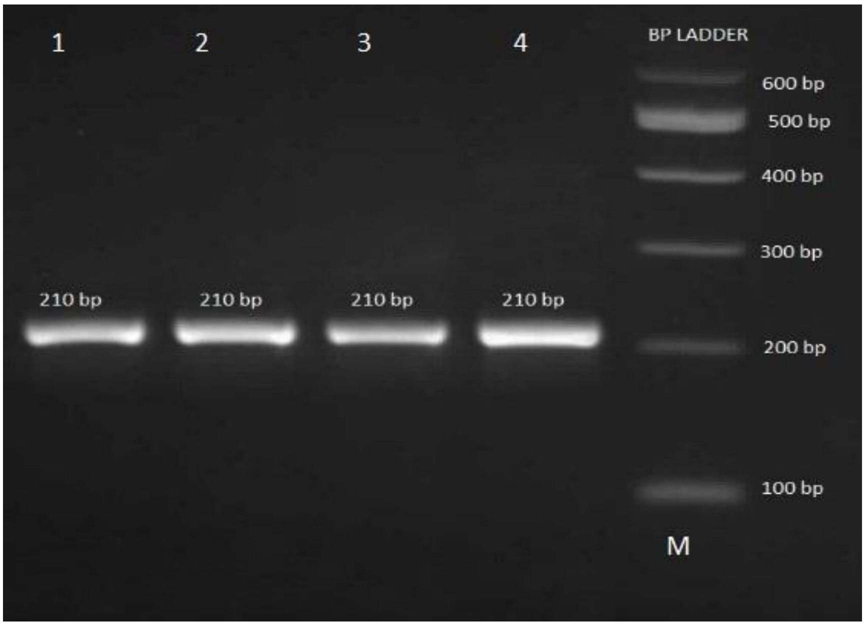


Fig. 5: Agarose gel electrophoresis showing PCR amplified product of exon-4 (191 bp) of GH gene

Lane 1-4: 191 bp, Lane M: 100 bp ladder

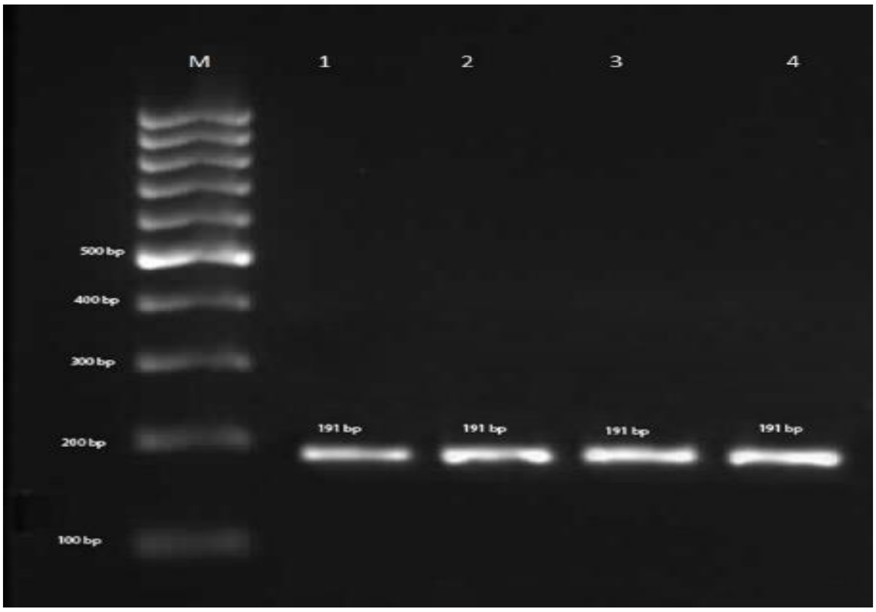


Fig. 6: Agarose gel electrophoresis showing PCR amplified product of exon-5 (480 bp) of GH gene

Lane 1-4: 480 bp, Lane M: 100 bp ladder

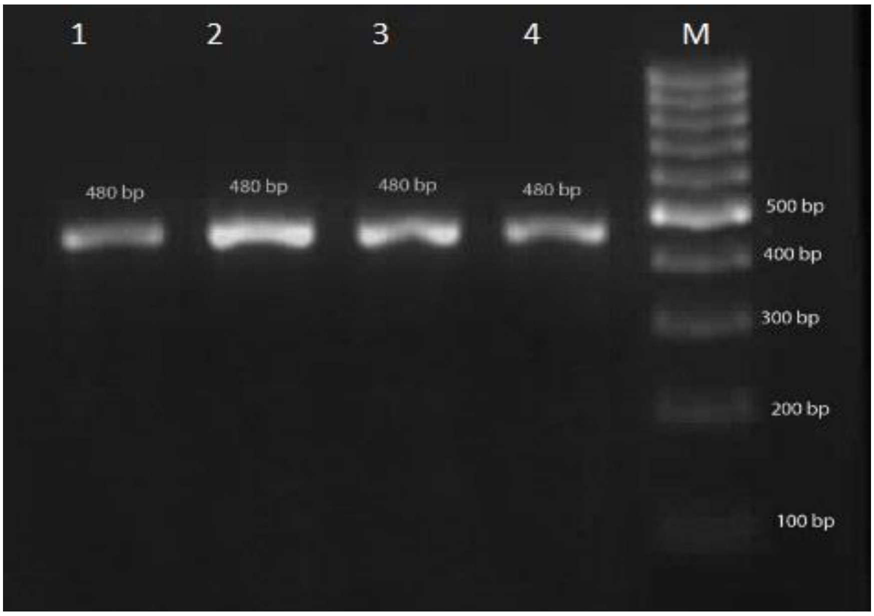


Fig. 7: Agarose gel electrophoresis showing PCR amplified product of 3' UTR (320 bp) of GH gene

Lane 1-5: 320 bp, Lane M: 100 bp ladder

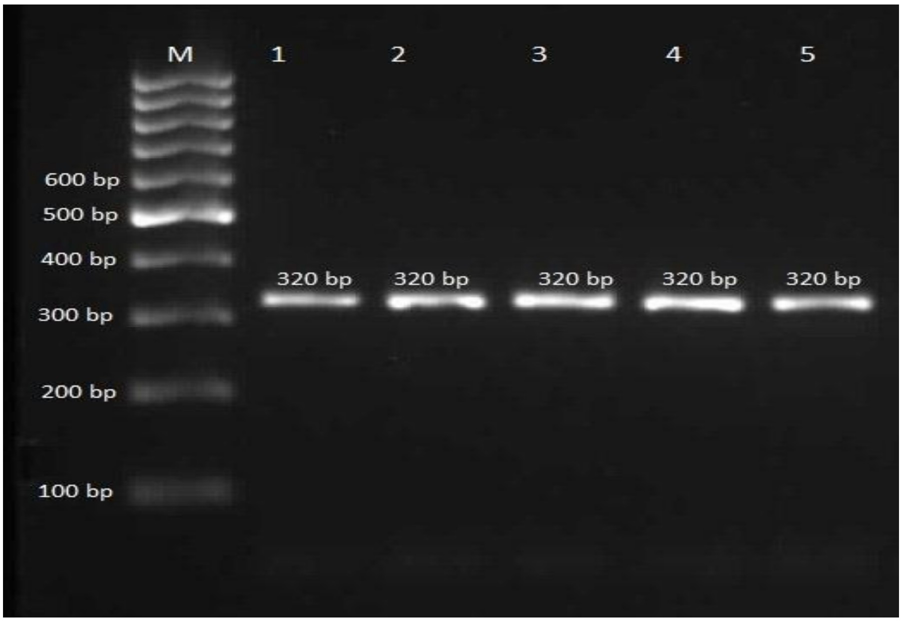


Fig. 8. Polyacrylamide gel electrophoresis showing SSCP patterns in 5' regulatory region (210 bp) of GH gene

Fig. 9. Polyacrylamide gel electrophoresis showing SSCP patterns in exon-4 (191 bp) of GH gene

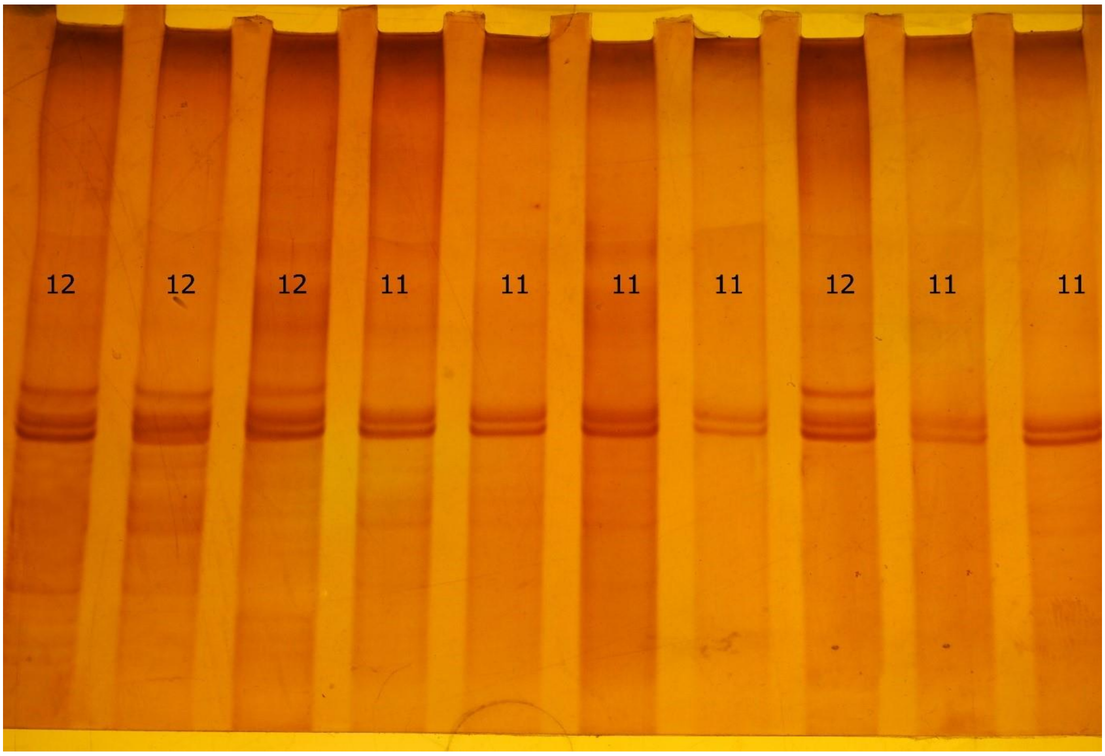


Fig. 10. Polyacrylamide gel electrophoresis showing SSCP patterns in exon-5 (480 bp) of GH gene

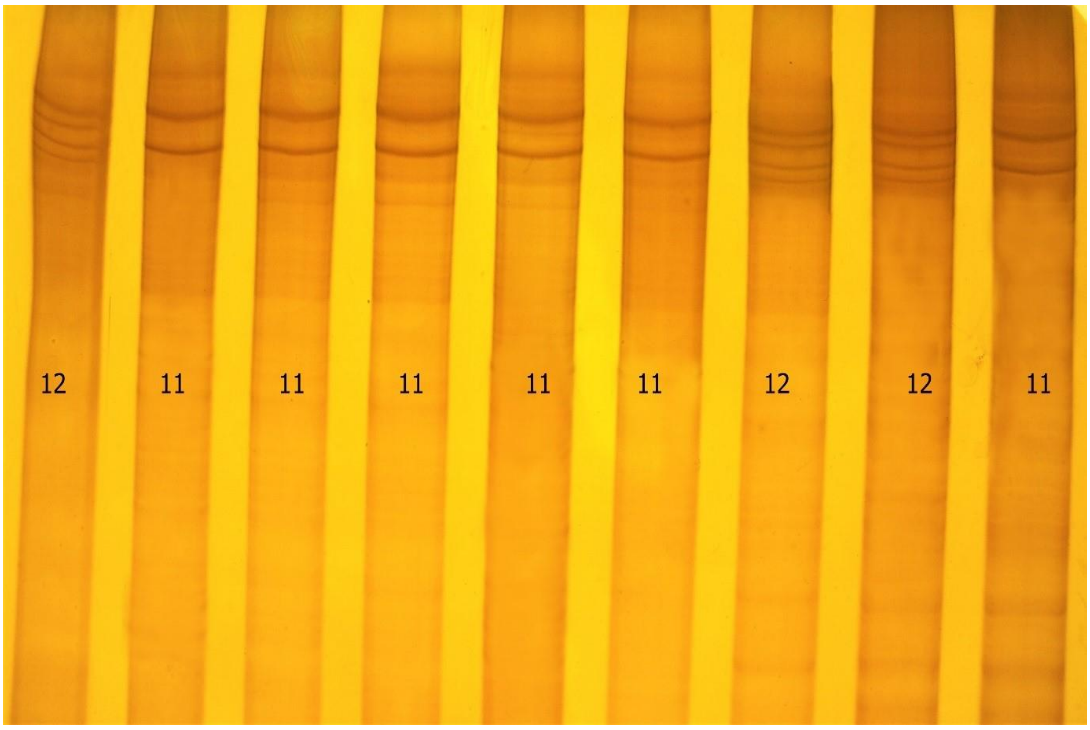
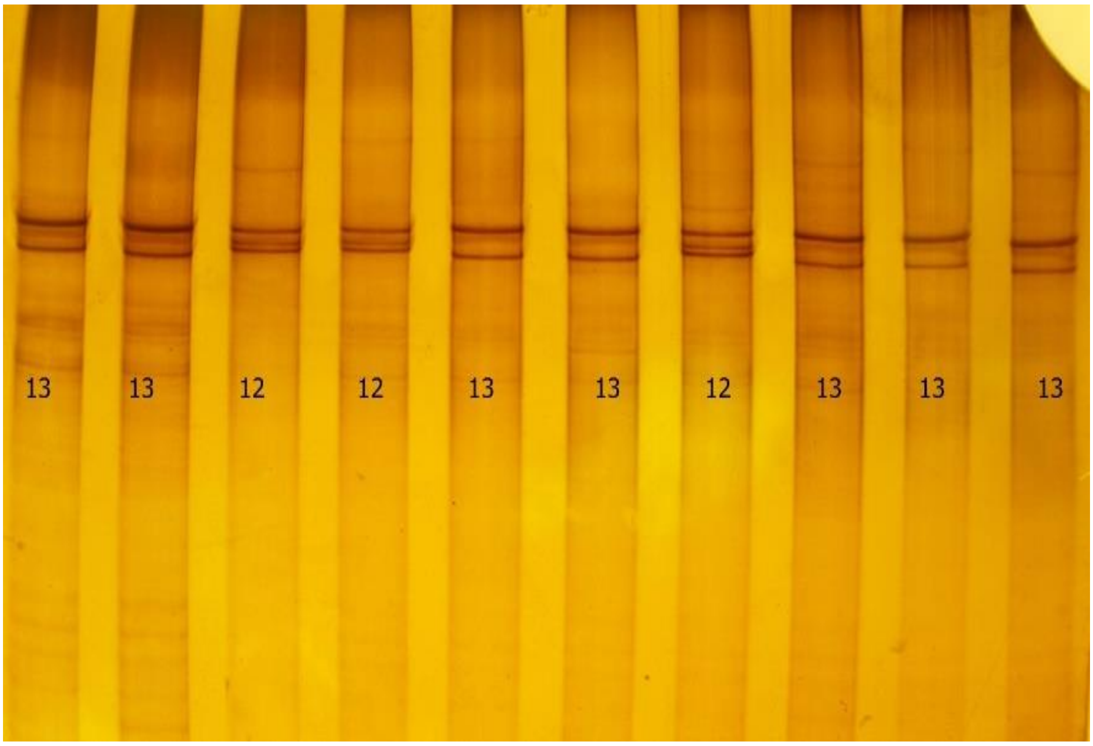


Fig. 11. Polyacrylamide gel electrophoresis showing SSCP patterns in 3' UTR (320 bp) of GH gene



4.6 Association of SSCP patterns of GH gene with body weight and morphometric traits

The observed PCR-SSCP patterns in different regions of GH gene were correlated with various morphometric traits such a body weight (BW),body length (BL),height at withers (HAW) and heart girth (HG) at 12 months of age. The results obtained are presented in Table 10, 11, 12 and 13.

In the region of 5' regulatory region three SSCP patterns were observed (**11,12** and **22)** and associated with body measurements and it was found that the mean BW, BL, HAW and HG in pattern **11** were 26.03 ± 0.42 kg, 72.00 ± 0.77 cm, 74.54 ± 0.30 cm and 75.97 ± 1.16 cm while with pattern **12** were 25.88 ± 0.35 kg, 70.81 ± 0.71 cm, 74.21 ± 0.29 cm and 75.76 ± 0.81 cm and for pattern **22** were 25.91 ± 0.32 kg, 71.18 ± 0.75 cm, 73.87 ± 0.29 cm and 75.44 ± 0.94 cm, respectively.

In the region of exon-4 two SSCP patterns were observed (**11** and **12**) and associated with body measurements and it was found that the mean BW, BL, HAW and HG in pattern **11** were 26.08 ± 0.25 kg, 71.93 ± 0.55 cm, 74.35 ± 0.25 cm and 75.96 ± 0.68 cm and in pattern **12** were 25.71 ± 0.35 kg, 70.75 ± 0.67 cm, 73.91 ± 0.21 cm and 75.32 ± 0.88 cm, respectively.

There were only two SSCP patterns (**11** and **12**) for the exon-5 portion of gene and the morphometric values of BW, BL, HAW and HG were 26.11 ± 0.27 kg, 71.68 ± 0.53 cm, 74.24 ± 0.21 cm, 75.52 ± 0.67 cm in pattern **11** . The animals with pattern **12** showed 25.50 ± 0.25 kg, 70.94 ± 0.74 cm, 74.00 ± 0.30 cm, 76.13 ± 0.89 cm for BW, BL, HAW and HG, respectively.

In case of SSCP in 3' UTR, the overall mean of BW, BL, HAW and HG were 25.75 ±0.23 kg, 71.16 ± 0.61 cm, 74.24 ± 0.23 cm, 75.10 ± 0.63 cm, for those showing pattern **12** of and in those with pattern **13** the values for BW, BL, HAW and HG were 26.22 ± 0.38 kg, 71.94 ± 0.53 cm, 74.06 ± 0.27 cm, 76.69 ± 0.93 cm, respectively.

However, the differences obtained between the different SSCP variants of growth hormone gene in body weight, body length, height at withers and heart girth were not statistically significant ($P \leq 0.05$).

Table 10. Association of SSCP patterns (genotypes) of 5' regulatory region with BW, BL, HAW and HG at 12 months age

Genotype Or pattern	Body weight			Body length			Height at withers			Heart girth			Number of animals
	male	female	Overall	male	female	overall	Male	female	overall	Male	female	overall	
11	26.73± 0.24	25.33± 0.72	26.03± 0.42	74.06± 0.50	69.93± 0.80	72.00± 0.77	75.18± 0.37	73.90± 0.34	74.54± 0.30	79.76± 0.44	72.18± 0.26	75.97± 1.16	12
12	26.88± 0.53	24.98± 0.28	25.88± 0.35	72.78± 0.90	69.03± 0.77	70.81± 0.71	74.30± 0.56	74.13± 0.28	74.21± 0.29	79.44± 0.27	72.41± 0.37	75.76± 0.81	21
22	26.04± 0.37	25.68± 0.37	25.91± 0.32	73.45± 1.04	71.01± 0.95	71.18± 0.75	74.65± 0.45	73.44± 0.31	73.87± 0.29	80.23± 0.43	72.83± 0.49	75.44± 0.94	17

Means are not significantly different ($P \leq 0.05$)

Table 11. Association of SSCP patterns (genotypes) of exon-4 with BW, BL, HAW and HG at 12 months age

Genotype Or pattern	Body weight			Body length			Height at withers			Heart girth			Number of animals
	male	female	Overall	male	female	overall	Male	female	overall	Male	female	overall	
11	26.40± 0.39	25.79± 0.32	26.08± 0.25	73.72± 0.52	70.36± 0.74	71.93± 0.55	74.92± 0.40	73.85± 0.27	74.35± 0.25	79.72± 0.24	72.66± 0.31	75.96± 0.68	30
12	26.70± 0.55	25.05± 0.36	25.71± 0.35	72.58± 1.06	69.53± 0.71	70.75± 0.67	74.13± 0.40	73.75± 0.25	73.91± 0.21	79.77± 0.41	72.35± 0.40	75.32± 0.88	20

Means are not significantly different ($P \leq 0.05$)

Table 12. Association of SSCP patterns (genotypes) of exon-5 with BW, BL, HAW and HG at 12 months age

Genotype Or pattern	Body weight			Body length			Height at withers			Heart girth			Number of animals
	male	female	Overall	male	female	overall	Male	female	overall	Male	female	overall	
11	26.72+ 0.3	25.60+ 0.34	26.11+ 0.27	73.70+ 0.50	69.98+ 0.68	71.68+ 0.53	74.61+ 0.37	73.94+ 0.23	74.24+ 0.21	79.61+ 0.23	72.07+ 0.29	75.52+ 0.67	35
12	25.95+ 0.49	25.20+ 0.22	25.50+ 0.25	72.28+ 1.28	70.05+ 0.81	70.94 0.74	74.70+ 0.53	73.54+ 0.30	74.00+ 0.30	80.08+ 0.46	73.50+ 0.26	76.13+ 0.89	15

Means are not significantly different (P ≤ 0.05)

Table 13. Association of SSCP patterns (genotypes) of 3' UTR with BW, BL, HAW and HG at 12 months age

Genotype Or pattern	Body weight			Body length			Height at withers			Heart girth			Number of animals
	male	female	Overall	male	female	overall	Male	female	overall	Male	female	overall	
12	26.45± 0.36	25.31± 0.26	25.75± 0.23	73.62± 0.76	69.61± 0.68	71.16± 0.61	74.72± 0.44	73.93± 0.23	74.24± 0.23	79.23± 0.22	72.48± 0.33	75.10± 0.63	31
13	26.58± 0.56	25.82± 0.53	26.22± 0.38	72.94± 0.66	70.83± 0.72	71.94± 0.53	74.53± 0.40	73.55± 0.31	74.06± 0.27	80.36± 0.28	72.62± 0.34	76.69± 0.93	19

Means are not significantly different (P ≤ 0.05)

CHAPTER-V

DISCUSSION

Growth hormone (GH), which is a 191-amino acid, single polypeptide chain that is synthesized, stored and secreted by the anterior pituitary gland. This hormone being an anabolic hormone, promotes body growth by enhancing growth of muscles, bones and organs in the body. Studies on polymorphism of growth hormone gene had gained much importance in recent years in livestock due to its possible use for improvement of growth traits through selection. Nellore sheep is an important breed to improve the income of farmers through wool, meat and skin. As Nellore sheep had gained lot of attention in recent times for meat production, the present study i.e studies on growth hormone gene polymorphism in Nellore sheep through PCR-SSCP with body weight (BW), body length (BL), height at withers (HAW) and heart girth (HG) traits was carried out in Nellore sheep. However, polymorphism studies in ovine growth hormone gene and its association with body weights and morphometric traits are scanty.

5.1 Isolation of genomic DNA and determination of its quantity and quality

The blood samples (approximately 10 ml) were collected from jugular vein using EDTA (2.7%) vacutainer tubes aseptically. The good quality genomic DNA was isolated through phenol-chloroform extraction method of Sambrook *et al.* (1989) .The Purity and concentration of isolated DNA was evaluated based on OD values (260:280) through Nano drop. The quality of the DNA was checked for any shearing by agarose gel electrophoresis (0.8%) and appeared as a single band. The DNA was diluted with nuclease free water to make the concentration between 50-100 ng/µl and used as a template in PCR reaction.

5.2 PCR amplification of different regions of GH gene in Nellore sheep

In the present study, DNA template of each sample were used to amplify different segments i.e 5' regulatory region (210 bp), exon-4 (191 bp), exon-5 (480 bp) and 3' UTR (320 bp) of the growth hormone gene with optimized conditions for each specific primers (Table 1).

5.3 PCR-SSCP of different regions of GH gene in Nellore sheep

The PCR-SSCP technique was used to detect the polymorphisms in 5' regulatory region, exon-4, exon-5 and 3' UTR of growth hormone gene.

5.3.1 PCR-SSCP of 5' regulatory region of GH gene

PCR-SSCP analysis of amplified product of the 5' regulatory region (210 bp) of GH gene revealed presence of three conformational patterns **11, 12** and **22**.Out of 50 unrelated samples, twelve samples showed pattern **11**, twenty one samples showed pattern **12** and seventeen samples showed pattern **22** corresponding to two allelic variants **1** and **2**.

These findings are in agreement with previous studies by Jia *et al.* (2014) who detected three conformational patterns (AA, AB and BB) in Small Tail Han and Tibetan sheep. However, Marques *et al.* (2006) in “Serra da Estrela sheep” and Lan *et al.* (2007) in Xinong Sannen, Laoshan, Guanzhong, Shaannan White, Guizhou White, Boer and Angora goats reported four SSCP patterns,while Kumar *et al*. (2008) reported a still higher polymorphism with eight conformational patterns (AA, AB, AC, AD, AE, AF, AG and AH) in the promoter region of growth hormone gene in Sirohi goats.

5.3.2 PCR-SSCP of exon-4 of GH gene

PCR-SSCP analysis of amplified product of the exon-4 (191 bp) of GH gene revealed only two conformational patterns **11** and **12**. Out of 50 unrelated samples, thirty samples showed pattern **11** and remaining twenty samples showed pattern **12** with two allelic variants **1** and **2.**

Current results were in agreement with previous studies by Bastos *et al.* (2001), Marques *et al.* (2006) and Moradi *et al.* (2016) who detected two conformational patterns using the SSCP analysis of exon 4 of the GH gene in Portuguese indigenous sheep breed "Churra da Terra Quente", "Serra da Estrela sheep" and Lori-Bakhtiari sheep, respectively. Jia *et al.* (2014) and Shiri *et al.* (2006) also observed three conformational patterns for exon 4 of this gene in four sheep breeds in China (Small Tail Han, Tibetan, German Merino and Polled Dorset sheep) and in Kordian sheep, respectively, while Moradian *et al.* (2013) reported five genotypes in Makooei sheep for exon-4 of GH gene

However, the results obtained were not in agreement with the previous reports of An *et al*. (2010) in the four Chinese goat populations (Xinong Saanen, Boer, F1 and F2 generations of Boer X Guanzhong dairy goats) who reported no polymorphism in exon-4.

5.3.3 PCR-SSCP of exon-5 of GH gene

PCR-SSCP analysis of amplified product of the exon-5 (480 bp) of GH gene revealed two conformational **11** and **12** patterns. Among 50 samples, thirty five samples showed Pattern **11** and fifteen samples showed pattern **12** on 12% polyacrylamide gel corresponding to two allelic variants **1** and **2.**

Current results were in agreement with previous studies conducted by Farag *et al.* (2016) who detected two conformational patterns on SSCP analysis of exon-5 of the GH gene in two pure breeds (Barki and Rahmani) and one crossbred (Rahmani x Awase) sheep. However, Tahmoorespur *et al.* (2011) in Baluchi sheep, Azari *et al.* (2011) in Dalagh sheep and Yousefi and Azari (2012) in Zel sheep observed three conformational patterns, while Marques *et al.* (2006) observed five different conformational patterns in “Serra da Estrela sheep” in exon-5.

5.3.4 PCR-SSCP of 3' UTR of GH gene

PCR-SSCP analysis of amplified product of the 3' UTR (320 bp) of GH gene revealed presence of polymorphism with two conformational patterns on 12% polyacrylamide gel. The results have shown two different SSCP patterns **12** and **13** corresponding to three allelic variants **1, 2** and **3 .**Out of 50 samples, thirty-one samples presented pattern **12** and nineteen samples presented pattern **13**.

The Current findings showed 3' UTR (320 bp) is less polymorphic than previous studies by Jia *et al*. (2014) and Marques *et al.* (2006) who detected four conformational patterns in 3' UTR of the growth hormone gene in four sheep breeds in China (Small Tail Han, Tibetan, German Merino and Polled Dorset sheep) and “Serra da Estrela sheep”, respectively. Bahrami *et al.* (2015) also reported five genotypes in the segment covering exon-5 and part of 3' UTR of this gene in Mehraban sheep, while Wicramaratne *et al*. (2010) reported six distinct band patterns in osmanabadi and four patterns in sangamneri goats.

5.4 Allelic and genotypic frequency

5.4.1 Allelic and genotypic frequency of 5' regulatory region of GH gene

PCR-SSCP of 5' regulatory region yielded three SSCP patterns **11, 12** and **22** corresponding to two allelic variants **1** and **2.** The allelic frequency of **1** and **2** were 0.45 and 0.55, respectively and genotypic frequencies were 0.24, 0.42 and 0.34 in **11, 12,** and **22**, respectively (Table 8).

5.4.2 Allelic and genotypic frequency of exon-4 of GH gene

PCR-SSCP of exon-4 yielded two genotypic patterns **11** and **12** corresponding to two allelic variants **1** and **2**. The allelic frequency of **1** and **2** were 0.80 and 0.20, respectively and genotypic frequencies were 0.60 and 0.40, respectively for pattern **11** and **12** (Table 8). However, Bastos *et al*. (2001) reported genotypic frequency as 0.725 and 0.275 in pattern I and II, respectively in “Churra da Terra Quente” sheep.

5.4.3 Allelic and genotypic frequency of exon-5 of GH gene

PCR-SSCP yielded two conformational pattern **11** and **12** in exon-5 indicating polymorphism in Nellore sheep with two alleles **1** and **2.** The allelic frequency **1** and **2** were 0.85 and 0.15, respectively and genotype frequencies of **11** and **12** were 0.70, 0.30, respectively (Table 8).

Farag *et al.* (2016) reported two SSCP conformational patterns (I&II) .Pattern I was found to be more frequent (83.3, 92.86 and 90%) than II (16.7, 7.14 and 10%) in Barki, Rahmani and crossbred sheep, respectively.

5.4.4 Allelic and genotypic frequency of 3' UTR of GH gene

The PCR-SSCP of 3' UTR yielded two different conformational patterns corresponding to three allelic variants **1, 2** and **3**. The allelic frequencies of **1, 2** and **3** were 0.50, 0.31 and 0.19, respectively and genotype frequencies were 0.62, 0.38 for **12** and **13**, respectively (Table 8).

5.5 Body weight and morphometry

In the current study, the least square means obtained for body weight, body length, height at withers and heart girth were 25.93 ± 0.20 kg, 71.46 ± 0.43 cm, 74.17 ± 0.17 cm, 75.70 ± 0.53 cm, respectively at 12 months of age.

The overall mean for bodyweight (BW) in the present study was 25.93 ± 0.20 kg which was in accordance with findings of Rao *et.al* (2002) (25.61 ± 0.53 kg in Nellore jodipi), howeve, lower values were reported by Reddy *et.al* (2009) (24.67 ± 0.56 kg in Nellore sheep), Jalajakshi *et.al* (2017) (24.67 ± 0.20 kg in Nellore brown sheep), and higher values observed by Dass (2008) (29.74 ± 0.28 kg in Pugal sheep), Kumar *et.al* (2017) (27.41 ± 0.18 kg in Nellore jodipi) ,Reddy *et.al* (2017) (27.06 ± 0.03 kg in Nellore brown) at 12 months of age.

In current study, the overall mean for body length(BL) was 71.46 ± 0.43 cm ,which was in consistent with findings of Rani *et.al* (2014) (71.14 ± 0.50 cm in Nellore sheep) ,Yadav *et al.* (2014) (71.6 to 78.5 cm in Deccani sheep ecotypes) and slightly lower than Mandal *et.al* (2008) (72.10 ± 0.20 cm in Muzzafarnagari sheep). However, lower values reported by Dass (2008) (63.20 ± 0.43 cm in Pugal sheep), Ravimurugan & Devendran (2009) (63.00 ± 0.48 cm in Ramnad white), Thiruvenkadan *et al.* (2011) (66.00 ± 0.40 cm in Mecheri sheep), Vani *et al.* (2017) (66.64 ± 0.33 cm in Nellore brown sheep) at 12 months of age.

The overall mean for height at withers (HAW) in the present study was 74.17 ± 0.17 cm ,which was higher than Vani *et al.* (2017) (72.03 ± 0.29 cm in Nellore brown), Dass (2008) (62.88 ± 0.31 cm in Pugal sheep),Thiruvenkadan *et al.* (2011) (67.00 ± 0.40 cm in Mecheri sheep). The present findings found lower measurements than Rani *et al.* (2014) (76.91 ± 0.40 cm in Nellore sheep). However, Gopal and Hari (2007) reported the values (73–79 cm in Muzaffarnagari sheep), Yadav *et al.* (2014) (68.7 to 76.5 cm in Deccani sheep).

In a similar way, the overall mean for heart girth (HG) in the present study was 75.70 ± 0.53 cm were in the range reported by Yadav *et al.* (2014) (74.5 to 78.1 cm in Deccani sheep ecotypes), Ravimurugan *et al.* (2010) (75.28 ± 1.43 cm in Kilakarsal sheep). The present investigation values for same trait were higher than those reported by Vani *et al.* (2017) (73.97 ± 0.30 cm in Nellore brown sheep) and lesser than Gopal and Hari (2007) (79–81 cm in Muzaffarnagari sheep), Rani *et al.* (2014) (78.04 ± 0.49 cm in Nellore sheep).

5.7 Association of SSCP patterns of GH gene with body weight and morphometric traits

The current work was carried out to study the association of SSCP patterns of growth hormone gene with body weights and different morphometric traits at 12 months of age in Nellore brown sheep.

The overall mean BW, BL, HAW and HG of Nellore brown sheep with pattern **11** in 5' regulatory region were 26.03 ± 0.42 kg, 72.00± 0.77 cm, 74.54 ± 0.30 cm and 75.97 ± 1.16 cm while with pattern **12** were 25.88 ± 0.35 kg, 70.81 ± 0.71 cm, 74.21 ±0.29 cm and 75.76 ± 0.81 cm and with pattern **22** were 25.91 ± 0.32 kg, 71.18 ±0.75 cm, 73.87 ± 0.29 cm and 75.44 ±0.94 cm, respectively. The present findings showed slight variation in body weights and morphometry traits. However, Min *et al.* (2005) reported that AA genotype of this region had significantly higher body weight in Boer and Boer x LuBei white goats at one year age than AB and BB genotype, while Kumar *et al.* (2008) identified significant association of SSCP patterns in promoter region with birth weight and non-significant association with BW at 3, 6 and 9 months of age. Wickramaratne *et al.* (2010) also identified that the GT pattern was associated with heavy body weight and GG with low body weight while there was no association of these patterns in 5' promoter region with body length and chest girth in Osmanabadi and Sangamneri goats. Jia *et al.* (2014) observed statistically significant differences between the SSCP genotypes within the 5' regulatory region in the scores of body weight, body length, and heart girth in Tibetan sheep.

In exon-4, mean BW, BL, HAW and HG in pattern **11** were 26.08 ± 0.25 kg, 71.93 ± 0.55 cm, 74.35 ± 0.25 cm and 75.96 ± 0.68 cm and in pattern **12** were 25.71 ± 0.35 kg, 70.75 ± 0.67 cm, 73.91 ± 0.21 cm and 75.32 ± 0.88 cm, respectively. The present study shows slight variation in body weights and morphometry in pattern **11** and pattern **12**. However Moradian *et al.* (2013) found significant differences in weights at weaning, six months of age and nine months of age among the SSCP genotypes. Jia *et al.* (2014) in Tibetan sheep also identified statistically significant differences in the scores of body weight, body length, wither height and heart girth between the genotypes within exon-4 region, while Dayal *et al.* (2016) reported that AC pattern of this region had significantly higher body weight at both 6 and 9 months of age than the CC pattern in Black Bengal goat.

In case of SSCP in exon-5, the overall mean in pattern **11** of BW, BL, HAW and HG were 26.11 ± 0.27 kg, 71.68 ± 0.53 cm, 74.24 ± 0.21 cm, 75.52 ± 0.67 cm and in pattern **12** were 25.50 ± 0.25 kg, 70.94 ± 0.74 cm, 74.00 ± 0.30 cm, 76.13 ± 0.89 cm, respectively. The present study shows slight variation in BW and other morphometric traits between patterns obtained. Lan *et al. (*2007) study in exon-5 reported little influence of different patterns of growth hormone gene on weight in one-year-old animals in 5 indigenous sheep breeds (xinongsannen, laoshan, Guanzhong, Shaannan White, Guizhou White),Kumar *et al*. (2008) studied the correlation between polymorphism in exon-5 of the growth hormone gene and growth traits in Sirohi goats. The animals with FF variant in exon-5 regions had significantly higher body weights at birth, nine months and six months of age, respectively. Tahmoorespur *et al.* (2011) studied growth hormone gene polymorphism in exon-5 and reported a significant effect of polymorphism on 6 months body weight. However, Yousefi and Azari (2012) investigated the association between SSCP genotypes of exon-5 of GH gene and body weights in Zel sheep. The statistical analysis revealed no significant differences between the genotypes studied.

In case of SSCP in 3' UTR, the overall mean in pattern **12** of BW, BL, HAW and HG were 25.75 ±0.23 kg, 71.16 ± 0.61 cm, 74.24 ±0.23 cm, 75.10 ± 0.63 cm and in pattern **13** were 26.22 ± 0.38 kg, 71.94 ± 0.53 cm, 74.06 ± 0.27 cm, and 76.69 ±0.93 cm, respectively. The study shows pattern **13** had slight higher BW, HG than pattern **12**. Jia *et al.* (2014) observed statistically significant differences in the scores of body weight, body length, wither height and heart girth in Polled Dorset and Tibetan sheep and non-significant differences in Small Tail Han and German Merino sheep among the SSCP patterns.

As discussed above, the differences were observed between the SSCP variants in the different regions of GH gene in body weight, body length, height at withers and heart girth. But these differences were not found to be statistically significant ($P \leq 0.05$).

This investigation indicates that SSCP analysis and its association with body weights and morphometry is a valuable tool for the identification of genetic polymorphism in the GH gene. The SSCP patterns (various genotypes) obtained in the present study for various segments of growth hormone gene and their relationship with body weights and other morphometric traits at 12 months age did not show significant differences among genotypes studied. To establish the real connection between genotypes and various traits if any requires large population of wide genetic base with accurate data.

CHAPTER-VI

SUMMARY

The present investigation was carried out in Nellore brown sheep to study the polymorphism in growth hormone gene though PCR-Single Strand Conformation Polymorphism (SSCP) and to elucidate if there is any association with various morphometric traits, body weights at 12 month age. 50 unrelated animals of Nellore brown sheep were chosen from various institutional farms viz, Livestock Research station, Mamnoor, Instructional Livestock farm complex, Rajendranagar of P.V. Narsimha Rao Telangana Veterinary University and a few from Shamshabad farmers of Rangareddy district. The genomic DNA was extracted from whole blood by standard phenol-chloroform method. Up on verifying the purity, quantity and quality of genomic DNA it was used for PCR. Several portions (5' regulatory region, exon-4, exon-5 and 3' UTR) of growth hormone gene assumed to be polymorphic were amplified with several sets of primers with optimized PCR cyclic conditions and reactions mix in thermal cycler .

The length of the various amplicons was confirmed by agarose gel electrophoresis (2%) with standard DNA marker (100 BP ladder).The amplified PCR products were a 210 bp of 5' regulatory region, 191 bp of exon 4, 480 bp of exon 5 and 320 bp of 3’UTR. The PCR products were denatured at 95ºC mixed with formamide and elelctrophoresed in 12% polyacrylamide gel to resolve their SSCP patterns.

PCR-SSCP of 5' regulatory region yielded three SSCP patterns **11, 12** and **22** corresponding to two allelic variants **1** and **2.** The allelic frequency of **1** and **2** were 0.45 and 0.55, respectively and genotypic frequencies were 0.24, 0.42 and 0.34 in **11, 12,** and **22** respectively.

PCR-SSCP of exon-4 yielded two genotypic patterns **11** and **12** corresponding to two allelic variants **1** and **2**. The allelic frequency of **1** and **2** were 0.80 and 0.20, respectively and genotypic frequencies were 0.60 and 0.40, respectively for **11** and **12** pattern.

PCR-SSCP yielded two conformational patterns **11** and **12** in exon-5 indicating polymorphism in Nellore sheep with two alleles **1** and **2.** The allelic frequency **1** and **2** were 0.85 and 0.15 respectively, genotype frequencies of **11** and **12** were 0.70, 0.30 respectively.

PCR-SSCP of 3' UTR yielded two different conformational patterns corresponding to three allelic variants **1, 2** and **3**. The allelic frequencies of **1, 2** and **3** were 0.50, 0.31 and 0.19 respectively and genotype frequencies were 0.62, 0.38 for **12** and **13** respectively.

In the current study, the least square means obtained for body weight, body length, height at withers and heart girth were 25.93 ± 0.20 kg, 71.46 ± 0.43 cm , 74.17 ± 0.17 cm , 75.70 ± 0.53 cm, respectively at 12 months of age in Nellore brown sheep.

Association of SSCP patterns of GH gene with body weight and morphometric traits at 12 months of age was carried out. The variation in BW, BL, HAW and HG among the SSCP patterns was studied to find the association between Single Strand Conformation Polymorphism and the body measurements.

The overall mean BW, BL, HAW and HG with pattern **11** in 5' regulatory region were 26.03 ± 0.42 kg, 72.00 ± 0.77 cm, 74.54 ± 0.30 cm and 75.97 ± 1.16 cm while with pattern **12** were 25.88 ± 0.35 kg, 70.81 ± 0.71 cm, 74.21 ± 0.29 cm and 75.76 ± 0.81 cm and with pattern **22** were 25.91 ± 0.32 kg, 71.18 ± 0.75 cm, 73.87 ± 0.29 cm and 75.44 ±

0.94 cm, respectively. The findings showed slight variation in body weights and morphometry traits in 5' regulatory region.

In exon-4, overall mean BW, BL, HAW and HG in pattern **11** were 26.08 ± 0.25 kg, 71.93 ± 0.55 cm, 74.35 ± 0.25 cm and 75.96 ± 0.68 cm and in pattern **12** were 25.71 ± 0.35 kg, 70.75 ± 0.67 cm, 73.91 ± 0.21 cm and 75.32 ± 0.88 cm, respectively. The present study clearly reveals less variation in BW, BL, HAW and HG in pattern **11** compared to pattern **12**.

In exon-5, the overall mean in pattern **11** of BW, BL, HAW and HG were 26.11 ± 0.27 kg, 71.68 ± 0.53 cm, 74.24 ± 0.21 cm, 75.52 ± 0.67 cm and in pattern **12** of BW, BL, HAW and HG were 25.50 ± 0.25 kg, 70.94 ± 0.74 cm, 74.00 ± 0.30 cm, 76.13 ±0.89 cm, respectively. The present study shows slight variation in BW and other morphometric traits between patterns obtained.

In 3' UTR, the overall mean in pattern **12** of BW, BL, HAW and HG were 25.75 ±0.23 kg, 71.16 ± 0.61 cm, 74.24 ± 0.23 cm, 75.10 ± 0.63 cm, and in pattern **13** BW, BL, HAW and HG were 26.22 ± 0.38 kg, 71.94 ± 0.53 cm, 74.06 ± 0.27 cm, 76.69 ± 0.93 cm, respectively. The study shows pattern **13** had slight higher BW, HG than pattern **12**.

The present investigation through PCR-SSCP shows 5' regulatory region, exon-4, exon-5 and 3' UTR of growth hormone gene in Nellore sheep is polymorphic. It indicates SSCP analysis is a valuable tool for the identification of genetic polymorphism in all the regions studied.The SSCP patterns (various genotypes) obtained in the present study for various segments of growth hormone gene and their relationship with body weights and other morphometric traits at 12 months age did not showed significant differences among gentypes.To establish the real connection between genotypes and various traits if any requires large population of wide genetic with accurate data.

However, as the studies on Single Strand Conformation Polymorphism and its association with BW and morphometric traits in Nellore sheep were not reported earlier and the present study was based on a limited sample size, further studies may be needed to confirm the association.

CHAPTER-VII

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APPENDIX – I

REAGENTS FOR THE DNA ISOLATION

RBC lysis buffer

1.6M Sucrose	20 ml
0.1M Tris (pH 7.5)	1 ml
0.5M $MgCl_2$	1 ml
Triton X- 100	1 ml
Sterile double distilled Water	up to 100 ml

1.6 M Sucrose

Sucrose	87.54 g
Distilled water	100 ml

0.1M Tris (pH 7.5)

Tris (1M)	1.21 g
Distilled water	100 ml

0.5M MgCl2

$MgCl_2$ (1M)	2.38 g
Distilled water	50 ml

DNA Extraction buffer

1M Tris (pH 8.0)	1 ml
5M Nacl	8 ml
0.5M EDTA (pH 8.0)	0.4 ml
Distilled water	up to 500 ml

1M Tris (pH 8.0)

Tris	12.11 g
Distilled water	100 ml

5M NaCl

NaCl	14.6 g
Distilled water	50 ml

0.5M EDTA

EDTA	18.61 g
Distilled water	100 ml

10% SDS

SDS	10 g
Distilled water	100 ml

3 M Sodium acetate (pH 5.2)

Sodium acetate	20.4 g
Distilled water	50 ml

APPENDIX – II

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

5x TBE Electrophoresis buffer

Tris base	54 g
Boric acid	27.5 g
EDTA	4.15 g
Distilled water	up to 1000 ml

Agarose (2%)

Agarose	2 g
TBE (1X)	100 ml

Ethidium Bromide

Ethidium Bromide	100 mg (2.5 mg/ml)
Distilled water	1 ml

6X Gel loading buffer

Bromophenol blue	0.0025 g
Sucrose	4 g
Distilled water	10 ml

APPENDIX- III

SINGLE STRAND CONFORMATION POLYMORPHISM

Preparation of Formamide dye

Formamide (95%)	9.5 ml
Bromophenol blue (0.025%)	25 mg
Xylene cyanol (0.025%)	25 mg
0.5M EDTA	400 µl
Distilled water	100 µl

Preparation of solutions/reagents for PAGE (12%)

Acrylamide: Bisacrylamide (49:1) solution

Acrylamide	49 g
Bisacrylamide	1 g
Distilled water	100 ml

Ammonium persulfate (APS) 10%

Ammonium persulfate	0.1 g
Distilled water	1 ml

Polyacrylamide gel (12%)

Acrylamide: Bisacrylamide (49:1)	9.6 ml
Glycerol	1.5 ml
APS (10%)	100 µl
TEMED	75 µl
1x TBE	28.3 ml

Preparation of solutions/reagents for silver-staining

Fixative / stop solution (10%)

Ethanol	50 ml
Distilled water	up to 500 ml

Oxidative solution (1%)

Nitric acid	5 ml
Distilled water	up to 500 ml

Silver-staining solution (0.1%)

Silver nitrate	0.5 g
Distilled water	up to 500 ml

Developer solution

Sodium carbonate	15 g
Formaldehyde	750 µl
Distilled water	500 ml