A Comparative Study on Expression Profile of HSP genes during different Seasons in Goat breeds

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COLLEGE OF BIOTECHNOLOGY

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Mathura-281001 (UP)

(2016)

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This is to certify that the thesis entitled "**A Comparative Study on Expression Profile of HSP genes during different Seasons in Goat breeds**" submitted by **Mr. Devendra Kumar**, Enrolment No. **B-1386/14** in partial fulfillment of the requirement for the award of **Master of Science** in **Biotechnology** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (U.P.)**, India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

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Place: Mathura

Date: ……. /……. /……. **(Devendra Kumar)**

ABBREVIATIONS

%	:	Percentage
&	:	And
<	:	Less than
=	:	Equal
>	:	More than
×g	:	Relative Centrifugal Force
≤	:	Less than equal to
≥	:	More than equal to
°C	:	Degree Celsius
°F	:	Degree Fahrenheit
µ	:	Micro
µg	:	Microgram
µM	:	Micro molar
1X	:	One time
A	:	Adenine
ADP	:	Adenosine di-phosphate
ATP	:	Adenosine tri-phosphate
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
BSS	:	Balanced salt solution
C	:	Cytosine
cDNA	:	Complimentary deoxy-ribonucleic acid
cm	:	Centimeter
Conc.	:	Concentration
Cp	:	Crossing point

Ct	:	Cycle threshold
CTR	:	Control
DEPC	:	Di ethyl pyro carbonate
DNA	:	De-oxy ribonucleic acid
DNase	:	De-oxy ribonucelease
dNTP	:	De-oxy nucleotide triphosphate
DPBS	:	Dulbacco's Phosphate Buffer Saline
EDTA	:	Ethylene di amine tetra acetic acid
ELISA	:	Enyme linked immuosorbent assay
ER	:	Endoplasmic reticulum
et al	:	Et alia
EtBr	:	Ethidium bromide
etc.	:	Et cetera
EU/mL	:	Endotoxin unit per milliliter
Fig.	:	Figure
For.	:	Forward
G	:	Guanine
g/L	:	Gram per liter
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
gm	:	Gram
gm/mL	:	Gram per milliliter
HCl	:	Hydrochloric acid
hrs	:	Hours
HSE	:	Heat shock elements
HSF	:	Heat shock factor
HSPs	:	Heat shock proteins
htpG	:	high temperature protein G

i.e.	:	That is
kb	:	Kilo base pair
kD	:	Kilodalton
KH_2PO_4	:	Potassium di-hydrogen phosphate
kPa	:	kilopascal
LPS	:	Lipopolysaccharide
ltr	:	Litre
M	:	Molar
mA	:	Milliampere
Max.	:	Maximum
mg	:	Milligram
Mg^{++}	:	Magnesium divalent ion
min	:	Minute
Min.	:	Minimum
ml	:	Milliliter
mM	:	Millimolar
mm	:	Millimeter
mmHg	:	Millimeter of mercury
mRNA	:	Messenger Ribo-nucleotide
Na_2HPO_4	:	Di-sodium hydrogen phosphate
NaCl	:	Sodium Chloride
NaOH	:	Sodium hydroxide
NCBI	:	National centre for biotechnology information
NFW	:	Nuclease Free Water
ng	:	Nanogram
ng/µl	:	Nanogram per microliter
nmol	:	Nanomole

NOS	:	Nitric Oxide Synthase
NTC	:	No template control
OD	:	Optical Density
PBD	:	Peptide binding domain
PBMCs	:	Peripheral blood mononuclear cells
PBS	:	Phosphate Buffer Saline
PCR	:	Polymerase Chain Reaction
pg	:	Picogram
pH	:	Hydrogen ion concentration
pmol	:	Pico mole
psi	:	Pounds per square inch
qPCR	:	Quantitative polymerase chain reaction
Rev.	:	Reverse
RNase	:	Ribonuclease
rpm	:	Rotation Per Minute
RR	:	Respiratory rate
rRNA	:	Ribosomal ribonucleic acid
RT	:	Rectal temperature
RT-PCR	:	Reverse Transcription Polymerase Chain Reaction
SD	:	Standard deviation
sec	:	Second
SEM	:	Standard error of mean
ß	:	Beta
T	:	Thymine
TAE	:	Tris Acetate EDTA
TBE	:	Tris Borate EDTA
THI	:	Temperature humidity index

TLR	:	Toll like receptors
Tm	:	Melting Temperature
Tris	:	Tris (hydroxyl methyl) Amino Ethane
U	:	Unit
UBQ	:	Ubiquitin
UV	:	Ultra Violet
V	:	Volt
V/cm	:	Volt per centimeter
V/V	:	Volume by Volume
viz.	:	Videlicet
W/V	:	Weight by Volume
α	:	Alfa
Δ	:	Delta
µg/ml	:	Microgram per milliliter
µl	:	Microliter

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CHAPTER-I

INTRODUCTION

Goats the "poor man's cow" are versatile animal, playing an important role in the livestock industry by fulfilling the economic, cultural, agricultural and even religious needs of human beings since very early period of human civilization (Mac Hug and Bradley, 2001). These are the most adaptable and widespread livestock species, ranging from the high altitude of Himalayas to that of deserts of Rajasthan and humid coastal areas of India (Rekib, 1998). The socio-economic values of goats rearing context to the livelihood security of the poor farmers are imperative than that of other livestock. Goats raised in semi-arid environmental conditions have to confront with multiple stressors (Sejian *et al.*, 2012 Chech reference) owing to extreme fluctuations in availability of quality and quantity of feed throughout the year (Martin *et al*., 2004). Goats can survive in all types of agro-climatic conditions by adapting the harsh environmental condition and could perform better than other larger domestic ruminants (Sejian *et al*., 2012; Devendra, 1990; King, 1983; Shkolnik and Silanikove, 1981).

The low input, high fecundity, easy marketing and unprejudiced social acceptance of the goat's products are few of many advantages of this enterprise that provides assured higher income. The climatic stress partitions the body resources including protein and energy at the cost of decreased growth, reproduction, production and health status. In the prevailing situation of progressive shrinkage of grazing resources, under nourishment, feed and water scarcity, inhumane transportation, handling and slaughter practices, frequent draught and famine, long distance migration, poor *en route* shelter, early disposal of kids and distress sale of animals are some of the important issues vexing stress to small ruminants and therefore affecting their productive competence. Environmental factors such as ambient temperature, solar radiation and humidity has direct and/or indirect effects on animal performance. A portion of the metabolizable energy used for production is diverted to assure thermal balance under uncomfortable environmental conditions. Therefore, livestock production could be affected by climate change in which heat stress is the major cause of production loss.

Climate change projections suggested that environmental temperature is expected to increase between 2.3 and 4.8°C globally by 2100 (IPCC, 2007). It has been estimated that global warming could reduce animal productivity by 25% in tropical and subtropical countries which accounts for more than half of the milk and meat production (Seguin, 2008). Exposure

to an elevated ambient temperature negatively affects the biological functions reflecting the impaired productive and reproductive traits (Marai *et al*., 2007).

The lower critical temperature for goats is not specified but the limits of thermo-neutrality for goats may be taken as a climatic environment having an air temperature of 13°C -27°C, relative humidity of 60-70 per cent and wind velocity of 5-8 Km/hr and a medium level of solar radiation (Mishra, 2009). In Indian subcontinent, heat stress is the most important climatic stress which adversely affects survival and productivity of livestock (Sejian *et al*., 2012). Several physio-biochemical responses are activated to counteract the effects of heat and cold stress and maintaining the homeostasis. Chemical pathways activated by different stressors result into either survival and adaptation or apoptosis of the cells depending its competence to the stressor (Buchman *et al*., 1993 and DeMeester *et al*., 2001). Stress represents the reaction of the body to stimuli that disturbs the normal physiological equilibrium or homeostasis, often with detrimental effects. Stress is revealed by the inability of an animal to cope with its environment, a phenomenon that is often reflected in the failure to achieve genetic potential for production traits (Dobson and Smith, 2000).

Thermal stress stimulates sort of complex responses which are fundamental for the cell survival (Sonna *et al*., 2002). Particularly in mammals, exposure to hypothermia or hyperthermia has been related to morphological and physiological modifications. Heat and other stressors cause the formation of protein aggregates that not only lacking native activity, but might cause direct damage to cellular membranes (Rochet *et al*., 2004). Protein aggregates can also seed and propagate misfolding and aggregation to other labile or aggregation-prone proteins in the cell (Gidalevitz *et al*., 2006). Wu (1995) reported that dramatic up regulation of the heat shock proteins (HSPs) is a key part of the heat shock response. Increased expression of HSPs is one of the most conserved stress response mechanisms. The synthesis of HSPs result during heat stress (Lindquist and Craig, 1988) and these HSPs protect cells from toxic effects of heat and other stressors (Pockley, 2001). The induction of HSPs is remarkably rapid and intense, as an emergency response.

HSPs are multigene families that range in molecular size from 10-150 kD and found in all major cellular compartments and named according to the molecular weight. For example Hsp60, Hsp70 and Hsp90 refer to families of HSPs on the order of 60, 70 and 90 kD in size, respectively (Li and Srivastava, 2004). They are highly conserved proteins present in all the cells of living organisms and are essential for cellular viability as these have major physiological roles in protein homeostasis (Ellis and Vander vies, 1991). Most of these proteins are ATPases and their general role is to regulate structure and quality of other proteins in cells. Stress proteins have ability to scrutinize various protein structures in cells

(Ellis, 2006). These proteins can distinguish between unfolded, misfolded and native proteins. The HSPs regulates the folding and unfolding of other proteins. HSPs interfere with several heat shock processes within cell organelles and proper functioning which are translocated to different compartments following stress induced synthesis. The increased expression of HSPs has also been attributed to the accumulation of abnormal cellular proteins associated with various diseases (Gao and Hu, 2008). Hsp70 is the major inducible member of the heat shock protein family. The levels of Hsp70 in cells have been correlated with tolerance to a wide variety of stresses which include environmental insults such as heat shock, heavy metals (Wagner *et al*., 1999), osmotic stress (Kurz *et al*., 1998) as well as physiologic stresses such as ischemia (Nowak *et al*., 1990; Kumar and Tatu, 2000) and oxidative stress.

These proteins have been extensively studied earlier and purified in most of the livestock species and different breeds of bovines (Kristensen *et al*., 2004; Lacetera *et al*., 2006). Adaptations to tropical climates, zebu breeds (*Bos indicus*) of cattle are able to better regulate body temperature in response to heat stress than European breeds (*Bos taurus*) (Beatty *et al*., 2006; Mehla *et al*., 2013). The expression level of HSPs was highest in buffaloes followed by Hostein-Friesian and Sahiwal cows during the heat stress (Kishore *et al*., 2013). Recombinant HSP70 protein may be used for the development of an assay for detection of thermal stress (Pawar *et al*., 2012). Higher expression of HSP90 has been demonstrated Sahiwal cows as compared to Frieswal cows under the both *in vitro* and in vivo heat stress (Deb *et al*., 2014 Chech reference). Zebu cattle (Tharparkar) are more adapted to tropical climatic condition than crossbreed cattle (Karan-Fries) (Singh *et al*., 2014).

Dangi *et al.,* (2012) demonstrated the expression of HSP60, HSP70, HSP90, and UBQ in peripheral blood mononuclear cells (PBMCs) during different seasons in three different age groups of goats of tropical and temperate regions. Sharma *et al*., (2013) reported an increased expression of HSP60 gene after administration of melatonin in heat stressed goats. HSP70 was reported to be the most sensitive and bi-phasic in response to temperature fluctuation in goats (Dangi *et al*., 2015). Paul *et al*., (2014) reported a possible correlation between TLR and HSP genes for playing a modulatory role in activation of immune system to combat the deleterious effect of thermal stress. Yadav *et al*., (2016) found that NOS family genes along with HSPs genes maintain the cellular integrity and homeostasis during thermal stress. Banerjee *et al*., (2013) analyzed the relative expression profile of HSP70 genes (HSPA8, HSPA6, HSPA1A, HSPA1L & HSPA2) and found that during summer, the relative expressions of all the HSP70 genes were higher in cold-adapted breeds (Gaddi and Chegu) than the heat-adapted breeds (Sirohi and Barbari) of goat.

India is the repository of 24 well defined goat breeds which are specific to a particular geographical location. In semiarid region many goat breeds are being reared by the farmers

viz. Sirohi, Jakharana, Barbari etc. Albeit, based on physio-biochemical parameters the adaptability of these breeds to semi-arid regions has been examined; however there is no gene expression-based study validating the adaptability of these breeds in various seasonal conditions. As the role of HSPs gene regulation in response to heat stress in goats is well documented but the comparative expressions of HSPs in indigenous goat breeds have not been yet utilized to study the adaptability in adverse climatic conditions. Therefore, the present study was intended with the following objective:

1. To examine the comparative expression profile of HSPs (HSP60, HSP70 and HSP90) during different seasons in goat breeds.

CHAPTER-II

REVIEW OF LITERATURE

2.1 CLIMATE CHANGE

Earth's average temperature has risen by 1.4 °F over the past century, and is projected to rise another 2.0 to 11.5 °F over the next hundred years. High ambient temperatures, direct and indirect solar radiation and humidity are some environmental stressing factors that impose strain on animals. The responses of animals to changes in environmental temperature emphasize the key difference between ruminant and non ruminant species in their comfort zones. Ruminants have wide comfort zones and a high degree of thermal tolerance so it is likely that climate resulting in an increase of a few degrees is not going to have any major effect on these animal performances (Al-Tamimi, 2007).

Despite having well developed mechanisms of thermo-regulation, ruminants do not maintain strict homeothermy under heat stress. There is unequivocal evidence that hyperthermia is deleterious to productivity, regardless of breed and stage of adaptation. The internal readjustment to maintain homeostasis in the face of external temperature changes is called an adaptation to the thermal environment. Among the domestic ruminant species, goats are the best adapted to harsh hot environments.

2.2 BODY DEFENSE AGAINST STRESS

2.2.1 Physiological mechanism

Respiration rate, pulsation rate and rectal temperature are the parameters which illustrate the mechanism of physiological adaptation. Several researchers studied physiological adaptation mechanisms such as rectal temperature, pulse rate and respiration rate in small ruminants (Sevi et al., 2001; Srikandakumar et al., 2003; Maurya et al., 2004; Marai et al., 2007; Otoikhian et al., 2009; Phulia et al., 2010, Sharma et al. 2013). Body temperature is good measure of heat tolerance in animals and represents the resultant of all heat gain and heat loss processes of the body. Rectal temperature is considered as a good index of body temperature even though there is a considerable variation in different parts of the body core at different times of the day (Srikandakumar et al., 2003). Rectal temperature of goats was found to be elevated with high environmental temperature in several studies (Devendra, 1987; Marai et al., 2007). Rectal temperature is considered as a good index of

body temperature even though there is a considerable variation in different parts of the body core at different times of the day (Srikandakumar et al., 2003). Rectal temperature of goats was found to be elevated with high environmental temperature in several studies (Devendra, 1987; Marai et al., 2007). Phulia et al., (2010) reported increase in rectal temperature and respiration rate from 38.97 °C and 43.66 to 39.35°C and 77.33 respectively when goats were kept for 6 hours in hot ambient temperature in summer. Pulsation and respiration rate per minute was found to be increased by the effect of environmental temperature. Heat loss via high respiration rate was reported as higher than that via other ways (Devendra, 1987). Increase in heart rate and pulse rate is attributed to two causes. One is the increase in muscular activity controlling the rate of respiration, concurrent with elevated respiration rate. Higher values of means of these parameters (RR, RT, HR) have been reported than that of values in thermo-neutral zone (Mc Dowell and Woodward, 1982; Al-Tamimi, 2007).

2.2.2 Molecular mechanism

It is widely accepted that changes in gene expression are an integral part of the cellular response to thermal stress. Some of these genes are affected by a wide variety of different stressors and probably represent a nonspecific cellular response to stress, whereas others may eventually found to be specific to certain types of stress. This response characteristically includes an increase in thermotolerance (i.e., the ability to survive subsequent, more severe heat stresses) that is temporally associated with increased expression of HSPs. Thermal-induced changes in gene expression occur both during hyperthermia as well as hypothermia. Commonly heat shock response is the induction of a group of proteins which were first termed as heat shock proteins because of their initial discovery in cells exposed to elevated temperatures. Ritossa in 1962 first observed cellular stress response in *Drosophilla buskii* salivary glands, which were exposed to high temperature and induced dramatic alterations in gene activity as judged by the changes in 'puffing' patterns observed in the salivary gland polytene chromosomes and similar was the finding with dinitrophenol or sodium salicylate treatment. Tissieres and Mitchell (1974) reported that the induction of these puffs coincided with the synthesis of small number of new proteins. It is now known that these small proteins are called heat shock proteins.

2.3 HEAT SHOCK PROTEINS (HSPs)

HSPs synthesis in response to thermal or chemical stress and the degree of conservation of HSPs at the level of nucleotide sequence and protein function have resulted in an emphasis on the conserved nature of the heat shock response (Nover and Scharf, 1991). Increased diversity of HSPs as well as accumulation of larger amounts of HSPs may contribute to survival in thermally stressful environments. Differences in HSP expression

have been found in closely related organisms which occupy different environmental niches. Several HSP families have been identified as molecular chaperones. Chaperones associate transiently with unfolded polypeptides, preventing premature folding of nascent polypeptide chains or aggregation of unfolded proteins, thus facilitating proper folding and or assembly of these polypeptides (Ellis, 1990). Different chaperones are found in different sub cellular compartments and cooperate in the translocation of proteins across intracellular membranes, a process which requires the translocated polypeptide to be maintained in an unfolded, extended configuration until it reaches the proper compartment. Constitutively expressed forms of HSP60, HSP70, and HSP90 perform this function in unstressed cells (Hendrick and Hart, 1993). Small HSPs, in the 20-30 kD range, have also been shown to function as molecular chaperones *in vitro* (Jakob *et al*., 1993). There is evidence that heat and other stressors cause the accumulation of damaged proteins, resulting in the induction of additional HSP synthesis (Edington *et al*., 1989; Hightower, 1991). Induced HSPs could also function as chaperones, facilitating the refolding of damaged proteins or targeting them for degradation. Thus HSPs would be a critical component of cellular defenses against stress induced proteotoxicity, a term applied to the deleterious effects of proteins damaged by chemical and physical stressors on cells.

HSPs are a large protein family consisting of both constitutively expressed and inducible proteins (Locke, 1997). HSPs are highly conserved cellular stress proteins present in every organism from bacteria to man (Neuer *et al*., 2000). They are conserved in protein coding as well as regulatory sequences (Pelham, 1982). *In vivo* and *in vitro* studies have shown that various stressors transiently increase production of HSPs as protection against harmful insults. HSPs level increased after environmental stresses, infection, normal physiological processes and gene transfer (Kiang and Tsokos, 1998). When denatured proteins were injected into frog oocytes, there was induction of HSP genes but normal proteins failed to induce HSP. Abnormal proteins act as eukaryotic stress signals and trigger the activation of heat shock genes (Ananthan *et al*., 1986). The production of large amounts of abnormal proteins activates transcription of HSPs in *E. coli* (Goff and Goldberg, 1985). The HSPs have been extensively studied, especially with regard to their cellular localization, regulation and functions (Lindquist and Craig, 1988; Hightower, 1991; Welch, 1992; Morimoto *et al*., 1994). HSPs are a group of evolutionarily conserved proteins that are conventionally, classified according to molecular size ranging from 10 to 150 kDa (Benjamin and McMillan, 1998). Cellular stress disturbs the tertiary structure of proteins and has adverse effect on cell metabolism. A number of studies have shown that HSP confers protection against cellular stresses including hyperthermia, hypoxia, ischemia and reperfusion which would otherwise lead to cell death (Liu and Steinacker, 2001).

2.3.1 FUNCTIONS OF HSPs

HSPs act as molecular chaperones by participating in proteins assembly without being part of the final protein structure (Ellis, 1987). HSPs contribute to cell survival by reducing the accumulation of damaged or abnormal polypeptides within cells (Parsell and Lindquist, 1993; Welch, 1992). They possess crucial role in intracellular transport, the maintenance of proteins in an inactive form and the prevention of protein degradation (Neuer *et al.,* 2000). They possess important protective role *in vivo* as they can protect heart and brain against ischemia and lungs and liver against sepsis (Marja, 1999). HSPs play crucial roles in cell cycle control, signaling and protection of cells against apoptosis (Zihai and Pramod, 2003). HSP 27, HSP 70 and HSP 90 proteins are predominantly antiapoptotic, whereas, HSP 60 is proapoptotic (Garrido *et al*., 2001). During the period of hyperthermia and shortly thereafter, HSPs become the predominant proteins synthesized by cells (Lindquist, 1986). Interestingly, most HSP genes lack introns (Lindquist,1986), which may facilitate their rapid expression and which may also help explain how they can be expressed in the presence of stressors (such as heat) that can interfere with RNA splicing.

2.3.2 HSPs: principal biochemical activities

a) HSPs help to prevent misaggregation of denatured proteins and assist the refolding of denatured proteins back into native conformations. Even in unstressed cells, some of the chaperonin HSPs play a role in the folding of nascent polypeptides into native conformations during protein synthesis. Additionally, their ability to stabilize proteins in specific conformations is used by a variety of normal cellular regulatory processes, such as cell cycle control, steroid and vitamin D receptor processing, and antigen presentation by cells with immune function. The prototypical chaperonin HSPs are the members of the HSP40, HSP60, HSP70 and HSP90 families of proteins.

b) HSP32R is the best example of regulation of cellular redox state, which, better known as heme oxygenase-1(HO-1) (Otterbein and Choi, 2000). This enzyme catalyzes the breakdown of heme to biliverdin, carbon monoxide and free iron (which is rapidly incorporated into ferritin). Biliverdin is subsequently converted to bilirubin, a potent antioxidant with cytoprotective effects. The release of free iron by HO-1 also leads to increased expression of ferritin, which is thought to exert its cytoprotective effect in part by sequestering prooxidant free iron (Otterbein and Choi, 2000).

c) Regulation of protein turnover (Parsell and Lindquist, 1993) an example is ubiquitin, which is expressed in unstressed cells, upregulated by heat shock and serves as a molecular tag to mark proteins for degradation by proteosomes.

2.3.3 TYPES OF HEAT SHOCK PROTEINS

HSPs are named according to their molecular weight. For example HSP60, HSP70 and HSP90 refer to families of HSPs on the order of 60, 70 and 90 kD in size, respectively (Li and Srivastava, 2004. (Table 1)

Table 1: Types of heat shock proteins

Size (kD)	Prokaryotic proteins	Eukaryotic proteins	Functions
10 kD	GroES	HSP10	Acts as tag for degradation of proteins like ubiquitin
20-30 kD	GrpE	HSPB group of HSP Eleven members in mammals including HSP27 or HSPB1	Assist protein folding without being the final part
40 kD	DnaJ	HSP40	Co-factor of HSP70
60kD	GroE, 60kDa antigen	HSP60	Involved in protein folding after its post-translational import to the mitochondrion/chloroplast
70kD	DnaK	The HSPA group of HSP including HSP 71, HSP70, HSP72, Grp78 (BiP), Hsx70 found only in primates	Protein folding and unfolding, Provides thermo-tolerance to cell on exposure to heat stress. Also prevents protein folding during post-translational import in to the mitochondria/chloroplast
90 kD	HtpG, C62.5	The HSP group includes HSP90, Grp94	Assist in the maturation of a select clientele of proteins and proper folding of proteins
100 kD	ClpB, ClpA, ClpX	HSP104, HSP110	Tolerance of extreme temperature

(Trivedi, 2010)

2.3.3.1 HEAT SHOCK PROTEIN 60 (HSP60)

Mammalian HSP60 was first reported as a mitochondrial P1 protein (Gupta, 1995). The amino acid sequence showed a strong homology to GroEL/HSP60's bacteria homolog. HSP60 in eukaryotes is considered typically a mitochondrial chaperone (also called Cpn60) which also occurs in the cytosol, the cell surface, the extracellular space and in the peripheral blood under normal physiological conditions (Cappello *et al*., 2008; Itoh *et al*., 2002). In

order for HSP60 to act as a signal it must be present in the extracellular environment. Chaperonin 60 can be found on the surface of various prokaryotic and eukaryotic cells, and can even be released from cells (Ranford *et al*., 2000). HSP60 has the capability of activating monocytes, macrophages and dendritic cells and also of inducing secretion of a wide range of cytokines (Hansen *et al*., 2003). HSP60 constitutes 15-30% of cellular proteins (Urushibara *et al*., 2007). Under normal physiological conditions, HSP60 is a 60 kD oligomer composed of monomers that form a complex arranged as two stacked heptameric rings (Cheng *et al*., 1990). This double ring structure forms a large central cavity in which the unfolded protein binds via hydrophobic interactions (Fenton *et al*., 1994). This structure is typically in equilibrium with each of its individual components: monomers, heptamers, and tetradeceamers (Habich and Burkart, 2007). Each subunit of HSP60 has three domains: the apical domain, the equatorial domain, and the intermediate domain. The equatorial domain contains the binding site for ATP and for the other heptameric ring. The intermediate domain binds the equatorial domain and the apical domain together (Ranford *et al*., 2000). It catalyzes the folding of proteins destined for the matrix and maintains protein in an unfolded state for transport across the inner membrane of the mitochondria (Koll *et al*., 1992). On the basis of all these studies it has been suggested that HSP60 provides a surface or workbench which binds to unfolded proteins, and through a series of ATP hydrolysis events, result in the proper folding of target polypeptide (Lubben *et al*., 1990).

2.3.3.2 HEAT SHOCK PROTEIN 70 (HSP70)

HSP70s are a family of ubiquitously expressed heat shock proteins. It is found in prokaryotes and eukaryotes (Tavaria *et al*., 1996; Yoshimune *et al*., 2002) and is mainly localized in the cytosol, mitochondria and endoplasmic reticulum and exhibit constitutive and inducible regulation. HSP70 gene family in bovines includes HSP70-1, HSP70-2, HSP70-3, and HSP70-4 gene. HSP70-1 is an intronless gene located on chromosome 23 and has 1926 nucleotides in goats (Gade *et al*., 2010). Molecular chaperones of HSP70 family are also essential for the cell to survive environmental stress including heat shock. Members of HSP 70 family are strongly upregulated by heat stress. HSP70 family is structurally and functionally conserved in evolution.

HSP 70 contains two distinct functional regions, a peptide binding domain (PBD) and the amino-terminal ATPase domain (ABD). Peptide binding domain contains a groove with an affinity for neutral, hydrophobic amino acid residues. Amino terminal/C-terminal domain – rich in alpha helical structure acts as a ‘lid’ for the substrate binding domain. When an HSP70 protein is ATP bound, the lid is open and peptides bind and release relatively rapidly. When HSP70 proteins are ADP bound, the lid is closed, and peptides are tightly bound to the substrate binding domain. Under normal conditions, HSP70 functions as ATP dependent

molecular chaperon that assist the folding of newly synthesized polypeptides, the assembly of multi protein complexes and the transport of proteins across cellular membranes. Under stressful conditions, elevated HSP70 levels allow cells to cope with increased concentrations of unfolded or denatured proteins (Panjwani *et al*., 1999).

2.3.3.3 HEAT SHOCK PROTEIN 90 (HSP90)

HSP90 is a molecular chaperone and is one of the most abundant proteins expressed in cells (Csermely *et al*., 1998). It is highly conserved and expressed in a variety of different organisms from bacteria to mammals – including prokaryotic analogue htpG (high temperature protein G) (Chen *et al*., 2006). It has been identified in the cytosol, nucleus and endoplasmic reticulum, and is reported to exist in many tissues (Kunisawa and Shastri, 2006).

There are two isoform of HSP90 in mammalian cells – HSP90α and HSP90ß. Recently, a membrane associated variant of cytosolic HSP90, lacking an ATP binding site, has been identified and was named as HSP90N (Grammatikakis *et al*., 2002). It consists of four structural domains (Pearl and Prodromou, 2000, 2001; Prodromou and Pearl, 2003) such as a highly conserved N-terminal domain in which crystal structures are available (Stebbins *et al*., 1997; Prodromou *et al*., 1997). A "charged linker" region that connects the N-terminus with the middle domain and middle domain is involved in client protein binding. It also increases the ATPase activity of HSP90. The C-terminal domain possesses an alternative ATP-binding site, which becomes accessible when N-terminal Bergerat pocket is occupied (Meyer *et al*., 2003; Panaretou *et al*., 2002). In unstressed cells HSP90 plays a number of important roles, which include assisting in folding (Buchner, 1999), intracellular transport, maintenance and degradation of proteins as well as facilitating cell signaling. It acts as a general protective chaperone (Miyata and Yahara, 1992; Wiech *et al*., 1992). HSP90 also participates in many key processes in oncogenesis such as self-sufficiency in growth signals, stabilization of mutant proteins, angiogenesis and metastasis (Fontana *et al*., 2002; Calderwood *et al*., 2006; Eustace *et al*., 2004; Whitesell and Lindquist, 2005; Sato *et al*., 2000). HSP90 is one of the most abundant proteins (1-2%) in the cytoplasm of unstressed cells where it performs housekeeping functions, controlling the stability, maturation, activation, intracellular disposition and proteolytic turn-over of a plethora of proteins generally termed as 'client proteins' (Taipale *et al*., 2010).

2.3.4 HEAT SHOCK PROTEINS AND THEIR ROLE IN VARIOUS DISEASES

Heat shock proteins function as molecular chaperones, preventing stress induced aggregation of partially denatured proteins and promoting their return to native conformations when favorable conditions pertain. HSP polypeptides assemble into dynamic oligomers which undergo subunit exchange and they bind a wide range of cellular substrates. As molecular

chaperones, the HSPs protect protein structure and activity, thereby preventing disease, but they may contribute to cell malfunction when perturbed. HSPs are molecular chaperones, storing aggregation prone proteins as folding competent intermediates and conferring enhanced stress resistance on cells by suppressing aggregation of denaturing proteins, actions associated with oligomerization and subunit exchange (Fu and Chang, 2004). Intracellular quantities and cellular localizations of HSPs change in response to development, physiological stressors such as anoxia⁄hypoxia, heat and oxidation and in relation to pathological status. HSPs interact with many essential cell structures and it follows from such promiscuity that functional disruption and inappropriate association of these molecular chaperones with substrates will foster disease.

2.3.5 HSPs EXPRESSION

Expression of HSPs can explain inter and intrapopulation differences in heat shock resistance. In Drosophila, several studies have been investigated the role of HSPs in adaptation to heat stress. HSP70 levels are higher during heat shock exposure and also show improved thermo-tolerance when receiving a thermal pretreatment in genetically engineered Drosophila lines with extra copies of HSP70 (Feder *et al*., 1996). However, looking at intra-population variation in heat shock resistance and HSP70, correlations depend on species and developmental stage. Krebs and Feder (1997) found a correlation between HSP70 and thermo-tolerance in larvae, but not in adults.

In mammalian cells, nonlethal heat shock produces changes in gene expression and in the activity of expressed proteins, resulting in what is referred to as a cell stress response (Lindquist, 1986; Jaattela, 1999). This response characteristically includes an increase in thermo-tolerance that is temporally associated with increased expression of HSPs. Heat-induced changes in gene expression occur both during hyperthermia as well as after return to normothermia. Heat shock proteins are intracellular molecules, involved in stabilizing cells during thermal exposure (Hendry and Kola, 1991). These HSPs have a protective function and a strong correlation between their induction and the induction of thermo-tolerance has been observed (Lindquist and Craig, 1988).

Upon stress the most prominent HSPs present in the nucleolus are the inducible HSP70 and HSP110 (Welch and Suhan, 1985). The fact that some HSPs translocate to the nucleolus suggests a specific and unique role in the repair and protection of these cellular structures (Collier and Schlesinger, 1986). Upon recovery after heat shock, HSP70 exit the nucleolus to accumulate back in the cytoplasm, more specifically in the perinuclear region, along the perimeter of the cell, and in association with large cytosolic phase dense structures (Welch and Feramisco, 1984; Welch and Suhan, 1986). Perinuclear condensation of HSP70

seems to coincide with reassembly of the centrosome and microtubuli, and also with the cytoplasmic distribution of ribosomes. This suggests that HSP70 plays a crucial role in the function of these organelles immediately after heat shock and during subsequent recovery phase (Brown *et al*., 1996). Proteins that lose their normal three-dimensional conformation provoke HSP synthesis through the activation of HSF. During and after heat shock cytosolic proteins normally aggregate and have a reduced solubility (Vidair *et al*., 1996). Along with its release during heat stress they themselves are subject to strict autoregulation by multiple molecular mechanisms (Lindquist, 1993). Heat shock protein chaperoning is a permanent cellular event during both nonstressed and stressed conditions. However, heat shock or other stresses, upregulation of the synthesis and translocation of various HSPs to other cellular compartments suggest that during evolution, tissues develop intrinsic defense mechanisms for resuing unfolding proteins in various cellular compartments.

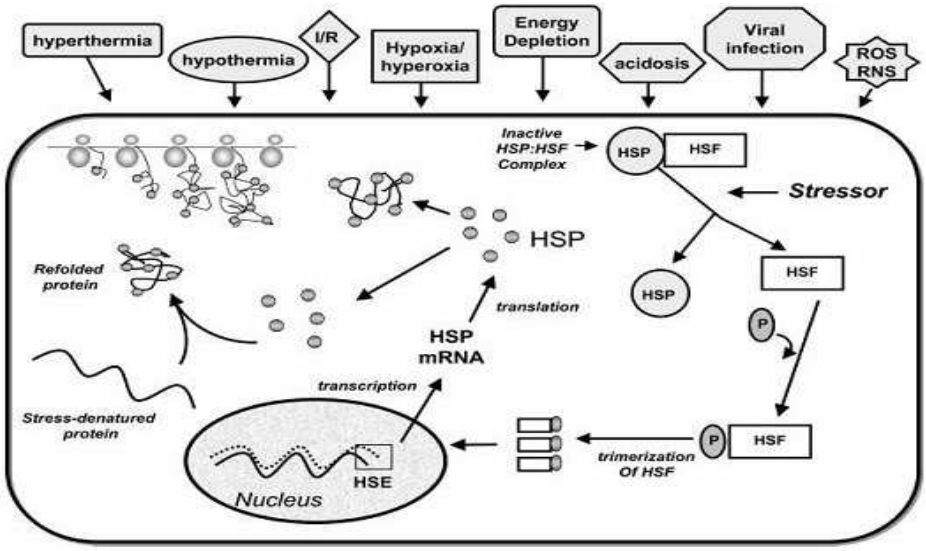


Fig.1. Mechanism of HSP genes expression

A summary of some of the major physiological signals that activate the inducible form of the heat shock protein (HSPs) synthesis (top) and a proposed mechanism for increased HSPs expression within a cell. Heat shock factors (HSFs), present in the cytosol, are bound by heat shock proteins (HSPs) and maintained in an inactive state. A broad array of physiological stimuli ("stressors") are thought to activate HSFs, causing them to separate from HSPs. HSFs are phosphorylated (P) by protein kinases and form trimers in the cytosol. These HSF trimer complexes enter the nucleus and bind to heat shock elements (HSE) in the promoter region of the HSP gene. HSP mRNA is then transcribed and leaves the nucleus for the cytosol, where new HSP is synthesized. Proposed mechanisms of cellular protection for HSPs include their functioning as molecular chaperones to assist in the assembly and translocation of newly synthesized proteins within the cell and the repair and refolding of damaged (e.g., stress- denatured) proteins. I/R, ischemia-reperfusion; ROS, reactive oxygen species; RNS, reactive nitrogen species. (Kregel, 2002)

Enhanced synthesis of HSPs was detected in highly purified T cells during heat stress (febrile temperatures less than or equal to 41°C), three major HSPs with approximate molecular weights of 110, 90 and 75 were detected in these T cell populations. Enhanced HSP synthesis reflected augmented transcription of HSP genes which was contingent on the continued presence of hyperthermic stress (Ciavarra and Simeone, 1990). Blake *et al*. (1990) demonstrated HSP gene expression in rats exposed to heat shock.

Lacetera *et al*.,2006); Patir and Upadhyay, 2007; Parmar *et al*. (2015) observed heat stress induced HSP70 expression in bovine lymphocytes. Among all HSP70 genes studied, a significant increase was observed with respect to HSPA1A, HSPA6, and HSPA8. Similarly, heat stress-induced upregulation of HSPA8, HSPA6, and HSPA1A gene expression was observed in human blood (Sonna *et al*., 2002; 2004). In Brownswiss cattle, increased HSP72 or HSPA1A mRNA levels in peripheral blood mononuclear cells due to heat stress have also been reported by Lacetera *et al*. (2006). Studies in camel shows that temperature elevation increases the level of constitutively expressed HSP70 in camel (Ulmasov *et al*., 1993; Garbuz *et al*., 2011). Though HSP70 genes are highly conserved across evolutionary lines, the HSP70 gene expression is species and breed-specific. The species-specific differences in HSP70 isoforms are most likely due to variations in thermal tolerance (Yamashita *et al*., 2004), and isoform expression may vary with regard to thermo-tolerance (Hightower *et al*., 1999).

Heat shock induces HSP70 in the bovine lymphocytes (Guerriero and Raynes, 1990; Kamwanja *et al*., 1994; Lacetera *et al*., 2006; Patir and Upadhyay, 2007; Parmar *et al*., 2015). A two hundred fold increase in HSP70 levels in serum and 2.5 fold increase in lymphocytes was observed after exposure at 42°C and 70% RH in buffaloes (Mishra *et al*., 2010). Characterization of HSP70 in buffalo (*Bubalus Bubalis*) suggested that recombinant HSP70 protein may be used for development of an assay for detection of thermal stress (Pawar *et al*., 2013). The mRNA level of HSP70 in lymphocytes was increased with increase in THI as well as temperature in dairy cows (Liu *et al*., 2010). The HSP70 concentration in Angus cattle increased from 0.07 to 0.25 µg/million cells when the temperature was enhanced from 38.5°C to 42.4°C whereas in Brahman cattle it increased from 0.07 to 0.26 µg/million cells when the temperature was enhanced from 38.5°C to 42°C (Kamwanja *et al*., 1994). In buffalo, higher intensity and duration of temperature exposure caused higher HSP70 induction in lymphocytes to maintain cellular homeostasis (Patir and Upadhyay, 2010). Irrespective of stocking density, transportation under hot, humid tropical conditions significantly increased HSP70 levels in the kidneys of goats (Zulkifli *et al*., 2010)

In vitro studies have indicated that HSP70 produced in heat stressed lung cells (Fargnoli *et al*., 1990), hepatocytes and liver (Heydari *et al*., 1995; Hall *et al*., 2000) and myocardium (Gray *et al*., 2000) provided protection from toxic effects of thermal stress.

DiDomenico *et al*. (1982) observed that once the cells are exposed to the temperature at which they are released, the cells are able to withstand any level of extremes of temperature. Heat shock proteins not only enhance heat tolerance but also give capacity to resist hypoxia, ischemia and inflammation. Further exposure to cellular toxins as heavy metals, endotoxins and reactive oxygen species, all imposing serious stress upon tissues and their composing cells (Luc *et al*., 2001). Increase in HSP72 in persons with spinal cord injury after 12 weeks leg cycling was observed (Darryn *et al*., 2002). In abdominal muscle, polyubiquitin mRNA levels increased during both hypo- and hyperosmotic stress (Jeffrey *et al*., 2002).

Adaptation with tropical climates, zebu breeds (Bos *indicus*) of cattle are better able to regulate body temperature in response to heat stress than European breeds (Bos *taurus*) (Gaughan *et al*., 1999; Beatty *et al*., 2006; Mehla *et al*., 2013). Expression level of HSP90 among Frieswal and Sahiwal cattle under *in vitro* and environmental heat stress were studied and a reported a higher expression in Sahiwal than Frieswal (Deb *et al*., 2014). The relative mRNA expression of inducible HSP70 genes (HSPA1A and HSPA2) were higher in Karan-Fries than Tharparkar. Zebu cattle (Tharparkar) are more adapted to tropical climatic condition than crossbreed cattle (Karan-Fries) and dermal fibroblast resistance to heat shock differed between breeds (Singh *et al*., 2014). Kishore *et al*. (2013) found that PBMCs can be used as an effective model to understand the heat stress response of different cattle types and buffaloes. The expression of HSPs in, Hostein-Friesian (HF) and Sahiwal cows in response to sublethal heat shock at 42°C indicated that HF cattle to be the most affected with the heat shock, whereas Sahiwal cattle were least affected. Among the HSPs, HSP70 was relatively more expressed followed by HSP60. The level of expression of HSPs throughout the time period of heat stress was highest in buffaloes, followed by HF and Sahiwal cows.

The mRNA expression of HSP70, HSP60, HSP90 and UBQ was significantly higher ($P<0.05$) during peak summer season as compared with peak winter season in both tropical and temperate region goats however, in the temperate region a non-significant difference of HSP70 expression between summer and winter seasons was noticed (Dangi *et al*., 2012; Sharma *et al*., 2013; Yadav *et al*., 2016). Melatonin administration in goat increased relative expression of HSP 60 manifold to alleviate heat stress (Sharma *et al*., 2013). HSP 70 was the most sensitive to temperature fluctuation of heat stress and played the most dominant role in protecting cells from damage caused by acute thermal stress and it could be used as an important molecular biomarker to heat stress in animals (Dangi *et al*., 2014). HSP expression pattern is biphasic or two-peak phenomenon in goats (Dangi *et al*., 2015). A possible correlation between TLR and HSP genes was reported to combat the deleterious effect of thermal stress and played an essential modulatory role in activation of immune system (Paul

et al., 2014). Nitric Oxide Synthase (NOS) family genes along with HSP genes maintain cellular integrity and homeostasis during thermal stress (Yadav *et al*., 2016).

Shilja *et al*. (2015) assessed the impact of heat and nutritional stress simultaneously on adaptive capability by behavioral and physiological responses, plasma HSP 70 level and HSP70 gene expression and indicated the significance of providing optimum nutrition to improve the adaptive capability of Osmanabadi goats to heat stress. The expression of HSP70 genes (HSPA8, HSPA6, HSPA1A, HSPA1L, and HSPA2) shows temperature sensitivity and seasonal variation. Relative expression of HSP70 genes varies markedly among the heat- and cold-adapted goat breeds with a moderate variation between breeds and shows a good response to increased or decreased ambient temperature. Rout *et al*. (2016) demonstrated the differential expression pattern of HSP70 gene expression in tissues and heat stress phenotypes in goat during peak stress period and found liver and brain tissues showed the highest gene expression at mRNA levels as compared to kidney, spleen and heart. HST individuals had higher levels of mRNA level expression than HSS individuals in all breeds. The Sirohi breed showed the highest (6.3-fold) mRNA expression levels as compared to the other three breeds, indicating the better heat stress regulation activity in the breed. Banerjee *et al*. (2013) used real time PCR to analyze the relative expression profile of HSP70 genes and found that the expression level of HSPA8 and HSPA1A was higher during both winter and summer. The expression level of HSPA6 and HSPA1L was higher only during summer. HSPA2 was observed to be downregulated during the summer and winter seasons. During summer, the relative expressions of all the HSP70 genes were higher in cold-adapted breeds (Gaddi and Chegu) than the heat-adapted breeds (Sirohi and Barbari). In Chegu, i.e., a cold-adapted breed, the expression of all the genes was higher as compared to the other cold adapted breed of goat, Gaddi, during summer. The HSPA8 and HSPA1A expression during winter in heat-adapted breeds was observed to be higher than cold-adapted breeds.

GAPS IN KNOWLEDGE

Limited information is available pertaining to expression profile and role of HSPs for amelioration of thermal stress respect to different seasons in Indian domestic animals in general and in goats in particular.

CHAPTER-III

MATERIALS AND METHODS

The proposed study was done by using a number of materials and methods which are described in this section. Standard protocols have been followed everywhere with slight modifications.

MATERIALS

3.1 EXPERIMENTAL ANIMALS

The experiment was carried out on three breeds *viz*. Barbari, Sirohi and Jakhrana. Five animals of each breed were used in the experiment

3.1.1 Description of genetic stock

(A) Barbari goat

Barbari is the medium size dual purpose goat breed. It is named for its origin place Berbera in British Somaliland in East Africa. This breed is distributed mainly in Etah, Etawah, Mathura, Agra and Aligarh districts of Uttar Pradesh and Bharatpur district of Rajasthan in India. White and short haired coat with brownish spots all over the body, short and straight horns, short and erect ears and a well set udder are the main characteristics of this breed (Fig. 1). The dam weight is averaged approximately 22-32 kg. The average lactation yield is 82±2 kg during a lactation period of 130 days which is similar to Indian dairy goat breeds and posses many desirable characters of body weight growth, prolificacy, reproductive efficiency. It is highly suited for stall-feeding, early maturing and non-seasonal breeder, and generally gives birth to twin and triplets. So, this breed is considered to be one of the best dual purposes among Indian goat breeds.

(B) Sirohi goat

Sirohi is native breed of Rajasthan. In India, it is distributed over Jaipur, Bhilwara, Tonk and Ajmer districts of Rajasthan and Palanpur in Gujarat and is also found in Uttar Pradesh. It is medium size dairy breed which body is covered with short and coarse hair, flat and leaf like drooping ears, curved horns with pointed tips and dark brown coated with tan patches (Fig. 2). Average weight of male and female is 48-50 kg and 25-30kg respectively. They started kidding in 19-20 months, twice in a year. Average milk yield is 65±2 over a

lactation period of 120 days. It is well suited for stall-feeding and mainly used for meat purpose.

(C) Jhakrana goat

Jhakrana is a good dairy type breed mostly used for milk production. In india, it is distributed over few surrounding villages near Behror of Alwar district of Rajasthan. It is large in size and predominantly black with white spots on ears and muzzle and have a highly developed udder (Fig. 3). Average weight of adult male is 53-55kg and adult female is 40-42 kg. Kidding is mostly single. Average dairy milk yield varies from 2.0-3.0 kg for a lactation length of about 180 days. This breed's skin is popular for tanning industry.

3.2 FEEDING, HOUSINNG AND MANAGEMENT OF THE ANIMALS

Animals were kept in well-ventilated pucca sheds under loose housing system in the experimental shed of Department of Veterinary Physiology, DUVASU, Mathura. Animals were offered concentrated diet with required amount of mineral mixture.

3.3 CLIMATOLOGICAL CONDITIONS

Samples were collected in three different climatic conditions in the month of January, March and May which is given in the (table 1).

Table 1:- Monthly mean of Meteorological observations

Month	Max. temp. (°C)	Min. temp. (°C)	Mean daily temp. (°C)	Vapour Pressure (mmHg)	Relative humidity (%)	Rainfall (mm)/wet days	Sun shine (hr)
January	23.42	7.56	15.49	10.84	74.54	0.00(0)	159.10
March	35.27	16.02	25.65	13.19	45.92	16.40(5)	275.30
May	44.53	26.23	35.38	16.42	33.18	13.60 (5)	275.90

Temperature humidity index (THI)

THI of months of January, March and May were calculated (Mader *et al*., 2006) using the formula:

$$THI = (0.8 \times T_{db}) + [(RH/100) \times (T_{db} - 14.4)] + 46.4$$

T_{db}- Dry bulb temperature, RH- Relative Humidity

3.4 CHEMICALS, EQUIPMENTS AND MISCELLANEOUS ITEMS:

All chemicals, equipments, laboratory wares and miscellaneous items used for the study are enlisted in annexure-I, while the details of buffers and solutions are given in annexure-II and that of enzymes and biologicals in annexure-III.

3.5 PRIMER SEQUECNE

To amplify the desired genes, specific primers were taken from the already published primer sequences (Dangi *et al*., 2012). These primers were further aligned by using PRIMER BLAST at NCBI and the details have been given in (table 2).

Table 2. Gene transcripts, primer sequences and resulting fragment size

Target gene	Sequence of nucleotide	size (bp)	EMBL
HSP 60	For: 5'-ACTGGCTCCTCATCTCACTC -3'	148	NM_001166609.1
	Rev: 5'-CTGTTCAATAATCACTGTCCTTCC -3'		
HSP 70	For: 5'-GACGACGGCATCTTCAAG -3'	132	FJ975769.1
	Rev: 5'-GTTCTGGCTGATGTCCTTC -3'		
HSP 90	For: 5'-GCATTCTCAGTTCATTGGCTATCC -3'	190	NM_001012670.1
	Rev: 5'-GTCCTTCTTCTCTTCCTCCTCTTC -3'		
GAPDH	For: 5' -AAGGCTGAGAACGGGAAACT -3'	101	XM_005680968.2
	Rev: 5'-TACTCAGCACCAGCATCACC-5'		

EMBL – accession number or reference of published sequence

3.6 EXPERIMENTAL DESIGN

Present study was carried out at the Department of Veterinary Physiology, College of Biotechnology and Central instrumentation Facility, DUVASU, Mathura and Central Institute for Research on Goats, Makhdoom, Farah, Mathura. Five goats each of three different breeds of semi-arid region were selected for the study *viz*. Barbari, Sirohi and Jhakrana. The study was conducted in three different seasons *viz*. Winter (January), Thermo-neutral (March) and Summer (May). All the animals selected for this study were clinically healthy and free from any physical injury or anatomical abnormalities. Selected goats were 2 to 2.5 years old and had an average body weight of 25-30 kg. All of the animals were regularly monitored and similar management inputs were provided during the experimental period. The climatologically data and physiological parameters during the experimental period were recorded. The blood samples were collected during three different seasons for RNA isolation, cDNA synthesis and quantitative analysis of gene expression. Recording of physiological parameters and collection of blood samples was done at 09:00 to 10:00 in all the seasons during experiment.

3.7 RECORDING OF PHYSIOLOGICAL PARAMETERS

The respiratory rate was recorded by observing movements at the flank region. The movement was observed for one minute and presented as respiratory rate/minute. The rectal temperature was recorded using digital thermometer by inserting it into the rectum of the animal and touching the rectal mucous membrane for one minute. The rectal temperature was presented in ºC.

3.8 METHODS

3.8.1 COLLECTION OF BLOOD SAMPLES

Blood samples were collected from jugular vein puncture of goats aseptically using NH Sodium heparinized vacutainer under sterile conditions. About three ml of blood was collected in the tube and inverted twice. Precautions were taken to minimize the effect of ribonuclease activity at the time of processing.

3.8.2 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)

a) Isolation of PBMCs was carried out in dust free, isolated room equipped with laminar flow having UV light facility. The room was regularly fumigated (using Potassium Permangnate and Formalin) and UV light was put on for two hours before beginning of the experiment. The histopaque which is usually kept at 8 - 15°C was brought to room temperature before starting the experiment.

b) Three ml Histopaque-1077 (SIGMA-ALDRICH) was taken in to fresh autoclaved 15ml conical centrifuge tube and sufficient time was allowed for Histopaque to reach room temperature.

c) Three ml blood was slowly layered upon histopaque-1077 keeping the centrifuge tube at 25-45° angle so that mixing of blood with histopaque did not occur.

d) Tubes were immediately centrifuged at 3000 rpm or 400 ×g for 30 min at 25°C.

e) Tubes were carefully removed from centrifuge machine without disturbing the layers. Following centrifugation layers of plasma, PBMCs, histopaque and RBCs were clearly visible (Fig. 4).

f) The upper plasma layer was slowly removed and placed in separate plasma collection vials for further biochemical analysis.

g) The opaque interface containing PBMCs was carefully transferred in to fresh 2ml micro-centrifuge tube avoiding carryover of the lower clear layer of Histopaque.

h) Equal amount of 1X DPBS pH (7.4) (Hi-Media) was added to the tubes and mixed gently by inversion and centrifuged at 1700 rpm or 250 ×g for 10 minutes at 25°C.

i) Supernatant was discarded and PBMCs pellet was re-suspended in 1X DPBS pH (7.4) and centrifuged at 1700 rpm or 250 ×g for 10 minutes at 25°C.

j) Supernatant was poured off and PBMCs pellet was re-suspended in cold 1X DPBS pH (7.4) and centrifuged at 1700 rpm or 250 ×g for 10 minutes at 25°C.

k) Supernatant was poured off and proceed for RNA isolation.

3.8.3 ISOLATION OF TOTAL RNA

After discarding the supernatant, PBMCs pellet was resuspended in500 µl of DEPC treated-PBS (PH 7.4) and PBMCs were counted using hemocytometer and one million cells suspension was taken for RNA isolation in a 2 ml nuclease free micro-centrifuge tube.

a) Sample homoginization/lysis of cells

- One million of Suspended cells were pelleted by centrifugation and resuspended in Ribozol RNA Extraction reagent (Amresco) and cells were lysed by passing them several times through the tip of pipette and incubated for 5-10 minutes at room temperature for complete dissociation of nucleoprotein complexes.

b) Separation of phases

- In each sample tube 200 µl of chloroform (amresco) was added and tubes were tightly secured.
- Tubes were shaken vigorously by hand for 15 second to mix and incubated for 2-3 minutes at room temperature.
- Tubes were centrifuged at 12000 ×g for 15 minutes at 4°C.

  Following centrifugation separated into three phases;

  a lower red, phenol-chloroform phase,

  a white interphase and

  a colorless, upper, aqueous phase.

- Fresh set of 1.5ml DEPC treated conical micro-centrifuge tubes were labeled.
- Only upper aqueous layer about 350 µl was transferred to 1.5ml labeled RNase free tube carefully by pipette without disturbing the white interphase to avoid contamination with DNA, protein, Lipid and carbohydrates that appears as flocculent material at interphase.

c) Precipitation of RNA

- RNA was precipitated by adding 500 µl of Isopropanol (Amresco) into each tube.
- Tubes were incubated for 10 minutes at room temperature and then centrifuged at 12000 ×g for 10 minutes at 4°C.
- A white gel like pellet of precipitated RNA was formed along the side & bottom of the tubes at this stage but pellet of very pure RNA may be nearly transparent and difficult to see.
- The supernatant was removed carefully without disturbing the RNA pellet.
- Pellet was washed by adding 1ml 75% Ethanol (Amresco) prepared with RNase-free water (Amresco) into each of the tube and mixed by vortexing.
- Centrifuged at 7500 ×g for 5 minutes at 4°C.
- Ethanol was carefully removed without disrupting the RNA pellet and briefly air-dried for 5-10 minutes.
- Depending on the size of the pellet RNA was re-dissolved in 15µl-50µl of Nuclease free water.
- RNA samples were stored at –80°C for further analysis.

3.8.4 DETERMINATION OF RNA CONCENTRATION AND PURITY:

The isolated RNA was checked for its purity and concentration. The purity of total RNA was checked by using the Eppendorf Biophotometer. One microliter of total RNA was used for absorbance, and absorbance at 260 nm and 280 nm wavelengths were recorded against nuclease free water as blank. RNA samples of A_{260}/A_{280} value more than 1.8 were used for cDNA synthesis.

3.8.5 CONFIRMATION OF RNA BY GEL ELECTROPHORESIS:

The quality and integrity of the total RNA was checked by using denaturing Agarose gel electrophoresis. For this purpose, 1.5 % w/v agarose suspension (low EEO) in 1X TBE buffer was made and heated in oven until the agarose was completely melted to give clear and transparent solution. Then it was cooled to about 50 to 60°C and ethidium bromide 0.5µl/ml was added and mixed gently. Gel was casted in casting tray (BIO-RAD) fitted with comb. Once gel has solidified, a few ml of 1X TBE running buffer was added and comb was removed carefully and gel was immersed in the electrophoresis tank filled with1X TBE buffer. Sample was prepared by mixing 5 µl of total RNA and 1.5µl of 6X loading dye and loaded in to gel. Electrophoresis was carried out at @ 5-6 volts/cm and visualized under UV

light. Two intact bands of 28s and 18s without smearing indicated good quality and intactness of RNA (Fig. 5)

3.8.6 DNase TREATMENT

DNase treatment was carried out using RNase-free DNase I, Amplification grade (SIGMA-ALDRICH) as per manufacturer instructions. The reaction was carried out in thermal cycler. Following reaction mixutre was prepared into RNase-free PCR tube:

Components of reaction mixture	Quantity
RNA in water	8 µl
10X Reaction Buffer	1 µl
DNase I, , 1 unit/µl	1 µl

a) Reaction mixture was incubated for 15 minutes at room temperature.

b) 1 µl of Stop solution (50mM EDTA) was added in to each reaction tube to bind calcium and magnesium ions to inactivate the DNase I.

c) Tubes were heated at 70°C for 10 minutes to denature both the DNase I and the RNA.

d) Tubes were chilled on ice.

3.8.7 SYNTHESIS OF FIRST STRAND cDNA:

The first strand cDNA was synthesized from the isolated total RNA using Rever Tra Ace® qPCR RT Master Mix (TOYOBO) following manufacturers instruction in thermal cycle. Synthesis of cDNA was performed by using (400 ng) of RNA that was reverse transcribed according to the manufacturer instruction. Reaction was carried out in 20 µl reaction mixture. Calculation was done by utilizing the concentration of total RNA checked from nanodrop reading (ng/µl) to take 400 ng of total RNA for each reaction and final volume was make by adding nuclease free water.

Components of reaction mixture	Quantity
5X RT Master Mix	4 µl
DNase treated RNA template	X µl (400 ng)
Nuclease Free Water	Y µl
Total Volume	10 µl

Reaction mixture was mixed and incubated at 37°C for 15 minutes followed by 50°C for 5 minutes. Reaction was stopped by incubating for 5 min at 98°C. The cDNA was stored at -40 to -20°C.

3.8.8 CONFIRMATION OF cDNA WITH GAPDH PRIMERS

cDNA was checked by PCR using Dream Taq PCR Master mix (Thermo Scientific) with GAPDH primers. The amplification of 101 bp for GAPDH gene fragment from the cDNA indicated that the synthesized cDNA from the total RNA by harvesting PBMCs was of good quality (Fig. 6)

3.8.9 OPTIMIZATION OF END POINT PCR

End point PCR conditions were optimized to amplify goat HSP60, HSP70, HSP90 gene sequences in gradient thermo cycler using Dream Taq PCR mater mix (thermo scientific). Factor specific primers were used for the amplification of genes. The annealing temperature was standardized using cDNA prepared from mRNA of goat PBMCs by PCR. The reaction was carried out at different annealing temperatures, primer concentrations and template (cDNA). The optimum temperature of 57°C for HSP60, 62°C for HSP70 and 60°C for HSP90 were found to be most suitable for annealing for respective primers and was used in subsequent polymerase chain reaction. The concentration of different component which were found suitable for the optimum amplification are as follows.

Components	**Quantity**
PCR master mix (thermo Scientific)	12.5 µl
Primer Forward (10pmol/µl)	1.0 µl
Primer Reverse (10pmol/µl)	1.0 µl
cDNA template	2.0 µl
Nuclease free water	8.5 µl
Final volume	25 µl

The above reactants were added to a nuclease free thin walled 0.2 ml PCR tubes prechilled on ice. The contents were gently vortexed and then spin down to collect at the bottom of tube by centrifugation. The reaction was carried out in a thermal cycler using the following cycling parameters that have been found optimum for amplification of gene fragments.

Step I : 95° C for 5 min for initial denaturation

Step II : 95°C for 60 sec for denaturation

Step III : 56°C for HSP60, 62°C for HSP70, 60°C for HSP90, 60°C for GAPDH and 55°C to 58°C for beta actin for 60 sec for annealing

Step IV : 72°C for 60 second for extension

II to IV repeated for 35 cycles.

Step V : 72°C for 5 minute for final extension.

3.8.10 AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCT

The confirmation of amplification of specific RT-PCR amplicon was done by agarose gel electrophoresis (appendix). 1.5% agarose was mixed with 30 ml 1X TAE buffer and melted in a microwave oven. When the molten gel had cooled to about 60°C, ethidium bromide was added to final concentration of 0.5µg/ml. The gel was mixed by gentle swirling and then poured into the gel casting tray fitted with the comb. The gel was allowed to solidify and the comb was removed. The PCR products were loaded into the wells. For the comparison, a 50 bp molecular weight gene ruler was gel electrophoresed in parallel to the RT-PCR amplicons. The gel was run at a voltage of 5-6 V/cm (distance between electrodes) till the running dye crossed at least two third of the gel. The bands were visualized under UV light and recorded on a gel documentation system (Fig. 9-11)

3.8.11 REAL TIME PCR

Quantitative Real-time PCR was performed with THUNDERBIRD® SYBR qPCR Mix by Light Cycler 480 instrument (Roche, Germany) for gene expression. Reaction setup was performed in area separate from nucleic acid preparation or PCR product analysis. Pipetting was done with sterile DEPC treated tips. Exposure of light to the qPCR master mix was minimized. Careful pipetting was done without creating bubbles to avoid interference in reading of fluorescence by the instrument. For each gene**,** samples were run in duplicates or triplicates. No template control (NTC) was put for gene quantification for checking the contamination in the reaction components other than the cDNA. To ensure the cDNA samples were not contaminated with genomic DNA, reactions were set up using 10 ng of non-reverse transcribed RNA in place of cDNA. Failure to generate a detectable signal signified the samples as DNA free. In negative control, only the real time master mix and primers were added along with relative curve control. The reactions were performed with amplification conditions. Total 1 µl of cDNA was taken. Following reaction mixture was prepared in 96 wells plate (Roche, Germany).

Reaction mixture

Components	**Quantity**
Nuclease free water	µl
THUNDERBIRD® SYBR qPCR Mix 25 µl	10 µl
Forward Primer (10 pmol)	1 µl
ReversePrimer (10 pmol)	1 µl
cDNA template	2 µl
Final Volume	20 µl

After prepraration of reaction mixture, plate was covered by adhesive foil. Touching of the plate without gloves was avoided. GAPDH was taken as housekeeping gene. The qPCR amplification programme was used as given in table 3.

Table 3. RT-PCR Cycling Condition

Detection format	**Block Type**	**Reaction Volume**
SYBR Green 1	96	20 µl
Programs		
Program Name	**Cycles**	**Analysis Mode**
Pre-incubation	1	None
Amplification	40	Quantification
High Resolution Melting	1	Melting Curve
Cooling		

Target temperature	**Acquisition mode**	**Time**	**Ramp rate (°C/s) (96-well)**	**Acquisitions (per °C)**
Pre-incubation (1 cycle)				
95°C	None	10 min	4.4	-
Amplification (40 cycles)				
95°C	None	10 sec	4.4	-
57°C for HSP60	None		2.2	-
62°C for HSP70	None		2.2	-
60°C forHSP90	None	15 sec	2.2	-
72°C	Single	20 sec	4.4	-
High resolution melting/dissociation protocol				
95°C	None	1 min	4.4	-
40°C	None	1 min	2.2	-
65°C	None	1 sec	1.0	-
95°C	Continuous	-	0.02	25
Cooling				
40°C	None	10 sec	2.2	-

The dissociation protocol was used to investigate the specificity of the qPCR reaction and the presence of primer dimmers. The amplification and denaturation data was acquired. After the run has ended, qPCR expression data for each target gene was extracted in the form of crossing point (Cp) or cycle threshold (Ct) values by using the "SYBR Green (with Dissociation Curve)" method of the real time machine.

3.8.12 NORMALIZATION AND DATA ANALYSIS

For normalization of expression data of major HSP genes, GAPDH (Housekeeping gene) gene was used as internal control gene. The analysis of mRNA expression data across different samples was based on crossing point (Cp) values. The Cp values of each gene were subtracted from the arithmetic mean of Cp values of GAPDH to calculate ΔCt. Results were expressed as mean ±SEM.

3.8.13 CALCULATION OF RELATIVE EXPRESSION

Optical data were collected at end of each extension step, and relative expression of PCR product was determined by the equation $\mathbf{2^{(-\Delta\Delta Ct)}}$ (Livak, 2001).

3.8.14 STATISTICAL ANALYSIS

Analysis of variance was performed to determine the presence or absence of significant differences in the analytical variables among different groups by one way ANOVA using SPSS 16.0 statistical software package. *P* values <0.05 were considered significant.

CHAPTER-IV

RESULTS

In this chapter outcome of the present study has been described which includes the climatological data of experimental period, physiological responses of different goat breeds, quantification and analysis of purity and integrity of RNA, primer optimization and cDNA confirmation by end point PCR and comparative mRNA expression of different genes in goat of different breeds during different seasons by quantitative real time PCR.

4.1 CLIMATOLOGICAL DATA

The THI during the months of January, March and May was 59.63, 72.1 and 81.63 respectively.

4.2 PHYSIOLOGICAL RESPONSES

4.2.1 Respiratory rate (RR) of goats during different seasons

Respiratory Rate of goats during different seasons is presented in the Table 5 and fig. 8. The RR of studied goat breeds was found to be statistically similar during winter season as compared to thermo-neutral. However, there was a non-significant decrease in RR of Barbari goats in winter as compared to thermo-neutral season. The RR of all studied goat breeds was significantly ($P \leq 0.05$) higher in summer as compared to thermo-neutral and winter season. Moreover, the increase in RR of Sirohi and Jhakrana goats was highly significant ($P \leq 0.001$).

Table 5: Physiological responses of goats during different seasons

Breed	Physiological Parameter	Winter	Thermo-neutral	Summer	*P* values
Barbari	**RR**	21.80 ± 1.31^{a}	24.40 ± 0.51^{a}	26.60 ± 0.24^{b}	0.005
Barbari	**RT**	37.91 ± 0.21^{a}	37.94 ± 0.18^{a}	38.61 ± 0.12^{b}	0.029
Sirohi	**RR**	25.00 ± 0.63^{a}	24.20 ± 03^{a}	28.80 ± 0.58^{b}	0.000
Sirohi	**RT**	37.97±0.21	37.71±0.15	37.80±0.16	0.581
Jhakrana	**RR**	24.00±1.14	24.20±0.37	28.80±0.58	0.001
Jhakrana	**RT**	37.92±0.18	37.78±0.19	37.65±0.12	0.555

The values in rows with different superscripts differ significantly ($P<0.05$)

n = 5

RR Respiratory Rate, RT Rectal Temperature

4.2.2 Rectal temperature (RT) of goats during different seasons

The RT of goats during different seasons is presented in Table 5 and fig. 9. The RT of all goat breeds was found to be statistically comparable (P>0.05) duringseasons.

4.3 RNA ISOLATION AND QUANTIFICATION

Blood samples were collected from healthy goats by jugular vein puncture using heparinized vacuetainer and PBMCs were separated by density gradient centrifugation using histopaque-1077 (SIGMA-ALDRICH) (Fig. 5).

Total RNA was isolated from PBMCs by standard protocol using RiboZol RNA Extraction reagent (Amresco) with slight modifications. The integrity of total RNA was checked on agarose (1.5%) gel using 1X TBE as electrophoresis buffer. Total RNA was in good yield in all the samples. The bands of 28s RNA and 18sRNA reflected the high quality of extracted total RNA (Fig. 6).

The purity and concentration of total RNA was checked in nanodrop Biophotometer (Eppendorf). Isolated RNA samples were free from the protein contamination as the OD 260: OD 280 values were more than 1.8. The concentrations of the RNA samples were in the range of 50-500 ng/µl.

4.4 cDNA SYNTHESIS AND ITS CONFIRMATION

DNase treated 400 ng of Total RNA was directly used for cDNA synthesis in thermal cycler using Rever Tra Ace qPCR RT Master Mix (TOYOBO) as per manufacturer instructions. cDNA integrity was checked by PCR using GAPDH gene amplification with already published primer sequence whose reaction conditions were already known. After running on agarose (1.5%) gel, single band of 101 bp was visualized (Fig.7). In order to ensure the amplification of specific fragment with higher yield, the PCR protocol was optimized with respect to reaction conditions as well as cycle parameters.

4.5 OPTIMIZATION OF END POINT PCR PROTOCOL

Primers were taken from already published literature (Dangi *et* al., 2012) and further primer sequences were aligned using Primer BLAST at NCBI. Gradient PCR that allow different temperature profiles to be programmed for each cavity in the cycler was used to optimize PCR conditions with respect to the primer annealing temperature. All samples were treated equally, but different annealing temperatures were used. The reaction conditions were optimized using different combinations of the primers, cDNA concentration for all the genes. PCR efficiency was analyzed by agarose (1.5 %) gel electrophoresis investigating the intensity and integrity of the product bands. It was observed that annealing temperature of 57°C for HSP60, 62°C for HSP70 and 60°C for HSP90 provided the best results. The

optimized concentrations that provided the best results were 12.5 µl of Dream Taq PCR master mix and 10 pmoles of each primer. Finally PCR was carried out in 25 µl volume of reaction mixture containing optimized concentrations of Dream Taq PCR master mix and primers, and 1 µl cDNA as template.

4.5.1 AMPLIFICATION OF HSP60, HSP70 AND HSP90 GENES

Following PCR, the amplicon length was checked by high resolution agarose gel electrophoresis using 1X TAE as running buffer and a 50 bp gene ruler was also electrophoresed in parallel to the amplicons for confirmation of specific size. As expected a single and specific band of 148 bp for HSP60 (Fig. 10), 132 bp for HSP70 (Fig. 11) and190 bp for HSP90 (Fig. 12) was amplified from the cDNA.

4.6 REAL TIME PCR

Expression analysis of HSP genes were carried out in different goat breeds by quantitative real time PCR. GAPDH gene expression was used as internal control for all the replicates. Quantification was performed by analyzing the Cp (crossing point) value in amplification reaction. The Cp is the point at which the amplified product is first visible in the data. Real time PCR was optimized using different dilutions of templates of cDNA. 1.0 µl of the template gave good results for all genes. After standardization real time PCR was performed for each gene taking all the samples. The melting curve showed only one peak. After the run has ended, crossing point (Cp) values were acquired by using the "SYBR Green (with Dissociation Curve)" method of the real time machine Light Cycler 480 (Roche, Germany). The PCR-product was identified by the characteristic melting curve (Fig. 12).

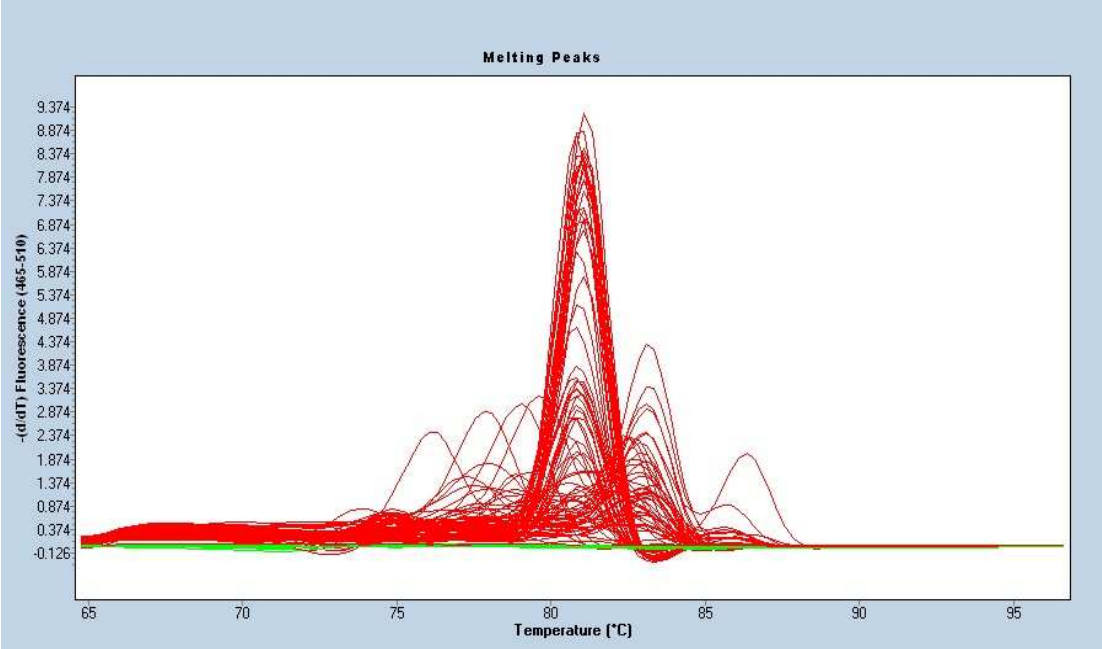


Fig. 13 Melting curve

4.6.1 Expression of HSP genes in Barbari goats during different seasons

The relative mRNA expression of HSP genes in Barbari goats during different seasons is presented in Table 6 and fig. 14. The relative mRNA expression of HSP60 during winter was differed non-significantly as compared to thermo-neutral season whereas, the relative mRNA expression of HSP60 was significantly (P=0.046) upregulated during summer season as compared to thermo-neutral. The relative mRNA expression of HSP70 in Barbari goats was differed non-significantly in winter season as compared to thermo-neutral season. It was found significantly higher (P=0.013) in summer season as compared to winter and thermo-neutral seasons. The relative mRNA expression of HSP90 did not differ significantly during winter season as compared to thermo-neutral season however during summer season the relative mRNA expression of HSP90 was highly upregulated (P<0.01) as compared to thermo-neutral and winter seasons.

4.6.2 Expression of HSP genes in Sirohi goats during different seasons

The relative mRNA expression of HSP genes in Sirohi goats during different seasons is presented in Table 6 and fig. 15. The relative mRNA expression of HSP60 during winter season was found to have non-significant difference as compared to thermo-neutral season whereas, it was highly (P<0.01) upregulted during summer season as compared to thermo-neutral and winter seasons. The difference relative mRNA expression of HSP70 was non-significant in winter and thermo-neutral seasons however, the mRNA expression of HSP70 was found to be significantly (P<0.01) upregulated during summer season in Sirohi goats. During winter season, the relative mRNA expression of HSP90 did not change significantly as compared to thermo-neutral however, it was significantly higher (P<0.01) during summer season as compared to winter and thermo-neutral season.

4.6.3 Expression of HSP genes in Jhakrana goats during different seasons

The relative mRNA expression of HSP genes in Jhakrana goats during different seasons is presented in Table 6 and fig. 16. The relative mRNA expression of HSP60 in Jhakrana goats was found to be non- significantly different in winter and thermo-neutral seasons whereas, it was found to be highly upregulated (P<0.01) during summer season. In Jhakrana goats, the difference in relative mRNA expression of HSP70 was found to be similar (P>0.05) during winter and thermo-neutral seasons however, was non-significant during summer as compared to thermo-neutral season. The relative mRNA expression of HSP90 was found to be significantly higher (P<0.01) during winter and summer seasons as compared to thermo-neutral season, however, the relative mRNA expression of HSP90 was highly upregulated during summer season as compared to winter season in Jhakrana goats.

Table 6: Relative mRNA expression of HSP genes during different seasons in goat breeds

Breed	Gene	Thermo-neutral	Winter	Summer	*P* values
Barbari	HSP60	1.09±0.22	1.01±0.17	1.88±0.30	0.046
Barbari	HSP70	1.01±0.08^{a}	0.94±0.14^{a}	1.58±0.17^{b}	0.013
Barbari	HSP90	1.05±0.14^{a}	0.83±0.09^{a}	3.39±0.41^{b}	0.000
Sirohi	HSP60	1.13±0.07^{a}	1.29±0.24^{a}	8.65±0.67^{b}	0.000
Sirohi	HSP70	1.08±0.04^{a}	0.79±0.06^{a}	3.40±0.23$^{\mathbf{b}}$	0.000
Sirohi	HSP90	1.07±0.07^{a}	1.01±0.17^{a}	4.52±0.40^{b}	0.000
Jhakrana	HSP60	1.10±0.22^{a}	1.51±0.42^{a}	5.51±0.98^{b}	0.001
Jhakrana	HSP70	1.09±0.13^{a}	0.84±0.06^{a}	2.79±0.23^{b}	0.000
Jhakrana	HSP90	1.02±0.09^{a}	2.34±0.29^{b}	6.33±0.38^{c}	0.000

The values in rows with different superscripts differ significantly ($P<0.05$)

n = 5

4.6.4 Fold change in expression of HSP genes during winter season

The fold change in relative mRNA expression of HSP genes in winter and summer seasons as compared to thermo-neutral season is presented in Table 7 and fig. 17. Thermo-neutral season values were used as calibrator to calculate fold change in relative expression of HSP genes. The change in relative mRNA expression of HSP60 was found to be non-significant in all goat breeds however, it was numerically higher in Jhakrana goats followed by Sirohi and Barbari goats.

During winter season the fold change in relative mRNA expression of HSP70 was statistically non-significant in all goat breeds however, it was found to be numerically higher in Barbari goats followed by Jhakrana and Sirohi goats.

The fold change in relative mRNA expression for HSP90 was found to be statistically non-significant ($P>0.05$) in Barbari and Sirohi goats however, the fold change in relative expression was highly significant ($P<0.01$) in Jhakrana goats as compared to Barbari and Sirohi during summer season.

4.6.5 Fold change in expression of HSP genes during summer season

The fold change in relative mRNA expression of HSP genes during summer season is presented in Table 7 and Fig. 18. The change in relative mRNA expression of HSP60 was found to be statistically higher ($P<0.01$) in Sirohi goats as compared to Barbari and Jhakrana goats. The fold difference in relative expression of HSP genes was highly significant ($P<0.01$) in Jhakrana goats as compared to Barbari.

The fold change in relative mRNA expression of HSP70 gene during summer was found to be significantly higher ($P<0.01$) in Sirohi and Jhakrana as compared Barbari goats however, the change in relative mRNA expression of HSP70 gene was similar in Sirohi and Jhakrana goats.

The fold change in relative mRNA expression of HSP90 genes during summer season was significantly higher in Jhakrana goats as compared to Barbari and Sirohi however, the fold difference in relative expression was statistically non-significant in Barbari and Sirohi goats however, its expression was higher in Sirohi as compared to Barbari goats.

Table 7: Fold change in relative mRNA expression of HSP genes in goat breeds during different seasons

Season	Gene	Barbari	Sirohi	Jhakrana	*P* values
Winter	HSP60	1.12±0.35	1.19±025	1.35±02	0.831
Winter	HSP70	0.99±0.23	0.74±0.06	0.82±0.12	0.542
Winter	HSP90	0.92 ± 0.25^{a}	0.97 ± 0.20^{a}	2.44 ± 0.42^{b}	0.007
Summer	HSP60	2.14 ± 0.64^{a}	7.72 ± 0.53^{c}	5.18 ± 0.37^{b}	0.000
Summer	HSP70	1.62 ± 0.23^{a}	3.16 ± 0.21^{b}	2.65 ± 0.27^{b}	0.002
Summer	HSP90	3.58 ± 0.68^{a}	4.23 ± 0.33^{a}	6.46 ± 0.63^{b}	0.010

The values in rows with different superscripts differ significantly ($P<0.05$)

n = 5

Fig. 2 Barbari Goat

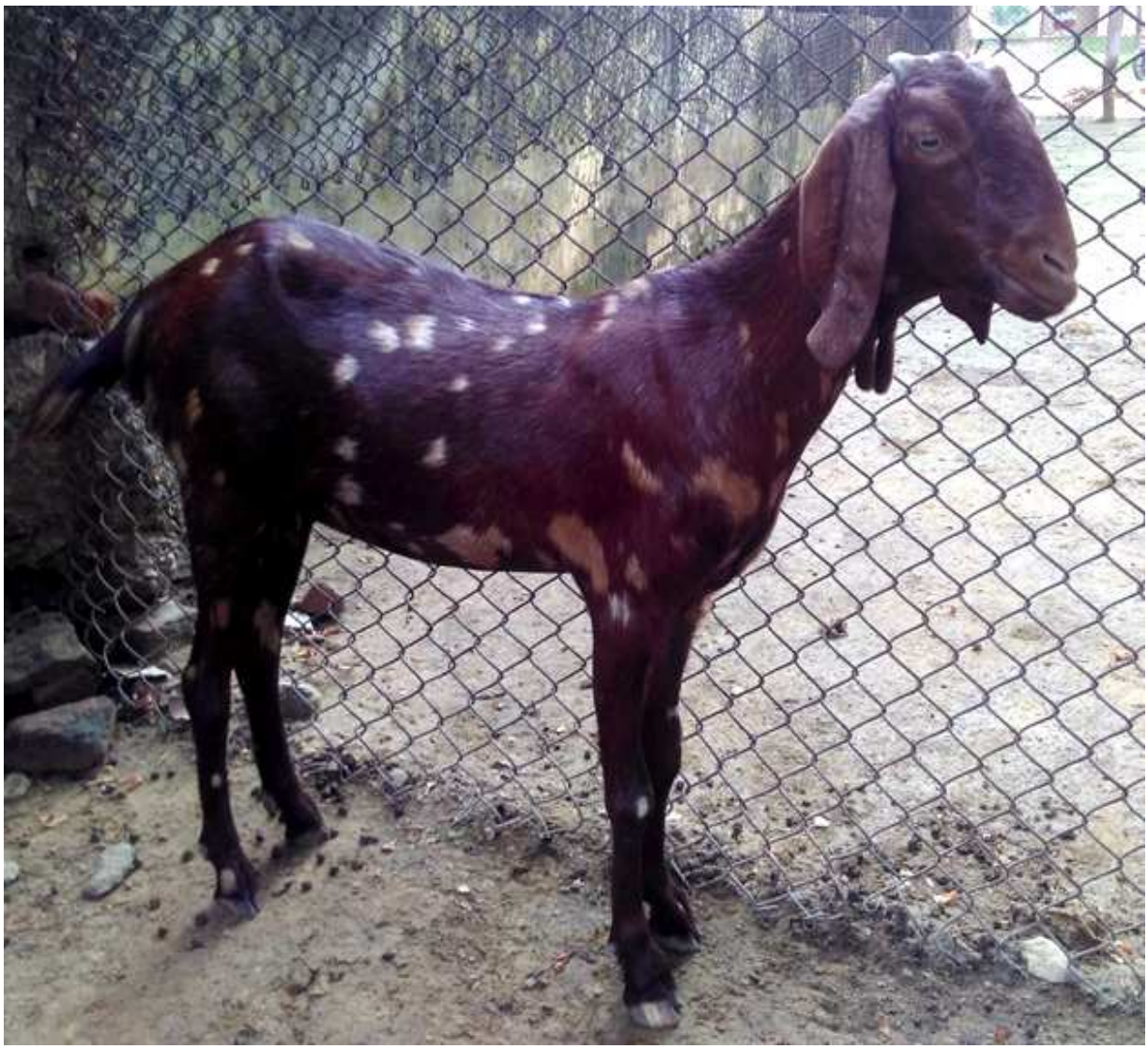


Fig. 3 Sirohi Goat

Fig. 4 Jhakrana Goat

Fig. 10 showing the amplification of HSP60 gene

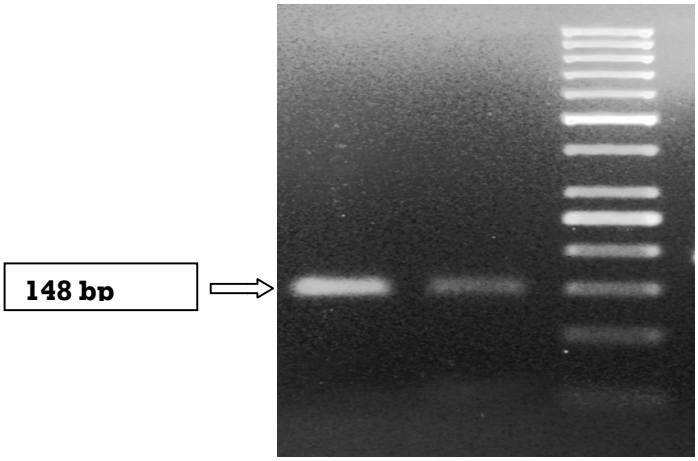


Fig. 11 showing the amplification of HSP70 gene

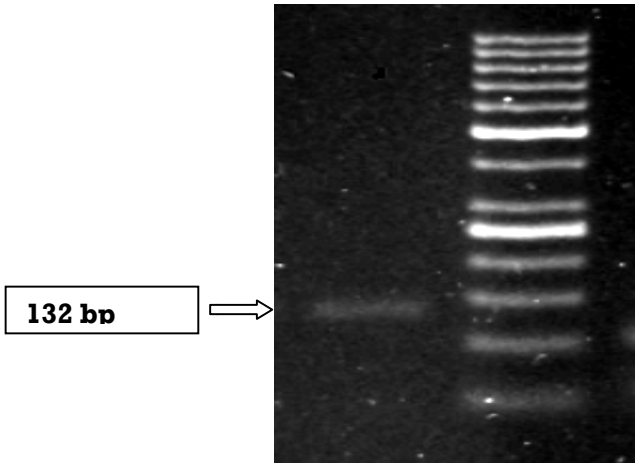


Fig. 12 showing the amplification of HSP90 gene

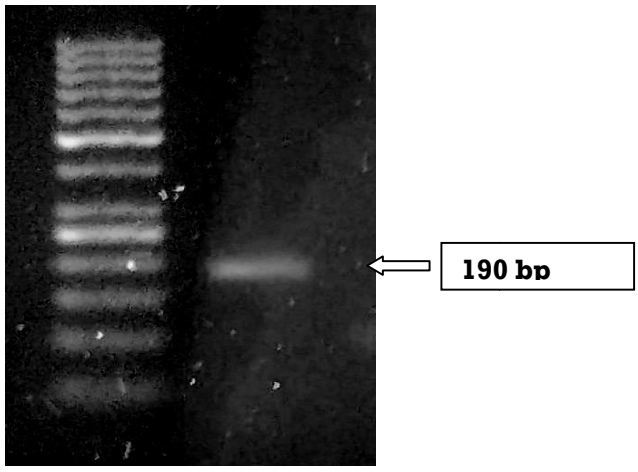


Fig. 8 Respiratory Rate of Goats during different seasons

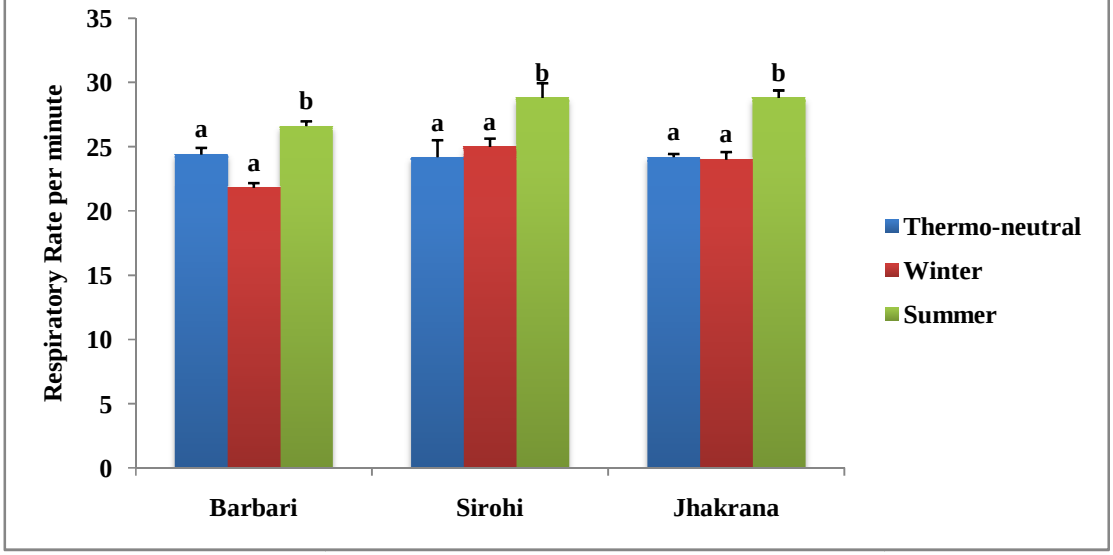


Fig. 9 Rectal Temperature of goats during different seasons

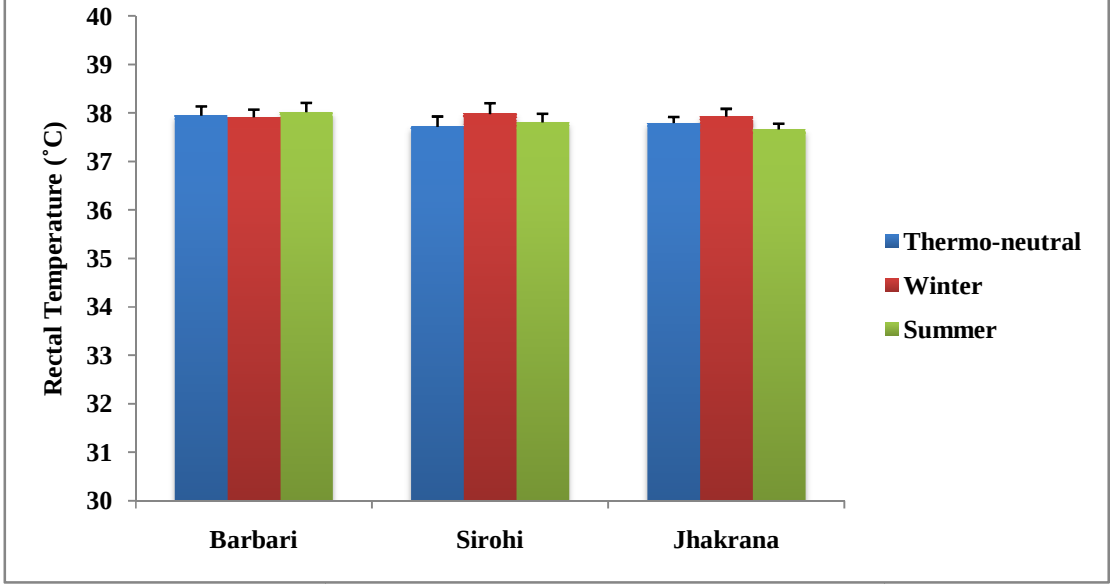


Fig. 14 HSP genes Expression in Barbari Goats

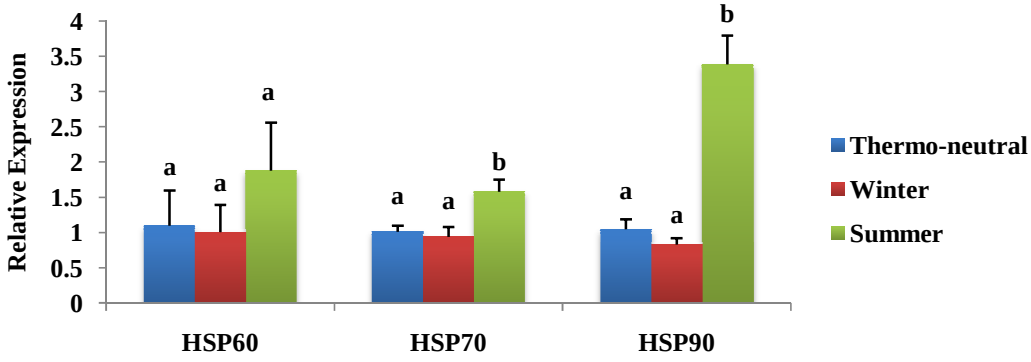


Fig. 15 HSP genes Expression in Sirohi Goats

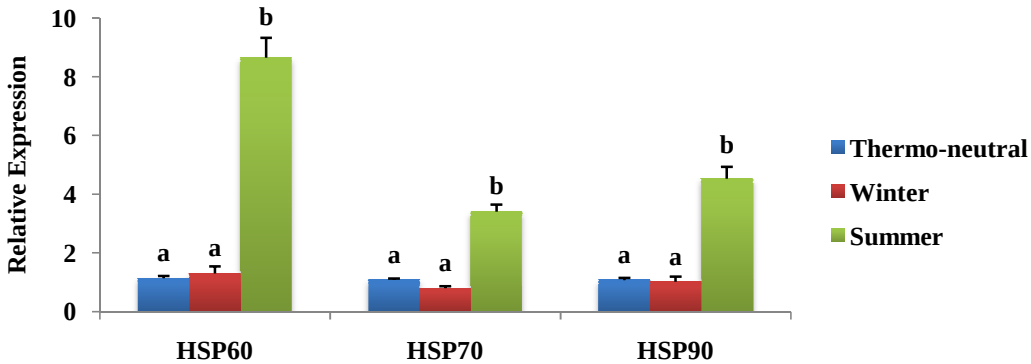
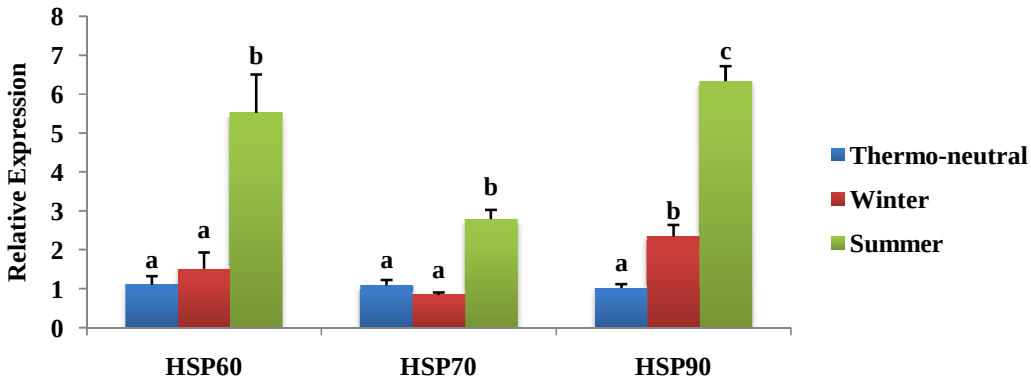


Fig. 16 HSP genes Expression in Jhakrana Goats



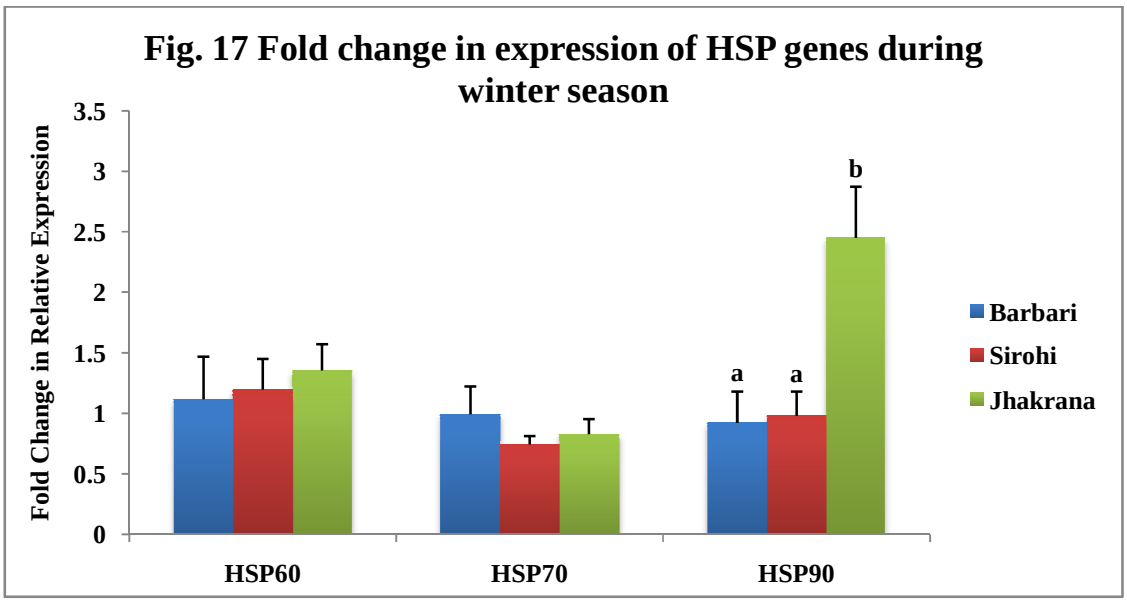


Fig. 17 Fold change in expression of HSP genes during winter season
Fold Change in Relative Expression
3.5
3
2.5
2
1.5
1
0.5
0
a
a
b
HSP60
HSP70
HSP90
Barbari
Sirohi
Jhakrana

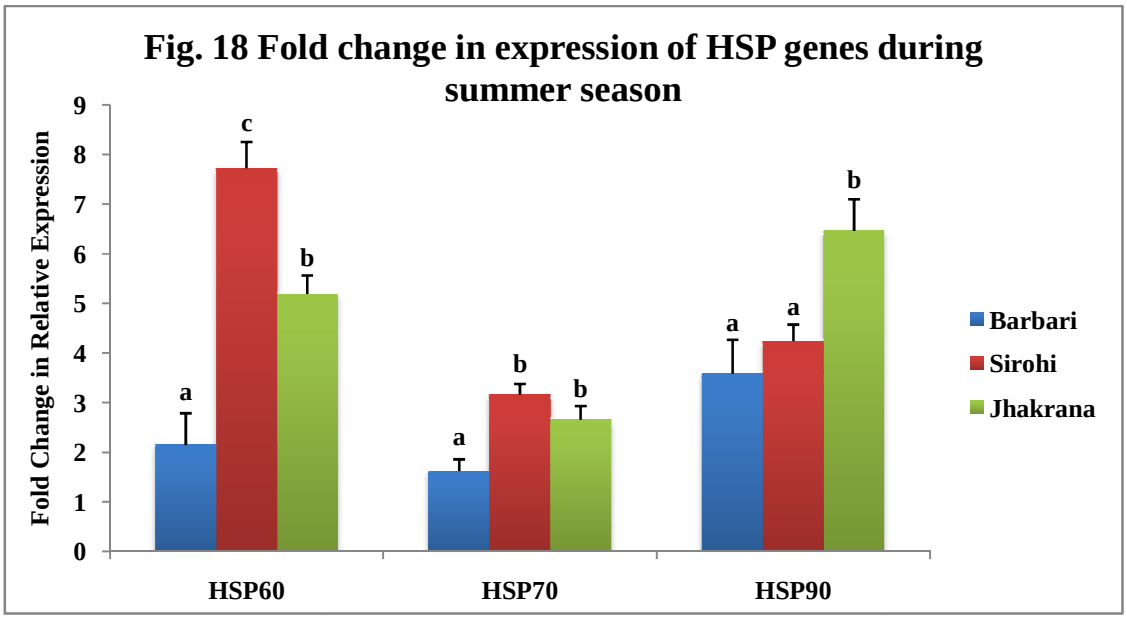


Fig. 18 Fold change in expression of HSP genes during summer season
Fold Change in Relative Expression
9
8
7
6
5
4
3
2
1
0
a
c
b
a
b
b
a
a
b
HSP60
HSP70
HSP90
Barbari
Sirohi
Jhakrana

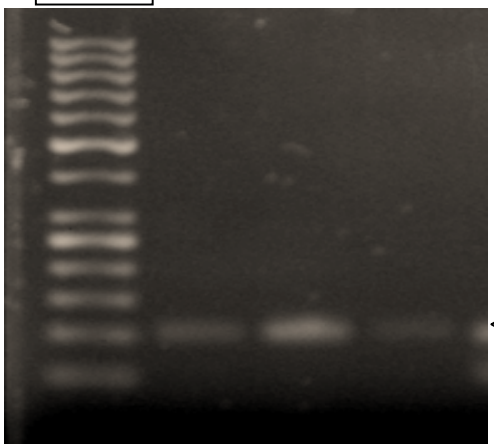


Fig. 7 cDNA confirmation with GAPDH gene

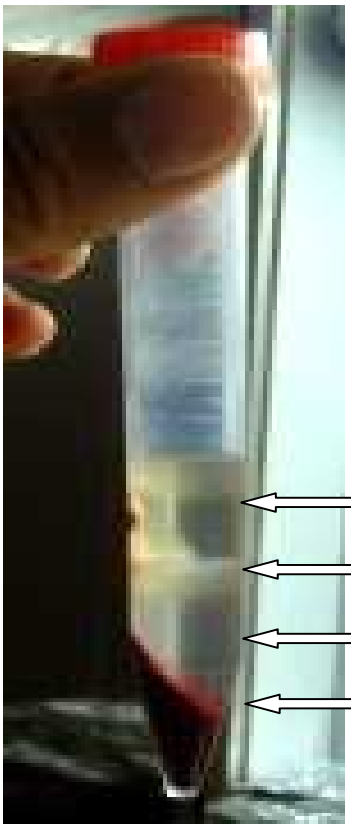


Fig. 5 PBMCs Separation

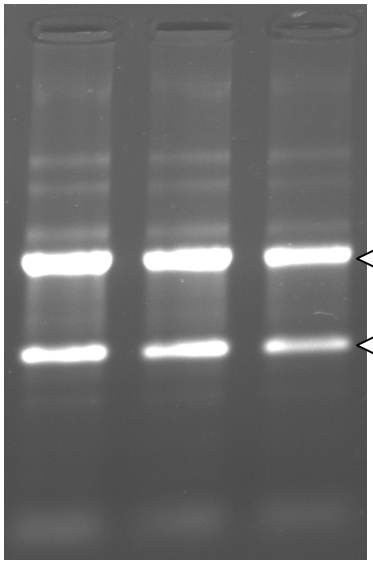


Fig. 6 RNA confirmation in gel electrophoresis

CHAPTER-V

DISCUSSION

Environmental factors such as temperature, humidity, solar radiation, wind velocity affect the livestock production. Environmental stresses reduce production parameters like growth, milk yield and reproduction in livestock leading to severe economic constraints (El-Tarabany and El-Bayoumi, 2015). The main physical factors affecting the dairy animals and other livestock are environmental temperature, relative humidity, rain fall, radient heat, atmospheric pressure UV rays, wind velocity and dust. The climatic factors directly affect the livestock by affecting the energy dynamics between the environment and the animal specially the heat loss and heat gain. In attempt to acclimatize with variation in climatic factors, several neuro-humoral responses, behavioral and physiological changes occur in animals to minimize the change in the normal physiological functions. The animals of different agro-climatic zones are naturally equipped with different mechanisms to acclimatize/adapt themselves for any change in their respective climate (Marai *et al*., 2007; Gupta *et al*., 2013).

Temperature Humidity Index (THI) during the experiment

The level of heat stress in animals cannot be measured commensurately using a single climatic variable and therefore temperature humidity index (THI) is used to measure the level of heat stress in different livestock animals. THI less than 72 is considered to be comfortable whereas THI between 75 to 78 is considered to be moderately stressful and, THI more than 78 is considered be extremely stressful. However, for Indian conditions the same THI may not be suitable to define heat stress levels. In present study, the experiment was carried out in winter (January), thermo-neutral (March) and summer (May). In terms of THI, the values were 59.63, 72.1 and 81.63 in January, March and May respectively. The THI values in respective seasons indicated that the January (winter) and March (thermo-neutral) months were comfortable whereas the month of May (summer) was extremely stressful.

PHYSIOLOGICAL RESPONSES

Respiratory Rate (RR)

Physiological changes are the preliminary responses to know that the animal is in stress condition. In physiological responses respiratory rate is considered to be the first observation during heat stress. Respiration rate is the most consistent of all the physiological responses studied and affected more by solar radiation than by other influences. Respiration rate (RR) has been used to evaluate the level of heat stress in goats (El-Tarabany *et al*., 2016;

Thomas *et al*., 2015). In present study, the RR during winter and thermo-neutral season i.e. during low and moderate THI was similar and comparable to the reference RR of adult goats (Pugh and Baird, 2012). In all the goat breeds, the RR during summer season i.e. during high THI, increased which indicated that animals were stressed. Under heat stress conditions, increased RR is an established mechanism for dissipating thermal load by evaporation (Hamzaoui *et al*. 2013). However, Devendra (1987) reported that changes in the respiration rate of heat-stressed goats could be attributed to the changes in metabolism and muscle activity.

The RR may vary in response to similar heat stress in different goat breeds however in present study, variation in respiratory rate was not observed in Barbari, Sirohi and Jhakrana goats. With increase in THI, the RR has been found to increase in different goat breeds by different researchers (Srikandakumar *et al*., 2003; Phulia *et al*., 2010; Thomas *et al*., 2015; El-Tarabany *et al*., 2016). Medeiros *et al*., (2015) used the respiratory rate as a parameter to study the comparative heat tolerance among African and European origin goat breeds and concluded that African goat breeds having less increase in RR after exposure to heat stress had better heat tolerance as compared to European origin goat breeds.

Rectal Temperature (RT)

Rectal temperature (RT) is an indicator of thermal stress and may be used to assess the environmental condition which can affect the growth, lactation, and reproduction of goats. To assess the animal stress and welfare rectal temperature is considered as gold standard (Falkenberg *et al*. 2014). Swenson and Reece (2006) reported that the RT in goats varied from 38.3 to 40.0°C and has been frequently used as an indicator of the body temperature of the animals. In present study, the RT did not change during moderate and high THI in all the breeds which was contrary to the other. The RT was found to be increased when the animal was subjected to hot climate (Popoola *et al*., 2014). Similar findings were reported in sheep (Marai *et al*., 2007) and goat (Srikandakumar *et al*., 2003; Phulia *et al*., 2010; Thomas *et al*., 2015; El-Tarabany *et al*., 2016). The increase in rectal temperature occurs only when the physical and physiological mechanisms of the animal fails to maintain homeothermy (Abdel-Hafez, 2002). In our study, no increment in RT even during high THI (summer) indicated that all the goat breeds were better adapted to heat stress and physical and mechanism activated in response to increased THI were sufficient enough to prevent any visible change in RT. It is important to note that in present study, during all the seasons the RT was recorded between 09:00 to 10:00 hrs and during these hours the heat load of goats was not sufficient enough to show an increase in RT. Moreover, all the breeds under the study are well adapted to semi-arid environment where day temperature is very high.

In present study, it was not possible to establish differential adaptability of Barbari, Sirohi and Jhakrana goat breeds on the basis of physiological parameters and therefore molecular markers of heat stress and stress sensitive chaperone genes were used to establish the differential adaptability of goat breeds.

HSP genes Expression

Despite animal's physiological and behavioral response to ameliorate the discomfort of thermal stress, there might be some molecular mechanisms to maintain their cellular homeostasis. Further, there are numerous intrinsic mechanisms in the cell which protect it from deleterious effects of environmental stress and maintain homeostasis, release of HSPs is one those mechanisms. HSP is considered as potential indicator of animal adaptation to harsh environmental stress and its expression has been correlated with resistance to stress (Feder and Hofmann, 1999). Increased diversity of HSPs as well as accumulation of larger amounts of HSPs may contribute to survival in thermally stressful environments. Differences in HSP expression have been found in closely related organisms which occupy different environmental niches. Several HSP families have been identified as molecular chaperones. Chaperones associate transiently with unfolded polypeptides, preventing premature folding of nascent polypeptide chains or aggregation of unfolded proteins, thus facilitating proper folding and or assembly of these polypeptides (Ellis, 1990). Different chaperones are found in different sub cellular compartments and cooperate in the translocation of proteins across intracellular membranes, a process which requires the translocated polypeptide to be maintained in an unfolded, extended configuration until it reaches the proper compartment. Constitutively expressed forms of HSP60, HSP70, and HSP90 perform this function in unstressed cells (Hendrick and Hart, 1993). There is evidence that heat and other stressors cause the accumulation of damaged proteins, resulting in the induction of additional HSP synthesis (Edington *et al*., 1989; Hightower, 1991). Induced HSPs could also function as chaperones, facilitating the refolding of damaged proteins or targeting them for degradation. Thus HSPs would be a critical component of cellular defenses against any type of stress.

Several studies in bovines, caprines and other species have concluded that HSP genes could be effectively used to study the heat tolerance or adaptability however; to the best of our knowledge, studies have been designed to study the heat tolerance between the goat breeds of tropical and subtropical region and, temperate region or between the goat of cold and heat adapted breeds. But, in present investigation the relative expression profile of HSP60, HSP70 and HSP90 was studied in Barbari, Sirohi and Jhakrana breeds of semi-arid region during three different seasons (with three different THI) to establish the comparative adaptability of these breeds.

HSP60

Mammalian HSP60 was first reported as a mitochondrial P1 protein (Gupta, 1995). HSP60 in eukaryotes is considered typically a mitochondrial chaperone (also called Cpn60) which also occurs in the cytosol, the cell surface, the extracellular space and in the peripheral blood under normal physiological conditions (Cappello *et al*., 2008; Itoh *et al*., 2002). HSP60 is also known to be one of the most important molecular chaperons under various stressful conditions (Oksala *et al*. 2006). In present study, the relative mRNA expression of HSP60 gene in PBMCs was similar during thermo-neutral and winter season (low and moderate THI) and the expression was comparatively higher during summer (high THI) in all the goat breeds under study. Results indicated that the increased HSP60 mRNA expression during summer season in goats could evoke its transcription in PBMCs to prevent cell from damaging effect of heat stress like denaturation of proteins and helps in refolding of proteins and prevents aggregation of denatured proteins. Dangi *et al*. (2012, 2014, 2015) reported that the mRNA expression of HSP60 was higher during peak summer season as compared with peak winter season in both tropical and temperate region goats however, in the temperate region a non-significant difference in HSP70 expression between summer and winter seasons was noticed. Relative expression of HSP60 gene was reported to increase in Barbari goats after exposure to higher temperature which provided initial protective effect. However, if the heat stressed animals were supplemented with antioxidant, the relative mRNA expression was reduced. Sharma *et al*. (2013) recorded upregulation of HSP60 gene during heat stress in Barbari goats and reported that administration of melatonin upregulated the HSP60 gene expression many folds. Kishore *et al*. (2013) exposed the PBMCs of Buffalo, Holstein Friesian (HF) and Sahiwal to sub-lethal temperature and found that increase in relative expression of HSP60 was minimum in case of Sahiwal cattle and concluded that Sahiwal was more heat tolerant than HF.

HSP70

HSP70s are a family of ubiquitously expressed heat shock proteins. It is found in prokaryotes and eukaryotes (Tavaria *et al*., 1996; Yoshimune *et al*., 2002) and is mainly localized in the cytosol, mitochondria and ER and exhibit constitutive and inducible regulation. HSP70 was found to be the most sensitive to temperature fluctuation, and it could be used as an important molecular biomarker to heat stress in animals (Dangi *et al*., 2015). In present study, the relative mRNA expression of HSP70 gene in PBMCs was similar during thermo-neutral and winter season (low and moderate THI) and the expression was comparatively higher during summer (high THI) in all the goat breeds under study. The increased relative expression of HSP70 gene during high THI may be attributed to the

protective function of the HSP70 protein. The mRNA expression of HSP70 gene was recorded to be upregulated during heat stress and high THI in goats (Dangi *et al*., 2012, 2014, 2015; Sharma *et al*., 2013; Mohanrao *et al*. 2014; Shilja *et al*., 2015; Yadav *et al*., 2016; Rout *et al*., 2016). Irrespective of stocking density, transportation under hot, humid tropical conditions significantly increased HSP70 levels in the kidneys of goats (Zulkifli *et al*., 2010). The expression of HSP70 genes (HSPA8, HSPA6, HSPA1A, HSPA1L and HSPA2) showed temperature sensitivity and seasonal variation and during summer, the relative expressions of all the HSP70 genes were higher in cold-adapted breeds (Gaddi and Chegu) than the heat-adapted breeds (Sirohi and Barbari) (Banerjee *et al*., 2013). In Chegu, i.e., a cold-adapted breed, the expression of all the genes was higher as compared to the other cold adapted breed of goat, Gaddi, during summer. The HSPA8 and HSPA1A expression during winter in heat-adapted breeds was observed to be higher than cold-adapted breeds. In present study, relative mRNA expression of HSP70 gene did not change during winter season in none of the breeds under study however, an increase in HSP70 gene expression was reported in winter (Banerjee *et al*. 2013) even in heat-adapted breeds (Sirohi and Barbari). On contrary, Yadav *et al*. (2016) did not observe any change in HSP70 expression during winter as compared to moderate season. Mohanrao *et al*. (2014) reported that expression of HSP70 gene increased in heat stressed PBMCs but did not change in cold stressed PBMCs *in vitro*.

In bovines, heat shock induced HSP70 in the lymphocytes (Guerriero and Raynes, 1990; Kamwanja *et al*., 1994; Lacetera *et al*., 2006; Patir and Upadhyay, 2007). A two hundred fold increase in HSP70 levels in serum and 2.5 fold increase in lymphocytes was observed after exposure at 42°C and 70% RH in buffaloes (Mishra *et al*., 2010). The mRNA level of HSP70 in lymphocytes was increased with increase in THI as well as temperature in dairy cows (Liu *et al*., 2010). In buffalo, higher intensity and duration of temperature exposure caused higher HSP70 induction in lymphocytes to maintain cellular homeostasis (Patir and Upadhyay, 2010). Kishore *et al*. (2013) exposed the PBMCs of Buffalo, Holstein Friesian and Sahiwal to sub-lethal temperature and found that increase in relative expression of HSP70 increased in all the animals. *In vitro* studies in dermal fibroblasts suggest that induced heat stress up-regulated the expression of all HSP70 genes (HSPA8, HSPA1A and HSPA2) at different temperatures in both Tharparkar and Karan-Fries cattle. At 40 and 44°C, the relative expressions of inducible HSP70 genes (HSPA1A and HSPA2) were higher in Karan-Fries than Tharparkar (Singh *et al*., 2014).

HSP90

HSP90 is a molecular chaperone and is one of the most abundant proteins expressed in cells (Csermely *et al*., 1998). It has been identified in the cytosol, nucleus and ER, and is reported to exist in many tissues (Kunisawa and Shastri, 2006). In present study, the relative

mRNA expression of HSP90 gene in PBMCs was similar during thermo-neutral and winter season (low and moderate THI) in Barbari and Sirohi breeds and the expression was comparatively higher during summer (high THI) in all the goat breeds under study. Unlike other genes, the relative expression of HSP90 during winter was higher as compared to thermo-neutral in Jhakrana goat breed. The mRNA expression of HSP90 gene was recorded to be increased during heat stress and high THI in goats (Dangi *et al*., 2012, 2015; Sharma *et al*., 2013). Dangi *et al*. (2012) and Yadav *et al*. (2016) reported that the mRNA expression of HSP90 was higher during peak summer season as compared with peak winter season in both tropical and temperate region goats. Relative expression of HSP90 gene was reported to increase in Barbari goats after exposure to higher temperature which provided initial protective effect. However, if the heat stressed animals were supplemented with antioxidant, the relative mRNA expression was reduced (Dangi *et al*., 2014). Kishore *et al*. (2013) exposed the PBMCs of Buffalo, Holstein Friesian and Sahiwal to sub-lethal temperature and found that increase in relative expression of HSP90 was minimum in case of Sahiwal cattle. Yadav *et al*. (2016) also did not observe any change in HSP90 expression during winter as compared to moderate season in accordance with the findings of the present study. Deb *et al*. (2014) reported a higher level of mRNA transcripts as well as protein concentration in Sahiwal cattle as compared to the Frieswal breed during high THI.

Comparative change in expression of HSP genes in goat breeds in winter and summer

In previous segments, discussion was based on the relative expression of genes in different breeds. However the information was not enough to come to a conclusion regarding the comparative stress tolerance (heat tolerance) of breeds under study. In present study, it was hypothesized that quantitative change in gene expression during winter and summer with respect to thermo-neutral season would provide a more appropriate data to compare the stress tolerance in different breeds under study. It was also hypothesized that the breeds with lesser change in relative gene expression during winter and summer season would be considered as more stress tolerant. The change in relative gene expression in adverse conditions has been used to determine the comparative heat tolerance in different breeds of cattle (Kishore *et al*., 2013; Singh *et al*., 2014 and Deb *et al*., 2014) and goat (Rout *et al*., 2016). Deb *et al*. (2014) and Rout *et al*. (2016) correlated the heat tolerance with more increase in relative HSP gene expression whereas, Kishore *et al*. (2013) and Singh *et al*. (2014) correlated heat tolerance with less increase in relative HSP gene expression.

In present study, the change in relative gene expression of HSP60 and HSP70 during winter season (cold stress) was similar in all breeds however, the change in expression of HSP90 was higher in Jhakrana as compared to Barbari and Sirohi breeds. The results indicated that the Barbari and Sirohi breeds were better adapted as compared to Jhakrana

breed under the prevailing cold temperature in semi-arid region. Our findings are in consonance with reports of Yadav *et al*. (2016). On contrary, Banerjee *et al*. (2013) reported an increase in HSP70 expression during winter in Sirohi and Barbari breeds however these finding were not compared with thermo-neutral seasons.

In present study, the change in relative gene expression of all the genes during summer season (high THI) was different in Barbari, Sirohi and Jhakrana breeds. The change in relative gene expression of HSP60 during high THI with respect to thermo-neutral season was 7.72, 5.18 and 2.14 folds in Sirohi, Jhakrana and Barabari respectively. The change in relative gene expression of HSP70 during high THI with respect to thermo-neutral season was 3.16, 2.65 and 1.62 folds in Sirohi, Jhakrana and Barabari respectively. Results with respect to change in HSP60 and HSP70 indicated that Barbari breed showed least change in gene expression as compared to that of gene expression during the thermal comfort season, followed by Jhakrana and Sirohi. Rout *et al*. (2016) also found the similar trend in HSP70 gene expression in the different tissues of heat stressed goat breeds. The change in relative gene expression of HSP90 during high THI with respect to thermo-neutral season was 6.46 4.23 and 3.58 folds in Jhakrana, Sirohi and Barabari respectively. The results with respect to change in HSP90 gene expression also suggested that there was minimum variation in Barbari breed as compared to Jhakrana and Sirohi breeds. As per our hypothesis a heat tolerant breed would have minimum change in HSP genes expression and therefore results of our study indicated that Barbari was most heat tolerant breed followed by Sirohi and Jhakrana.

Kishore *et al*. (2013) exposed the PBMCs of Buffalo, Holstein Friesian and Sahiwal to sub-lethal temperature and found that increase in relative expression of HSP70 was minimum in case of Sahiwal cattle and concluded that Sahiwal was more heat tolerant than HF. *In vitro* studies in dermal fibroblasts suggest that induced heat stress up-regulated the expression of all HSP70 genes (HSPA8, HSPA1A and HSPA2) at different temperatures in both Tharparkar and Karan-Fries cattle. At 40 and 44°C, the relative expressions of inducible HSP70 genes (HSPA1A and HSPA2) were higher in Karan-Fries than Tharparkar (Singh *et al*., 2014). On the basis of this study it was concluded that zebu cattle (Tharparkar) dermal fibroblasts are more adapted to tropical climate condition than crossbreed cattle (Karan-Fries).

CHAPTER-VI

SUMMARY AND CONCLUSION

Goats are the most adapted and geographically widespread livestock species and play an important role in rural economy. The goat can survive in all types of agro-climatic conditions where larger domestic animals cannot. Livestock species experiences stress of varying degree due to exposure to thermal challenges like hypothermia or hyperthermia. Environmental stressors affect behavioral and physiological measures such as sweating, panting, drinking water by regulating their metabolic rates. Despite animal's physiological and behavioral response to ameliorate the discomfort of thermal stress, there may be some molecular mechanisms to maintain their cellular homeostasis. In Indian conditions goats are prone to thermal stress when they are left outside for grazing during most of the day time where ambient temperature is high or low depending on the seasons.

Under thermal stress, transcriptional activation and accumulation of a set of proteins called heat shock proteins (HSPs) are highly conserved proteins present in all the cells of living organisms and essential for cellular viability as these have major physiological roles in protein homeostasis. HSPs are named according to their molecular weight for example HSP60, HSP70 and HSP90 are 60, 70 and 90 kD in size respectively. It has been well identified that the synthesis of HSPs is increased following exposure to elevated temperatures and account for 1-2% of total protein in unstressed cells which increased to 4-6% of cellular proteins when exposed to heat stress.

The present study was planned to examine the comparative expression profile of HSPs (HSP60, HSP70 and HSP90) during different seasons in goat breeds. Blood samples were collected from each breed *viz.* Sirohi, Jhakrana and Barbari during winter (January), thermo-neutral (March) and summer (May) season. Selected animals were 2 to 2.5 years old and had an average body weight of 25-30 kg. All of the animals were regularly monitored and similar management inputs were provided during the experimental period. The climatological data and physiological parameters during the experimental period were recorded. Recording of physiological parameters *viz.* respiratory rate, rectal temperature and collection of blood samples was done at 09:00 to 10:00 hours in all the seasons during experiment.

PBMCs were separated by density gradient centrifugation from collected blood samples. Total RNA was isolated by RiboZol RNA extraction reagent (Amresco). Concentration and purity was verified in nanodrop using Biophotometer. RNA samples having the $A_{260/280}$ values more than 1.8 were used further. The integrity of the Total RNA

was checked by agrose gel (1.5 %) electrophoresis and bands of 28s and 18s indicated good quality and intactness of RNA. Total RNA was treated with DNase I (Sigma) and reverse transcribed into cDNA using Rever Tra Ace qPCR RT master mix (TOYOBO). The integrity of the cDNA was checked by PCR with GAPDH primers and desired band of 101 bp was obtained. End point PCR was standardized using different annealing temperatures, factor specific primers and synthesized cDNA to amplify HSP60, HSP70 and HSP90 gene sequence. Products of desired length 148 bp for HSP60, 132 bp for HSP70 and 190 bp for HSP90 were confirmed by agrose gel (1.5 %) electrophoresis.

Quantitative Real time PCR was performed with Thunderbird SYBR qPCR Mix in Light Cycler 480 (Roche, Germany) real time machine. GAPDH gene expression was used as internal control. Quantification was performed by analyzing the Cp value in amplification reaction. Expression data was normalized using internal control GAPDH. Relative expression of HSPs (HSP60, HSP70 and HSP90) was analyzed by Livak method ($2^{(-\Delta\Delta Ct)}$). The thermo-neutral season values were used as calibrator for calculating relative mRNA expression. During the experimental period the temperature humidity index (THI) in winter (January), thermo-neutral (March) and summer (May) was 59.63, 72.1, 81.63 respectively.

The respiratory rate was found to be increased ($P \leq 0.001$) during summer season in all of goat breeds however the rectal temperature was found to be similar ($P > 0.05$) during all the season. The relative mRNA expression of HSP genes was higher in all of the goat breeds during summer season as compared to winter and thermo-neutral season. The fold change in relative mRNA expression of HSP60 and HSP70 genes was found to be similar ($P > 0.05$) in all of the breeds during winter season. HSP60 expression was significantly higher ($P < 0.01$) in all goats however, it was higher in Sirohi during summer season followed by Jhakrana followed by Barbari goats. The relative mRNA expression of HSP70 was found to be similar during winter. During summer season it was significantly higher ($P < 0.01$) in Sirohi and Jhakrana goats. The fold change in relative expression of HSP90 was found to be similar ($P > 0.05$) in Barbari and Sirohi goats however, the expression of HSP90 was significantly higher ($P < 0.01$) in Jhakrana goats during both winter and summer season.

The change in relative gene expression of HSP60 and HSP70 during winter season (cold stress) was similar in all breeds however; the change in expression of HSP90 was higher in Jhakrana as compared to Barbari and Sirohi. The change in relative gene expression of all the genes during summer season (high THI) was different in Barbari, Sirohi and Jhakrana breeds. The change in relative gene expression of HSP60 during high THI with respect to thermo-neutral season was 7.72, 5.18 and 2.14 folds in Sirohi, Jhakrana and Barabari respectively. The change in relative gene expression of HSP70 during high THI with respect to thermo-neutral season was 3.16, 2.65 and 1.62 folds in Sirohi, Jhakrana and Barabari respectively.

The change in relative gene expression of HSP90 during high THI with respect to thermo-neutral season was 6.46 4.23 and 3.58 folds in Jhakrana, Sirohi and Barabari respectively. As per our hypothesis a heat tolerant breed would have minimum change in HSP genes expression and therefore results of our study indicated that Barbari was most heat tolerant breed followed by Sirohi and Jhakrana.

CONCLUSIONS

On the basis of findings of present study we concluded that

1) During summer season (high THI) the RR increased in all the breeds indicating that all the goat breeds were under stress. However, physiological parameters could not be utilized to conclude about the comparative heat tolerance of the different breeds.

2) Expression of HSP genes (HSP60, HSP70 and HSP90) increased during summer season (high THI) as compared to thermo-neutral season in all the goat breeds. In Jhakrana breed, the HSP90 expression was increased during winter season as compared to thermo-neutral season. Increased expression of HSP genes during adverse condition indicated that these genes were involved in maintaining cellular homeostasis in goats.

3) The change in expression of HSP genes during summer and winter seasons with respect to thermo-neutral season in different breeds indicated that there was minimum change in expression of HSP genes in Barbari breed followed by Sirohi and Jhakrana. It can be concluded that Barbari breed possessed better heat tolerance followed by Sirohi and Jhakrana.

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Title of the thesis	**:**	**A comparative study on expression profile of HSP genes during different seasons in goat breeds**

ABSTRACT

Goats are most adapted species to all type agro-climatic conditions and play important role in rural economy. Livestock species experiences stress due to thermal challenges. Environmental stressors such as hypothermia or hyperthermia affect behavioral, physiological and molecular mechanisms. HSPs are highly conserved groups of proteins that expressed under various kinds of stresses and considered as potential indicator for animal adaptation. HSPs are group of well conserved proteins across the species that are expressed under various kinds of stresses and these are considered as potential indicator of animal adaptation. Present study was study was conducted in three different seasons *viz.* winter (January), Thermo-neutral (March) and summer (May) to examine comparative expression profile of HSP genes (HSP60, 70 and 90). Five animals from each breed of semi-arid region were selected for this study *viz.* Barbari, Sirohi and Jhakrana. All of the animals were regularly monitored and similar management inputs were provided during the experimental period. The climatological data and physiological parameters during the experimental period were recorded. Recording of physiological parameters *viz.* respiratory rate, rectal temperature and collection of blood samples was done at 09:00 to 10:00 hours in all the seasons during experiment. During the experimental period the temperature humidity index (THI) in winter (January), thermo-neutral (March) and summer (May) was 59.63, 72.1, 81.63 respectively. Blood samples were collected and PBMCs were separated. Total RNA was isolated and reverse transcribed to cDNA. Real time PCR was applied to investigate the relative mRNA expression of HSP genes. The respiratory rate was found to be higher ($P<0.01$) during summer season as compared to winter and thermo-neutral season in all the goat breeds. The rectal temperature was found to be similar during all seasons. Expression data showed significant increase in mRNA expression of HSP genes during summer season as compared to winter and thermoneutral season. Fold change in relative mRNA expression of hsp60, HSP70 and HSP90 was significantly higher ($P<0.01$) in Sirohi breed as compared to Jhakrana and Barabri. It can be concluded that Barbari breed possessed better heat tolerance followed by Sirohi and Jhakrana.

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ANNEXURES

Annexure-I

CHEMICALS, EQUIPMENTS, LAB WARE AND MISCELLANEOUS ITEMS

All chemicals used in present study were obtained from different companies like Sigma-Aldrich, Thermo Scientific, Amresco, TOYOBO, Hi-Media, Genetix as per requirement.

❖ **CHEMICALS**

Histopaque 10771	SIGMA-ALDRICH
Dulbacco's Phosphate Buffer saline	Hi-Media
DEPC	SIGMA-ALDRICH
Ribozol	Amresco
Chloroform	Amresco
Isopropyl Alcohol	Amresco
Ethanol	Amresco
Nuclease free water (DEPC treated)	Amresco
Nuclease free water	Thermo scientific
Sodium hydroxide	Amresco
Hydrochloric Acid	Amresco
Agarose	Amresco
Tris base	Amresco
Boric Acid	Amresco
EDTA	Amresco
Acetic Acid	Amresco
Xylene cyanol FF (XCFF)	Hi-Media
Glycerol 100% water free	Hi-Media
Bromophenol blue	Hi-Media
Methanol	Hi-Media
Primers	Imperial Life Sciences
Agarose (Low EEO)	Amresco
Ethidium bromide	Hi-Media
Nuclease iliminater	Amresco

❖ **EQUIPMENTS**

Major equipments used were as follows:

Agarose gel electrophoresis apparatus	BIO-RAD
Microcentrifuge	REMI

Non refrigerated Centrifuge)	REMI
-40°C Deep freezer	REMI
-80°C Deep freezer	REMI
Vortexer	GeNei
Biophotometer	Eppendorf
Real time PCR	Roche Light Cycler 480
Microwave Oven	IFB
Double distillation apparatus	PERFIT
Weighing balance	KERN
Table top centrifuge	REMI
Gel casting apparatus	BIO-RAD
Hot air oven	Viometra/SONAR
Laminar airflow	Viometra/SONAR
Micropipettes (all ranges)	Eppendorf research
pH meter	Systronics
Power supply power pack	BIO-RAD
Laboratory Refrigerator	Whirlpool
Thermal cycler	peQLab-primus96 Advanced
Water bath	MACFLOW
Shaker	MACFLOW

❖ **LAB WARES**

For RNA work, RNase-free plastic wares and glassware were used, and they were thoroughly treated with 0.1% DEPC overnight at 37 °C and next day DEPC was evaporated by incubating overnight in hot air oven at 55°C. It was further autoclaved to make it DNase and RNase free before use. For PCR and other DNA related work plastic wares were autoclaved [121°C for 25 minutes at 101.3 kPa (15 psi)] and then used.

a. GLASS WARES

Beakers	Borosil
Conical flasks	Borosil
Measuring cylinders	Borosil
Pipettes	Borosil
Reagent bottles	SCHOTT

b. PLASTIC WARES

Micro centrifuge tubes	(1.5 & 2.0 ml)	AXIVA
Centrifuge tubes (15 ml)		AXIVA
Centrifuge tubes (50 ml)		AXIVA

PCR tubes (0.2 ml)	AXIVA
Microtips (all ranges)	AXIVA
Real time PCR plates	Roche

c. MISCELLANEOUS

Adhesive tapes

Parafilm

Autoclavable lable

Scissors

Stethoscope

Thermometer

Cello tape

Thermometer

Tissue paper

Stickers

Needles

pH paper

Racks

Blotting paper

Cotton

Threads

Filter papers

Marker pens

ANNEXURE-II

BUFFERS AND REAGENTS

The details of the media, buffers and other solutions used in this study are given in this section. All reagents/chemicals used for preparation of various buffers and solutions were of molecular biology grade and prepared using autoclaved distilled/MilliQ water, whereas the buffers and reagents used for RNA work were prepared in diethylpyrocarbonate (DEPC) treated distilled water or nuclease free water.

REAGENTS FOR PBMCS ISOLATION

1. Histopaque-10771

Histopaque -10771 is an endotoxin solution of polysucrose and sodium diatrizoate with a density of 1.0771 g/mL. This medium was used to recovery of mononuclear cells from whole blood. Histopaque-10771 is a sterile solution of polysucrose, 57 g/L, and sodium diatrizoate, 90 g/L. Density: 1.076–1.078 g/mL Endotoxin: £0.3 EU/mL pH: 8.8–9.0

2. DPBS (Dulbacco's Phosphate Buffer Saline)

DPBS is a synthetic mixture of inorganic salts known as a physiological or BSS. The function of a salt solution is:

a) To maintain the medium within physiological pH range.
b) To maintain intracellular and extra cellular osmotic balance

REAGENTS FOR RNA ISOLATION

1. Ribozol

Ribozol™ RNA Extraction Reagent which contains Guanidinium thioisocynate and phenol is a single phase phenol solution which is optimized for isolation of total RNA from a variety of cells and tissues. Directly disruption or homogenization in Ribozol RNA extraction reagent inhibits RNase activity to minimize the degradation of all classes of RNA. Procedure is simple and effective for RNA isolation which includes homogenization, phase separation, RNA precipitation, RNA washing and solubilization.

Caution

Working with Ribozol some precaution must be taken because it contains Phenol which is highly corrosive and cause severe burns. Wear gloves protective clothing and safety glasses when handling phenol. All manipulations should be carried out in a chemical hood. Areas that come in contact with Ribozol should be rinsed with large volume of water.

2. Chloroform

Chloroform is used to recover the aquous phase of nucleic acids. In the extraction procedures chloroform forms a biphasic mixture.

3. Isopropyl alcohol

Isopropyl alcohol is a common reagent used for nucleic acid purification. Less volume of isopropyl alcohol is needed for ethanol precipitation.

4. 75% Ethanol

Ethanol (75%) prepared in DEPC treated water is used as a washing solution to precipitate RNA to collect a clean RNA pellet after discarding the supernatant that contained contaminating salts and proteins.

5. Nuclease free water

Nuclease free water (thermo scientific) is used to dissolve RNA pellet.

BUFFERS

1. 0.5M EDTA solution

EDTA disodium salt	18.61 g
Double Distilled Water	up to 100 ml

Adjust pH to 8.0 using NaOH pellets

Autoclave and store at room temperature

Functions:

Chelates Mg++ ions

Protects from nucleases

Makes plasma membrane fragile

2. 1M Tris

a) Using Tris HCl

Tris HCl	15.76 g
Distilled Water	up to 100 ml

Adjust pH to 8.0 with NaOH pellets and Autoclave

b) Using Tris Base

Tris base	12.11 g
Double distilled water	up to 80 ml

Allow the solution to cool to room temperature before making final adjustment of pH and autoclave

Adjust pH at 8.0 to 7.6 using HCl

3. 10 X TAE Buffer

1 M Tris HCl (pH 8.0)	450 ml
0.5M EDTA (pH 8.0)	10 ml
Distilled water	40 ml
Total volume	500 ml

4. PBS

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Distilled water	up to 1000 ml
Adjust pH to 7.4 with HCl	
Autoclave and store at room temperature	

5. 5X TBE Buffer

Tris base	5.4 gm
Boric acid	2.75 gm
0.5M EDTA	2 ml
Autoclaved distilled water	up to100 ml
Autoclave and store at room temperature	

6. 6X loading dye

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in H_2O	30%
Mix and store at 4°C	

Function

1) Increase the density of the sample ensuring that the DNA drops evenly into the well.
2) Add color to the sample, which help in loading process.
3) Dye that moves in an electric field towards the anode at predictable rates.

Bromophenol blue migrates through agarose gel approximately 2.2 fold faster than xylene cyanol FF, independent of the agarose concentration.

7. Ethidium bromide (10mg/ml)

Ethidium bromide	10 mg
Autoclave distilled water up to	1 ml
Wrap in aluminium foil	

ANNEXURE-III

ENZYMES AND BIOLOGICALS

All the enzymes and biologicals were purchased from thermo Scientific, TOYOBO, SIGMA-ALDRICH

1. PRIMERS

Lyophilized content form of primer were firstly dissolved in nuclease free water for preparing Stock primer – 100 pmol/µl by vortex following centrifugation and kept overnight at 4˚C to complete dissolve.

Working primer

Add 20 µl from stock in 180 µl of nuclease free water

2. DNase I

DNase I (Sigma) is an endonuclease isolated from bovine pancreas that digests double and single stranded DNA into oligo and mononucleotides. Amplification Grade DNase I has been purified to remove RNase activity, and is suitable for illuminating DNA from RNA preparations prior to sensitive applications such as RT-PCR (Reverse Transcriptase – Polymerase Chain Reaction).

3. c-DNA synthesis kit

Rever Tra Ace® qPCR RT Master Mix (TOYOBO) contains 5X RT Master Mix that contains highly efficient reverse transcriptase "Rever Tra Ace®", RNase inhibitor, oligo dT primer, random primer, dNTPs, $MgCl_2$ buffer optimized for synthesis of short-chain cDNA suitable for real-time PCR.

4. PCR Master Mix

Dream Taq PCR Master Mix **(**thermo scientific)

5. qPCR Mix

THUNDERBIRD SYBR qPCR Mix **(**TOYOBO)

6. 50 bp DNA Ladder (thermo Scientific/MBI Fermentas)

It contains 13 discrete fragments in bp, starting from 1031 bp and ends at 50 bp.

CURRICULUM VITAE OF STUDENT

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Academic Qualifications

Degree	University /Board	Year Passing	Percentage of marks	Subjects
Masters (M.Sc.)	Veterinary University DUVASU, Mathura	2016	7.94 OGPA	Major Specialization-Biotechnology
Graduation (B.Sc.)	DBRAU, Agra	2013	61.25%	Zoology, Botany, Chemistry
Intermediate	Board of High School and Intermediate Education, U.P	2010	66.40 %	General Hindi, English, Physics, Chemistry, Biology
High School	Board of High School and Intermediate Education, U.P	2008	60.83 %	Hindi, English, Mathematics, Science, Social Science, Drawing

EXTRA-CURRICULAR ACTIVITY

1. Actively participated as a volunteer in successful organization of various national conferences and seminar held at the university

MEDALS/HONOURS RECEIVED :

LIST OF PUBLICATION :

UNDERTAKING OF COPY RIGHT

I **Devendra Kumar,** Enrolment No. **B-1386/14** undertake that I give copy right to DUVASU, Mathura of my thesis entitled "**A Comperative Study on Expression Profile of HSP Genes during Different Seasons in Goat breeds.**"

I also undertake that patent, if any, arising out of research work conducted during the program shall be filed by me only with due permission of the competent authority of DUVASU, Mathura (UP).

Kumar
25/07/16

Signature of the student