MOLECULAR CHARACTERIZATION OF *HSP70-1* GENE IN BEETAL GOAT

By

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CERTIFICATE–I

This is to certify that the thesis entitled **"Molecular characterization of *HSP70-1* gene in Beetal goat"** submitted in partial fulfillment of the requirements for the degree of **Masters in Animal Genetics and Breeding,** is a record of bonafide research, carried out by **Mr. Kashif Dawood Khan**, Registration No. **J-18-MV-518** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

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"IN THE NAME OF ALMIGHTY THE MOST GRACIOUS,
THE MOST BENEFICENT AND THE MOST MERCIFUL"

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Needless to say, all omissions and errors are mine.

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ABSTRACT

Title of the Thesis	:	**Molecular characterization of *HSP70-1* gene in Beetal goat**
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ABSTRACT

HSP70 play a variety of functions in the cells and are responsible for cytoprotection under stress conditions. The present study was conducted to carry out molecular characterization of *HSP70-1* gene in Beetal goat and analyzing genetic diversity through sequences available in the databases. Genomic DNA was extracted from whole blood of Beetal goat. *HSP70-1* gene was amplified using specific primers designed by Primer 3 software. The amplified PCR product (1917 bp) was sequenced by Sangers dideoxy chain termination method with "Primer Walking". A partial cDNA sequence of goat *HSP70-1* gene of 1839 bp encoding 613 amino acids was obtained. Comparison of nucleotide and deduced amino acid sequences of Beetal goat *HSP70-1* gene was done with different species of animals. The comparison of Beetal goat *HSP70-1* partial cDNA sequence demonstrated 99.08, 98.10 and 97.66 percent homology with sheep, buffalo and cattle, respectively which indicates close evolutionary relationship and high sequence homology among the species. Deduced amino acid sequence of 613 residues of Beetal goat *HSP70-1* gene was 98.83 and 98.99 percent similar to cattle and buffalo, respectively. The phylogenetic tree drawn by MEGA X software at nucleotide level showed conserved nature of *HSP70-1* gene. The pair wise distance between sequences aligned with ClustalW method was estimated by MEGA X software. Maximum divergence from Beetal partial cDNA was observed with chicken CDS and minimum with sheep. Z test based on relative abundance of synonymous and non synonymous substitution indicates purifying selection and not positive selection operating on *HSP70-1* gene.

Key words: *HSP70-1* gene, PCR, Sequencing, Homology, Beetal goat

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

%	Percent
°C	Degree Celsius
µ	Micron
µg	Microgram
µl	Microlitre
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
Bp	Base pairs
CDS	Coding sequences
Da	Daltons
d_N	Nonsynonymous substitutions
DNA	Deoxy ribonucleic acid
dNTPs	deoxynucleotide triphosphates
d_S	Synonymous substitutions
EBr	Ethidium bromide
EDTA	Ethylene diamine tetra acetate
EEO	Electroendosmosis
et al.,	Any other people
HSP	Heat shock proteins
Kb	Kilo base
KDa	Kilo Daltons
$Mgcl_2$	Magnesium chloride
min	Minutes

ml	Millilitre
MSA	Multiple sequence alignment
NCBI	National Center for Biotechnology Information
nm	Nanometer
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribo nucleic acid
TBE	Tris borate EDTA
T_m	Melting temperature
UV	Ultraviolet
V	Volt

CHAPTER - I

INTRODUCTION

Goat (*Capra hircus*) popularly known as Poor Man's cow is one of the earliest domesticated species found in Indian subcontinent since 9000 B.C (Gupta, 2004). Rearing of goat is an important economic operation for poor and marginal farmers of developing countries such as India. According to 20th National Livestock census report 2019, total goat population in India is 148.88 million goats which show an increase of 10.14% over previous Livestock census (2012). About 27.7% of total livestock is contributed by goats. Rajasthan has the highest number of population among Indian states. Out of the total number Jammu and Kashmir holds about 1.98 million goats (http://dahd.nic.in/division/provisional-key-results-20th-livestock census).

Among domestic ruminants, goats are less liable to environmental stress as they exhibit water conservation capability, lower basal metabolism, higher rate of respiration, higher temperature of skin, higher sweating rate, constant heart rate and cardiac output (Robertshaw, 1968 and Shkolnik *et al*., 1972). But in Indian conditions goats are usually left for grazing during day time and are more susceptible to heat stress because of high ambient temperature which could unfavorably affect their energy efficiency and productivity.

Beetal one of the heaviest dairy type breed of goat found in Northern India. These animals are characterized by large size, long drooping ears and roman nose. The breed is found in the Gurdaspur and Amritsar districts of Punjab (Tantia *et al*., 2001). Due to high body weight, large body size, good milking potential and reasonable fecundity rate, Beetal is highly preferred breed for intensive goat farming in tropical and sub-tropical regions of the world (Khan and Ashfaq, 2012). Goat flocks are mainly stationery and browsing is the main source of feed and fodder for these goats.

Among different environmental stresses, heat stress is the major concern for the livestock sector. In a tropical country like India, temperature in the summer reaches around 37–45°C which affect the thermal comfort zone of the animals. Further, the

productive performances of animals including the milk yield, growth and reproductive efficiency are negatively impacted due to thermal stress (Pragna *et al*., 2017).

When the cells are exposed to heat stress, it may show changes in most of its original functions, such as translation, transcription and protein synthesis (Negri *et al*., 2013). There are specific proteins which show altered expression under the adaptive cellular mechanism such as slick hair gene and heat shock proteins (HSPs) (Bhat *et al*., 2016). Among these proteins, HSP are well-established stress proteins which acts as molecular chaperones and prevents the misfolding and misappropriation of proteins within the cells (Shiber and Ravid, 2014). Further, HSPs are also identified to be the confirmatory biomarker for assessing the thermo-tolerance of the livestock species (Sejian, 2013; Archana *et al*., 2017). These identified biomarkers may pave way for development of thermo-tolerant breeds through marker assisted selection breeding program which can adapt and produce optimally in the current climate change scenario (Sejian *et al.,* 2017).

Heat shock proteins are ubiquitous, occurring in all organisms from bacteria and yeast to humans (Kregel, 2002). These proteins can be categorised into different families according to their molecular weight like HSP110, HSP100, HSP90, HSP70, HSP60, HSP40 and small HSP families (Feder and Hofmann, 1999). HSPs play important physiological roles in normal conditions and situations involving both systemic and cellular stress. HSPs were first discovered in 1962 (Ritossa, 1962), have strong cytoprotective effects that are involved in many regulatory pathways (Hightower, 1991; Lindquist and Craig, 1988).

Many functional roles for HSPs are known, but the mechanisms for these multiple functions are not entirely understood. It has been postulated that the determination of these mechanisms would permit the design of more precise ways to combat cellular stress in a variety of clinical conditions such as immunologic diseases, cancer, cardiovascular diseases and aging (Benjamin and Mcmillan, 1998; Garrido *et al*., 2001; Hall *et al*., 2000; Moseley, 2000; Stojadinovic *et al*., 1997; Kiang and Tsokos, 1998). HSPs also offer the potential to be used as markers of cellular injury and for diagnostic and therapeutic purposes.

Among all HSPs, HSP70 is a vital molecular chaperone of prime significance to all mammalian cells. HSP70 proteins are the most abundant among all the HSPs. One of the first physiological functions related to HSP70 was acquired thermotolerance defined as the ability of cell or organism to become resistant to heat stress after a prior sub lethal heat exposure (Landry *et al*., 1982; Landry and Chretien, 1983; Li *et al*., 1983; Li and Werb, 1982; Mizzen and Welch, 1988; Moseley, 1997). HSP70 can be found in different cellular compartments (nucleus, cytosolic, mitochondrial, endoplasmic reticulum, etc (Flaherty *et al*., 1990). There exist a number of proteins in HSP70 family. Some of them are present only under stress conditions, while some are present in cells under normal growth conditions and are not thermal inductive (constitutive) (Flaherty *et al*., 1990; Snutch *et al*., 1988). HSP70 have been found to play a neuroprotective role in several models of neuro degeneration both in vivo and in vitro. In addition to this, HSP70 directly inhibits cellular apoptosis.

HSP70 family of gene in goats comprises of *HSP70-1*, *HSP70-2*, *HSP70-3* and *HSP70-4* gene. *HSP70-1* is an intronless gene. It is positioned on chromosome 23 of bovine and consists of 1926 nucleotides. Restriction fragment length polymorphism (RFLP) study in goat revealed the position of *HSP70* genes within the major histocompatibility complex class I (Cameron *et al*., 1990). The spontaneous expression of *HSP70* protein is mostly because of transcription of vital gene, *HSP70-1* (Christians *et al*., 1997). HSP70 protein of goat has a molecular weight of 70190.56 Dalton and consists of 641 amino acids, out of which 92 are strongly acidic (-) amino acids and 82 are strongly basic (+) amino acids. Further, hydrophobic amino acids are 220 and 151 amino acids are polar in nature (Gade *et al*., 2010).

Limited reports are available regarding the role of *HSP70-1* during heat stress in goat. Considering the importance of *HSP70-1* gene in providing thermotolerance, the present work has been undertaken with the following objectives.

1. Molecular characterization of *HSP70-1* gene of Beetal goat.

2. Analysing genetic diversity through sequences available in the databases.

CHAPTER - II

REVIEW OF LITERATURE

2.1 Heat shock protein (HSP)

Heat shock proteins (HSP) are a family of proteins expressed in response to a wide range of biotic and abiotic stress. They are thus also referred to as stress proteins (Legare *et al*., 2004). Heat shock proteins (HSPs) are highly preserved polypeptides, lodging almost in any cell. They participate in maintaining protein homeostasis and cell survival (Sharma and Masison, 2009). These proteins are induced by heat and many other stresses like infection; inflammation; exercise; exposure to toxins like ethanol, arsenic, heavy metals, and pesticides; starvation; water stress; oxygen deprivation; cold; and salinity (Waters *et al*., 1996; Boston *et al*., 1996; Vierling, 1991). HSPs have been classified into several classes based on their molecular weight, such as HSP90 (85-90 kDa), HSP70 (68-73 kDa), HSP60, HSP47, and small HSPs (12-43 kDa) (Park *et al*., 2007). Recently, a new classification has been approved where a combination of letters and numbers was used. For example; HSP70 family was given letter A and the family members were denoted with numbers like *HSPA1*, *HSPA2*, and *HSPA3* (Kampinga *et al*., 2009). In addition, HSPs are classified according to their expression behavior. Some HSP members are constitutively expressed inside the cell and are crucial for quality control of protein folding and, therefore, referred to as housekeeping HSPs while expression of other proteins is strongly increased after stress conditions, hence referred to as inducible HSPs. Moreover, HSPs can be functionally distinguished according to ATP requirement. HSPs of high molecular weight like HSP70, HSP90 and HSP100 have ATPase activity and act in an ATP dependent fashion and are, therefore, called foldases, whereas HSPs with lower molecular weight like sHSPs are ATP-independent and are called "holdases" (Carra *et al*., 2017; Shin and Tan, 2016). Other aspect concerning HSPs classification is their intracellular localization. For example, among HSP70 or HSPA family; *HSPA1L* and *HSPA2* are localized in the cytosol while *HSPA9*, also called Mortalin, is localized in the mitochondria and *HSPA5* or BiP is localized in the endoplasmic reticulum (ER).

2.2 Heat shock protein70 (HSP70)

HSP70 kDa family is most conserved family of heat shock proteins (HSPs). It includes 13 genes in human and 4 genes in bovine (Grosz *et al*., 1992; Gallagher *et al.,* 1993; Kampinga *et al*., 2009). *HSP70* is a member of HSPs family, which are spread in all living organisms, whether eukaryotic or prokaryotic (Sharma *et al.,* 2009; Kampinga *et al.*, 2010). There are several functions of the HSP70, which have been extended to their participation in the immunological defense against infectious diseases and cancers (Srivastava *et al*., 1998). The HSPs can be potent adjuvants for eliciting immune responses and are powerful inducers of antitumor immunity (Tamura *et al*., 1997). It has been shown that virus and tumor derived HSP70s specifically immunize against the cells from which the HSP70s were derived (Udono and Srivastava, 1993). The role of HSPs is not only as chaperone but it also acts as cytokine, a chaperokine, which has the ability to activate the pro-inflammatory cytokines (Srivastava, 2002; Asea, 2002). The expression of *HSP70* gene has been positively correlated with variation in thermo tolerance in different organism. *HSP70* family members have many similar proteins which have similar gene products such as *HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA4, HSPA5, HSPA6* and *HSPA8* (Kampinga *et al*., 2009).

Table 2.1: Different members of *HSPA* (*HSP70*) family (Kampinga *et al*., 2009)

Gene name	HSP protein	Alternative name
HSPA1A	HSPA1A	*HSP70*-1; HSP72; HSPA1
HSPA1B	HSPA1B	*HSP70*-2
HSPA1L	HSPA1L	hum70t; hum70t; Hsp-hom
HSPA2	HSPA2	Heat-shock 70kD protein-2
HSPA5	HSPA5	BiP; GRP78; MIF2
HSPA6	HSPA6	Heat shock 70kD protein 6 (*HSP70*B′)
HSPA7	HSPA7	Heat shock 70kD protein 7
HSPA8	HSPA8	HSC70; HSC71; HSP71; HSP73
HSPA9	HSPA9	GRP75; HSPA9B; MOT; MOT2; PBP74; mot-2
HSPA12A	HSPA12A	FLJ13874; KIAA0417
HSPA12B	HSPA12B	RP23-32L15.1; 2700081N06Rik
HSPA13b	HSPA13b	Stch
HSPA14	HSPA14	*HSP70*-4; *HSP70*L1; MGC131990

HSP70 protein of goat possesses molecular weight of 70190.56 Da. and 641 amino acids. Out of 641 amino acids, 92 are strongly acidic (−) amino acids and 82 are strongly basic (+) amino acids. 220 amino acids are hydrophobic and 151 amino acids are polar in nature. In goat, the Isoelectric point of HSP70 protein is 5.611 and charge of −8.829 at pH 7.0 (Gade *et al*., 2010). However HSP70 protein of sheep possesses molecular weight of 70293.63 Da having 641 amino acids of which 91 are strongly acidic (-) amino acids and 83 are strongly basic (+) amino acids. Hydrophobic amino acids are 221 and polar amino acids are 151. In sheep, the Isoelectric point of HSP70 protein is 5.924 and has a charge of -6.662 at pH 7 (Pawar *et al*., 2013). Molecular structure of HSP70 contains 44-kDa fragment (amino acid residues 1-386) at N-terminus and contains 4 domains forming 2 lobes with a deep cleft between. 18-kDa fragment (amino acid residues 384-543) contains two 4-stranded antiparallel β-sheets and single α-helix (Kiang and Tsokos, 1998). 10-kDa fragment (amino acid residues 542-640 for HSP70) at C-terminus conserves EEVD terminal sequence. The N-terminal 44-kDa domain is ATPase domain; 18-kDa domain is peptide-binding domain; C-terminal 10-kDa fragment carries highly conserved EEVD terminal sequence, which is present in all eukaryotic HSP70 and HSP90 (Hightower *et al.,* 1994).

In *E.coli HSP70* is a single-copy heat inducible gene (Bardwell and Craig, 1984), whereas in eukaryotes *HSP70* is a complex, multigene family. The mouse genome contains at least two *HSP70* genes whose expression is limited to heat shocked or stressed cells (Lowe and Moren, 1986; Hunt and Calderwood, 1990). The gene, *HSP70-1* in mice shows 91% nucleotide homology to a human *HSP70* gene (Hunt and Calderwood, 1990). Four *HSP70* genes have been identified from bovine genomic sperm library (Grosz *et al*., 1992). Fluorescence *in situ* hybridization localized *HSP70*-1 and *HSP70*-2 gene to chromosome 23 band 22, *HSP70-3* gene to Chromosome 10 band 34, and *HSP70*-4 gene to chromosome 3 band 13 (Gallagher *et al*., 1993). Screening of phage library of bovine genomic DNA for hybridization with human *HSP70* cDNA probe revealed that there is syntenic conservation of *HSP70* genes in cattle and humans (Grosz *et al*., 1992). In pig, four sequences related to the heat shock proteins *HSP70* were localized on chromosome 7 and 14 (Nunes *et al*., 1993). Radioactively labeled Drosophila *HSP70* gene probe were used for the isolation of *HSP70* gene from chicken

genome (Morimoto *et al*., 1986). The overall structure of chicken *HSP70* gene is similar to that of Drosophila (Holmgren *et al*., 1979) and human (Hunt and Morimoto, 1985) *HSP70* genes. Chicken *HSP70* gene is transcribed as a primary transcript of 2.6 Kb that contains a single open reading frame that can encode a protein of predicted size of 70 kDa. The chicken *HSP70* gene sequences show 73% homology with the Drosophila *HSP70* gene sequence and 80% homology with human *HSP70* gene (Morimoto *et al*., 1986). It has been established in mouse *HSP70* gene knock out models that cytosolic *HSP70* family members regulate the cellular stress response where as other HSPs are involved in housekeeping and tissue-specific tasks (Daugaard *et al*., 2007).

Madhusudan, 2007 examined that buffalo *HSP70* cDNA sequence and deduced amino acid sequence has 97.8% and 94.4% homology with that of cattle *HSP70*-1 DNA sequence and deduced amino acid sequence respectively.

Dodamani *et al*., 2017 studied PCR-SSCP analysis of *HSP70* gene in Kenguri breed of sheep to characterize *HSP70* gene. The fragments consisting exon 1, 2, 3, 4 and 5 were of 490, 469, 525, 307 and 352 bp size. The exons 2, 3 and 4 of *HSP70* gene showed monomorphism with similar pattern in all the 48 animals studied. Two unique SSCP patterns with a pattern frequency of 0.1875 and 0.8125 respectively were observed in fragment 1 comprising exon 1. Two SSCP patterns with a pattern frequency of 0.3541 and 0.6458 were observed in fragment 5 comprising exon 5 of *HSP70* gene. The analysis of fragment 1 comprising exon 1 revealed T170C (Methionine Threonine), A210G (Arginine Glycine) and G504A (Glycine Arginine) amino acid substitution showing transition while for fragment 5 compromising exon 5 the observed polymorphisms at G2033C (Glycine Alanine) amino acid substitution showed transversion.

Habib *et al*., 2018 studied molecular detection of polymorphism of heat shock protein 70 (*HSP70*) in the semen of Arabi rams. DNA was extracted and then polymerase chain reaction (PCR) amplified sequencing, BLAST analysis and multiple sequence alignment was carried out. The results of nucleotides sequence analysis and the Multiple Sequence Alignment (MSA) was compared with heat shock protein *HSP70* gene of sheep in the gene bank, suggesting presence of two new haplotypes, haplotype G1 and haplotype G2, all mutations were silent except the mutation in site 514 was missense

appeared in G1. It concluded that the Arabi rams in Iraq have new polymorphism of *HSP70* gene which can help cope with difficult environmental conditions.

The genes encoding *HSP70*s may be with introns or intronless, are chromosomally dispersed, and may be expressed constitutively or inducible by different cellular stress (Lindquist and Craig, 1988; Sambrook *et al*., 1989). Most eukaryotes have a large family having at least five *HSP70*s related genes. These genes reveal close homology sequence at the level of amino acid (Ito *et al*., 1998). *Bos taurus* contains at least three different regions which include *HSP70* genes in the genome. One of the regions containing two tandemly array *HSP70* sequences were named as *HSP70*-1 and *HSP70*-2. The other two regions containing a single *HSP70* sequences were named as *HSP70*-3 and *HSP70*-4. Few reports are available regarding the role of *HSP70* during heat stress in goat.

2.3 HSP70 gene variability in other species

Pawar *et al*., 2012 studied molecular characterization of *HSP70* gene from buffalo. Genomic DNA was isolated and used for PCR amplification of *HSP70* gene. PCR product having size of 1,926 bp was cloned in pGEM-T easy vector and sequenced. 1,926 bp long ORF of *HSP70* gene encoding 641 amino acids in buffalo was obtained on sequence analysis. The amino acid sequence showed 98% similarity with *Bos indicus*, *Capra hircus, Bos taurus, Bos grunniens* and 90-95% similarity with *Sus scrofa*, *Camelus dromedarius, Canis familiaris, Felis catus* and *Homo sapiens*.

Mathew *et al*., 2013 studied the molecular cloning and characterization of *HSP70* gene in chicken. In this study, intron less gene of chicken *HSP70* was amplified, cloned in E.coli and characterized. The *HSP70* gene contains 1,905 bp ORF. Sequence analysis of *HSP70* gene using MEGA 4 and DNA STAR software showed a high sequence homology among different species indicating that the gene is evolutionarily conserved. Synonymous substitution (d_S) in the *HSP70* gene was higher than non-synonymous substitution (d_N), suggesting that the gene is not under positive selection and that no advantageous mutations had any significant role in its evolutionary adaptation.

Kerekoppa *et al*., 2015 studied molecular characterization of the *HSPA1A* gene by single-strand conformation polymorphism in Holstein Friesian and Deoni cattle. Genomic DNA was extracted from 94 blood samples and was subjected to polymerase chain reaction single-strand conformation polymorphism analysis, which revealed 14 band patterns in Deoni cattle and 8 band patterns in HF crossbreds. Sequence data were analysed using BioEdit software for detecting single nucleotide polymorphisms. Sequence analysis showed 12 single nucleotide polymorphisms in the coding region of the *HSPA1A* gene, which included 5 transitions (G456A, A972G, A1098G, C1766T, and G1788A) and 2 transversions (C312G and G2033C) in Deoni cattle and 2 insertions (C at positions 574-575 and 624-625), 2 transitions (A480G and A1098G) and 1 transversion (C312G) in HF crossbred cattle. The study indicated a high degree of genetic variability in the *HSPA1A* gene in the cattle populations under study.

Morimoto *et al.,* 1986 characterized *HSP70* gene in chicken and found that chicken *HSP70* cDNA sequence is 80% identical to human *HSP70* cDNA sequence and 73% identical to Drosophila *HSP70* cDNA sequence. However, chicken HSP70 deduced amino acid sequence is 80% identical to human HSP70 deduced amino acid sequence but only 71% identical to Drosophila HSP70 amino acid sequence.

Kano *et al*., 2004 revealed that the amino acid sequence of *HSP70* gene in canine showed 90-95% similarity with bovine, mouse and human HSP70 proteins.

2.4 Heat shock protein70-1 (*HSP70-1*) gene

HSP70 family of gene in bovines includes *HSP70-1, HSP70-2, HSP70-3,* and *HSP70-4* gene. *HSP70-1* is an intron less gene which is located on chromosome 23 of bovine (BTA 23) and consists of 1926 nucleotides. Similarly goats also possess four *HSP70* genes out of which *HSP70-3* gene sequence has been characterized in Shiba goat by Luengrattana *et al.,* 2000. In his study he found that goat HSP70-3 was highly identical (up to 98.2%) to the bovine HSP70-3 protein, and only 6 out of 636 amino acids were different. The other difference was that the goat HSP70-3 protein had insertion of 5 amino acids which were not observed in the bovine HSP70-3 protein. The Restriction fragment length polymorphism (RFLP) study in goat shows *HSP70* genes are all located within 800 kilobase (kb) of the Major histocompatibility complex class I loci (Cameron *et al*., 1990).

The entire nucleotide sequence of goat *HSP70*-1 gene shows 99.4% homology with sheep (partial), 96.3% homology with buffalo, 97.5% with yak, 97.8% with cattle, 94.4% with horse, 95.3% with pig and 94.1% with human which suggest close evolutionary relationship (Gade *et al*., 2010).

Pawar *et al*., 2013 studied expression, characterization and purification of heat shock protein 70 (*HSP70*) from sheep and goat. 1926 bp fragment of *HSP70* (*orf*) was amplified, cloned, sequenced and characterized in sheep and goat. DNA was isolated from lymphocytes and was used for PCR amplification of *HSP70* gene, cloned in pGEM-T easy vector and sequenced. Sequence analysis revealed 1926-bp long open reading frame of *HSP70* gene encoding 641 amino acids in these species. The predicted amino acid sequence of *Capra hircus* showed 98% identity with *Ovis aries, Bos indicus, Bubalus bubalis* and more than 90% indentity with *Canis familiaris, Sus scrofa* and *Homo sapiens* HSP70 protein sequences.

Fatima *et al*., 2019 studied molecular characterization of *HSP70*-1 gene of Sindh ibex (*Capra aegagrus blythi). HSP70*-1 gene was amplified, sequenced and data was analyzed. The results of homology analysis showed 99.93% similarity with sequences of goat and 99.35% similarity with sheep (partial). This was confirmed by constructing phylogenetic tree which showed that Sindh ibex, sheep and domestic goat share a common ancestor.

Elzarei *et al*., 2017 studied comparison of sequence of *HSP70-1* gene between Aradi and Damascus goats with the reported one of Yunnan black goat (*Capra hircus)* and identified that Damascus breeds are more mutant to *HSP70-1* gene sequence when compared to other breeds. The results also revealed an addition of A, T and G nucleotides at positions 935, 984 and 997 in Damascus goats. However, Aradi goats did not differ in these positions from Yunnan black goats. It could be concluded that *HSP70*-1 gene sequence varied between Damascus and Aradi breeds and Damascus breed showed more mutation in DNA sequence at 3 positions, Aradi breed did not show any variation with respect to that published for *Capra hircus.*

Sharma *et al*., 2012 studied characterization of constitutive *HSP70*-1 (*HSPA1A*) gene in buffalo embryos. RNA obtained from 8 to 16 cells stage IVP embryos was used for PCR amplification of *HSPA1A* gene. Agrose gel (1.2%) electrophoresis clearly revealed 1124 and 1107 bp fragments of *HSPA1A* gene and positive recombinant clones were further confirmed by colony and plasmid PCR. The nucleotide sequence and predicted amino acid sequence of *HSPA1A* gene was aligned and compared with *HSP70*-1 cDNA sequences of different species such as, *Bos taurus* (NM_174550.1), *Bos grunniens* (DQ022675.1), *Capra hircus* (FJ975769.1), *Sus scrofa* (NM_213766.1), *Canis lupus familiaris* (NM_001003067.1), *Rattus norvegicus* (NM_031971.2), *Mus musculus* (BC151107.1) and *Homo sapiens* (NM_005345.5. Buffalo *HSPA1A* gene showed 98% homology with bovine (*Bos taurus* and *Bos grunniens*), 97% with *Capra hircus*, 95% with *Sus scrofa* and *Canis lupus familiaris*, 94% with human, 93% with *Mus musculus*, and 92% with *Rattus norvegicus* which indicates close evolutionary relationship. Phylogenetic analysis revealed that *HSPA1A* is a highly conserved gene (91–98% homology among mammalian species) having an open reading frame of 1926 bp encoding 641 amino acids. Phylogenetic analysis showed that bovine, human, rat and mice are derived from different ancestors according to their closer evolutionary relationship. Cattle and buffalo might have evolved from a common ancestor, pig positioned in between and diverged early from the bovine ancestors. Buffalo *HSPA1A* gene has a separate place, closer to bovine but having different lineage.

Similar results was shown by Gade *et al*., 2010 deduced amino acid sequence of 641 amino acids of goat *HSP70* gene was 100% similar to sheep (partial), 95.9% similar to buffalo, 98.6% similar to cattle, 98% to pig, 98.4% to yak, 98.1% to horse, and 97.7% similar to human sequence. The unrestrained *HSP70* expression is principally a result of the transcription of the *HSP70*-1 locus (Christians *et al*., 1997). Further, Ramunno *et al*., 2005 stated that characterization of *HSP70*-1 locus is playing an important role to pediment phylogenic relationship among specific species. The molecular characterization of *HSP70*-1 gene will be helpful for deriving phylogenic relationship among different species and for determining expression and identifying new functions among the related species.

CHAPTER - III

MATERIALS AND METHODS

3.1 Chemicals, enzymes, biologicals, equipments and laboratory wares

3.1.1 Chemicals

Molecular biology grade water (Sigma), Agarose special low EEO (Himedia), Distilled water, Ethanol (99.9% absolute), 10X TBE (Himedia) etc were used.

3.1.2 Enzymes and biologicals

DNASure blood mini kit, forward and reverse primers, GeneDireX Kplus DNA Ladder RTU (Ready-to-use), PCR master-mix, DNA loading dye, Ethidium bromide (EBr) etc. were used.

3.1.3 Equipments

Vertical deep freezer (Siemens), Vertical autoclave (Relitech), Serological water bath (Macro-Scientific works), Electronic balance (Shimadzu, Japan), refrigerated centrifuge (Biogen), UV-transilluminator (Genetix), vortex shaker (JSGW), thermocycler (Eppendorf, USA), horizontal electrophoresis apparatus (MAC), micropipettes (Eppendorf) were used.

3.1.4 Glass and plastic ware

Routine glasswares like volumetric flask, beaker, measuring cylinder, test tubes, PCR tubes, pipettes (Borosil, Germany), 1.5 ml microcentrifuge tube (Tarsons) and microtips (Tarsons) were used in the study.

3.2 Source of Blood samples

3.2.1 Blood samples for molecular studies

Molecular study was conducted on blood sample taken from Beetal goat kept at Govt. Dairy Goat farm Rajbagh, Kathua Jammu, which is located at the longitude

75.52°E and the latitude of 32.37°N. It has an average elevation of 393 metres (1,289ft). Kathua has a monsoon-influenced humid subtropical climate. It generally experiences extreme rainfall during the monsoon being on the windward side of Sivalik hills. Because of its proximity to rivers, the climate is moderate to very hot in summers and mild to very cold in winters. Summers are hot and the temperature may reach 41 degrees, while in winters, the temperature can dip to 2 degrees. The annual rainfall is around 1,300 millimetres, mainly in monsoons and winters.

3.2.2 Collection of blood samples

Five (5) ml blood sample was taken from the jugular vein of Beetal goat aseptically in sterile K3 EDTA coated vacutainer (Vacutech, Labtech disposables, India). The tube was tightly capped and inverted gently few times to facilitate thorough mixing of blood with the anti-coagulant. After proper labelling, the sample was kept in an ice-box containing ice-packs and transported to the laboratory for further analysis **(Figure 3.1)**.

3.2.3 Isolation of genomic DNA

Genomic DNA was isolated from the venous blood samples by DNASure Blood Mini Kit (Nucleo-pore). Following protocol was used for isolation of genomic DNA.

1) 25 µl of Proteinase K was taken into a 1.5 ml microcentrifuge tube. Then 200 µl of blood was added to it.

2) 200 µl buffer GB3 was added to the samples and the mixture was vortexed vigorously for 15 s. The sample was incubated at 70°C for 10-15 min.

3) 210 µl ethanol (96-100%) was added to sample and vortexed again.

4) The mixture from step 3 was transferred into the DNASure Blood Mini Column placed in a collection tube and was centrifuged for 1 min at 11,000 x g. The collection tube was discarded along with flow- through.

5) The DNASure Blood Mini Column was placed into a fresh collection tube of 2 ml and 500 µl of buffer GBW was added to it. It was then centrifuged for 1 min at 11,000 x g. Collection tube was discarded along with flow- through.

Figure 3.1: Collection of blood sample from Beetal goat

6) The DNASure Blood Mini Column was placed into a fresh collection tube of 2 ml and 600 µl Buffer GB5 was added. It was then centrifuged for 1 min at 11,000 x g. Flow-through was discarded and collection tube was reused.

7) The DNASure Blood Mini Column was placed back into the collection tube and centrifuged for 1 min at 11,000 x g.

8) The DNASure Blood Mini Column was placed in a 1.5 ml microcentrifuge tube and 100 µl preheated Buffer GBE (70°C) was added to it. Buffer was directly dispensed onto the silica membrane. It was incubated at room temperature for 1 min and then centrifuged for 1 min at 11,000 x g.

9) **Storage of the elute with purified DNA:** The elute contains pure genomic DNA and the samples were kept at -20^{o}C for long term storage, and at 2-8°C for short-term storage.

3.3 Checking the concentration, quality and purity of genomic DNA

3.3.1 Quality of genomic DNA

DNA quality was checked through 0.8% horizontal submarine agarose electrophoresis as detailed below:

a) The gel casting plate was sealed with adhesive tape and placed on a leveled table surface. The comb was placed appropriately.

b) Agarose 0.8% (w/v) was boiled in 1X TBE buffer (pH 8), cooled to 55˚C and then ethidium bromide (1%) was added (5 µl/100 ml).

c) The gel was gently poured into the casting tray avoiding bubble formation and was allowed to solidify at room temperature. After solidification, the comb and adhesive tape were removed.

d) The gel casting tray was submerged in gel tank of electrophoresis unit having 1X TBE buffer in it.

e) Sample was carefully loaded into the wells. Each well was charged carefully with 5.0 µl DNA mixed with 1.0 µl of 6X gel loading dye.

f) Electrophoresis was carried out at 75 volts for 60 min and then gel was visualized under UV trans-illuminator.

The genomic DNA samples having good quality (intact bands with no smearing) were used for further analysis.

3.3.2 Estimation of DNA concentration and its purity

The purity of genomic DNA stock samples were quantified by using Nano-drop spectrophotometer (ND-1000) at 260 nm and 280 nm using the conversion that one absorbance unit at 260 nm wavelength equals 50 µg DNA per ml. The ultraviolet (UV) absorbance was checked at 260 nm and 280 nm for determination of DNA concentration and its purity. The optical density (OD) value at 260 nm gave the amount of nucleic acid present in given sample whereas OD at 280 nm gave the amount of protein present in given sample. Purity of DNA was judged on the basis of OD ratio at 260/280 nm. Samples having OD ratio (260/280 nm) of 1.7 to 1.9 were considered for further study. Concentration of DNA was estimated using the following formula:

$$\textbf{DNA concentration (µg/µl)} = \frac{OD_{260} \times \text{Dilution factor} \times 50}{1000}$$

Where, Dilution factor was 50.

1 OD value at 260 nm corresponds to 50 ng ds DNA/µl.

3.4 Primers

Specific primers for goat *HSP70-1* were designed based on available sequence of goat *HSP70-1* (Accession number: FJ975769) gene using Primer3 software available at NCBI.com. The amplicon size was of 1917 bp.

Table 3.1: Primer sequence used in present study

Gene	Primer name	Sequence	Melting temperature (T_{m°)
HSP70-1	***HSP70-1*** **Forward**	**CGAAAAACATGGCTATCGGC**	**66.3**
	HSP70-1 **Reverse**	**CCACCTCCTCAATGGTGGG**	**66.7**

3.5 Polymerase chain reaction (PCR)

In order to optimize the PCR conditions, different concentrations of PCR master-mix containing (Taq DNA Polymerase, dNTPs, $MgCl_2$) and primers (forward and reverse) was tried. The standardised reaction mixture concentrations of the different components which give optimum result for *HSP70-1* gene is presented in **Table 3.2.**

Table 3.2: Composition of PCR Reaction mixture for *HSP70-1* gene

S.No.	Content	Volume	Concentration
1.	PCR master mix	12.5 µl	-
2.	Forward Primer	0.25 µl	25µM
3.	Reverse Primer	0.25 µl	25 µM
4.	Distilled water	10.5 µl	-
5.	$Mgcl_2$	0.5 µl	-
6.	Template DNA	1 µl	50ng / µl
	Total	**25 µl**	**-**

3.5.1 PCR cycle conditions

Amplification was carried out in Thermal cycler (Eppendorf, USA). Different annealing temperatures were tried to optimize PCR cycle condition. Finally, annealing was standardized with respect to *HSP70-1* gene as presented in **Table 3.3**.

Table 3.3: Optimised PCR conditions for *HSP70-1* gene

S.No.	Steps	Temperature	Time
1.	Initial denaturation	94˚ C	5 min
2.	Denaturation	94 ˚C	1 min
3.	Annealing	50˚ C	1 min
4.	Extension	72˚ C	1 min 30 secs
5.	Repeat Steps 2 to 4	35 cycles	
6.	Final Extension	72 ˚C	10 min
7.	Hold	4˚C	-

3.6 Agarose gel electrophoresis of polymerase chain reaction product

The PCR product was analyzed by running on 1.5 % agarose gel in 1X TBE buffer. Ethidium bromide was added in the agarose (1% solution @ 5µl/100 ml). The run was performed at constant voltage at 80 V for 45 minutes. Along with the test samples GeneDireX Kplus DNA Ladder RTU (Ready-to-use) was also run in one lane. The amplified product was visualized as a single compact band under UV transilluminator and documented by gel photography.

3.7 Sequencing of PCR product

After the confirmation, one PCR product was selected and was sent for Sanger sequencing with "Primer Walking" at AgriGenome Labs Pvt. Ltd Cochin India. Primer walking is a step-by-step approach to sequencing long DNA templates from end to end that overcomes the inability of the Sanger chain termination method to read more than a few hundred bases in a single reaction. After an initial round of sequencing from a known sequence at one end of the template, each subsequent round is initiated from a new primer, which is based on the end of the sequence obtained from the previous reaction.

3.8 Sequence Analysis

The obtained sequence of Beetal *HSP70-1* was subjected to BLAST analysis in order to confirm that the obtained sequence is of *HSP70-1* or not. The obtained sequence

of *HSP70-1* gene of Beetal goat was analysed by MEGA X software with other reported CDS (Coding sequences) *HSP70-1* of different livestock species obtained from the NCBI (National Center for Biotechnology Information).

Following analysis were performed

3.8.1 Multiple Alignment of Beetal *HSP70-1* gene with coding sequence of different species by ClustalW method.

Multiple sequence alignment was done by ClustalW method using MEGA X software. Multiple sequence alignment can be looked at as an extension of the pair-wise alignment. The first step in multiple sequence alignment is pair-wise alignment of all the sequences. ClustalW is a progressive alignment program and is based on dynamic programming (DP) methods. ClustalW produces the best match for the selected sequences, and arranges them so that the identities, similarities and differences can be observed.

3.8.2 Construction of Phylogenetic tree

The Phylogenetic tree was constructed using Neighbor-Joining method (Saitou and Nei, 1987), based on the aligned sequences. To assess the reliability of a phylogenetic tree, MEGA provides the Boot strap test with 1000 replicates. This test uses the bootstrap re-sampling strategy.

3.8.3 Estimating Evolutionary Distances Using Pair wise Distance

The evolutionary distances between each pair of sequences were estimated by computing the proportion of nucleotide differences between the sequences. The evolutionary distances can also be calculated based on the proportion of amino acid differences. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al.*, 2004). During each comparison, the number of changes (base substitutions and insertion/ deletion events) are counted and presented as a proportion of the overall sequence length. These final estimates of the difference between all possible pairs of sequences are known as pair wise distance which measures the estimates of evolutionary divergence between sequences.

3.8.4 Nucleotide and Amino Acid Compositions

The relative frequencies of the four nucleotides (nucleotide composition) and that of the twenty amino acid residues (amino acid composition) were computed for all the sequences used. From these tables the G + C content can easily be computed.

3.8.5 Z test for Test of Neutrality

Z test was conducted in order to test whether positive selection is operating on a gene or not. Analyses were conducted using the Nei-Gojobori method (Nei and Gojobori, 1986). It was done by comparing the relative abundance of synonymous (d_S) and non synonymous substitutions (d_N) that have occurred in the gene sequences.

The null hypothesis is that H_0: $d_N = d_S$

Alternate hypothesis are

HA: (a) $d_N \neq d_S$ (test of neutrality).

(b) $d_N > d_S$ (positive selection).

(c) $d_N < d_S$ (purifying selection).

CHAPTER - IV

RESULTS

This section has depicted the outcome of present study which covers the Molecular characterization of *HSP70-1* gene in Beetal goat as per the objectives of the study. The results have been presented under following headings:

4.1 Isolation of genomic DNA

4.2 Checking of concentration, purity and quality of DNA

4.3 Primer designing

4.4 Polymerase chain reaction (PCR) standardization

4.5 Sequencing of PCR product

4.6 Confirmation of sequence by BLAST

4.7 Sequence analysis

4.1 Isolation of genomic DNA

The genomic DNA was isolated from whole blood of Beetal goat as per the protocol of DNASure Blood Mini kit (Nucleo-pore) and was stored at -20°C.

4.2 Checking of concentration, purity and quality of DNA

4.2.1 Concentration of DNA

Concentration of DNA was calculated spectrophotometrically by taking optical density (OD) at 260 nm. A total of 50-60 µg of DNA was obtained from the 200µl of the blood.

4.2.2 Purity of DNA

Purity of DNA was checked using Spectrophotometer by taking ratio of optical densities at 260 nm and 280 nm. All DNA samples with OD ratios ranging from 1.7 to 1.9 were used for further analysis.

4.2.3 Quality of DNA

Horizontal agarose gel electrophoresis was performed to check quality of DNA using 0.8% agarose. The gel was photographed by gel documentation system. The genomic DNA samples having good quality without smearing were used for further analysis **(Plate 1)**.

4.3 Primers

In the present study, specific primers for goat *HSP70-1* gene designed by Primer3 software were used to amplify gene of interest and presented in **Table 3.1.**

4.4 Polymerase chain reaction (PCR) standardization

PCR was carried out in a final volume of 25 µl. Reaction was carried out in PCR tubes containing volume of 1 µl of genomic DNA, 0.25 µl each of forward and reverse primers, 0.5 µl of $MgCl_2$, 12.5 µl of PCR master-mix, and 10.5 µl of distilled water. The same was presented in **Table 3.2.**

4.4.1 PCR amplification

Amplification was performed in Eppendorf thermal cycler using optimized PCR conditions. Cyclic conditions standardized for PCR amplification included one cycle of initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 1.5 min) followed by final extension at 72°C for 10 min as presented in **Table 3.3**.

4.4.2 Visualization of the amplified product

To visualize the amplified product, 1.5% agarose gel containing ethidium bromide @ 5 µl/100ml of agarose gel was used. A GeneDireX Kplus DNA Ladder RTU (100-10K base pairs) was used to know the size of PCR product. The amplified PCR product was visualized under UV transilluminator and documented by gel documentation system. The amplified product was observed as a single band of 1917 bp **(Plate 2)**.

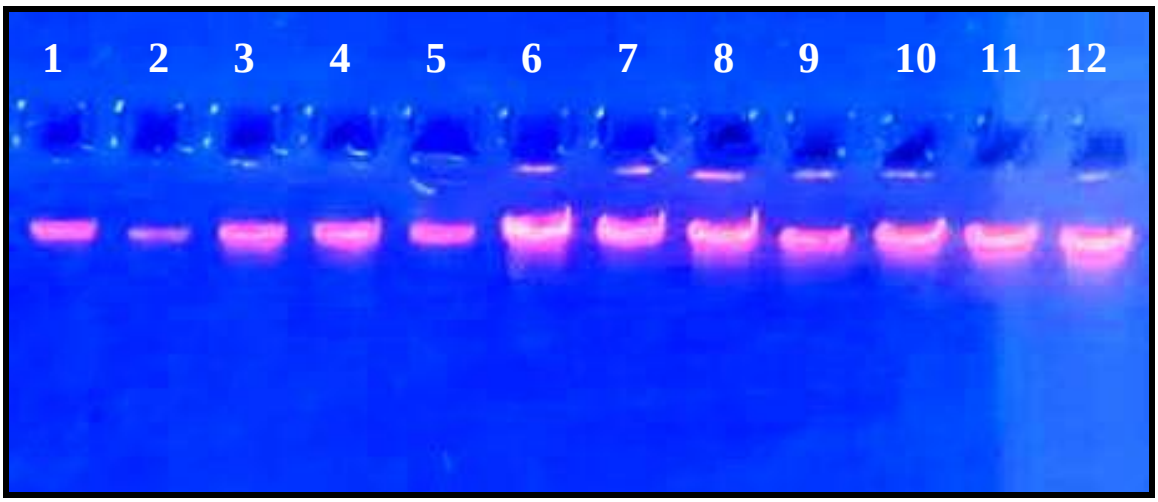

Plate 1: Quality of DNA at horizontal submarine (0.8%) gel electrophoresis

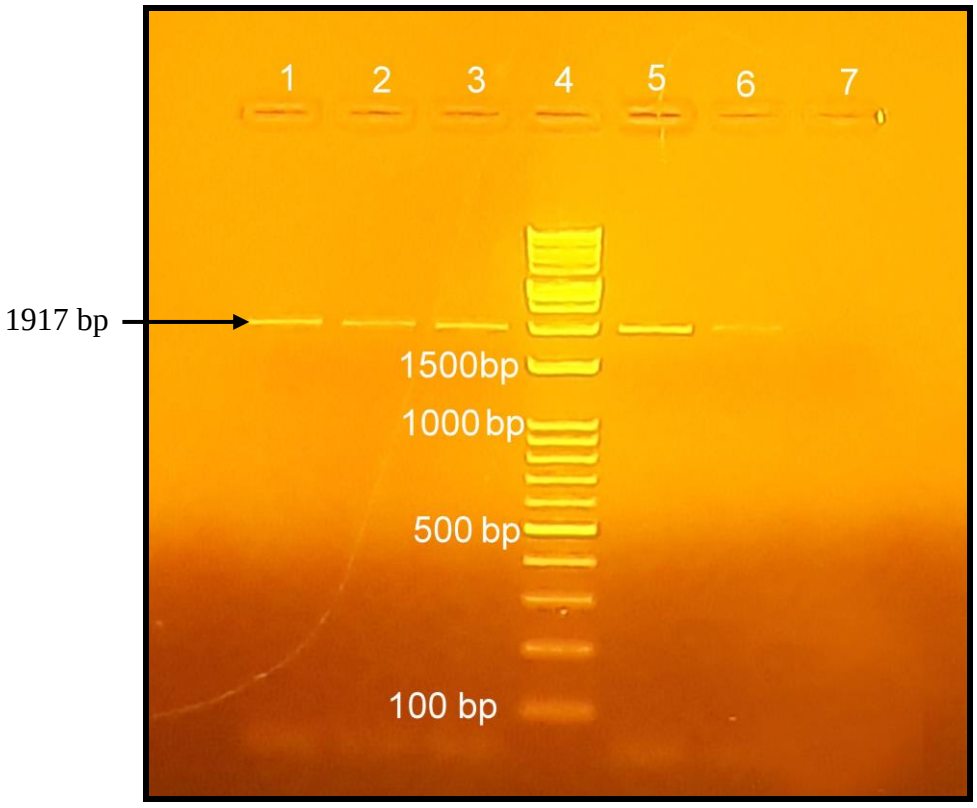

Plate 2: Amplified PCR product of *HSP70-1* gene in Beetal goat at 1.5% agarose gel electrophoresis.

Lane 1-3 and 5-6: Amplified PCR product of 1917 bp

Lane 4: DNA Ladder (GeneDireX Kplus DNA Ladder RTU)

Lane 7: Negative (-ve) control

4.5 Sequencing of PCR product

After the confirmation, one PCR product was selected and was sent for Sanger sequencing by "Primer Walking" at AgriGenome Labs Pvt. Ltd. Cochin India. The amplified PCR product was of 1917 bp. However as the PCR product was directly sequenced, approximately 40-50 bp from both side (from forward and reverse primers) clean sequence was not obtained and hence sequence of 1839 bp was obtained. The sequence obtained was compared to the gene sequence for the HSP70 protein of goat, sheep, cattle, yak, buffalo, pigs, chicken and humans available at GenBank *(http://www.ncbi.nlm.nih.gov).*

4.6 Confirmation of sequence by BLAST

The obtained sequence of Beetal *HSP70-1* was subjected to BLAST analysis in order to confirm that the obtained sequence is of *HSP70-1* or not. Following BLAST, different sequences of *HSP70-1* gene of different species were retrieved. This confirms that the sequence obtained is of *HSP70-1*.

4.6.1 Percentage Identity of *HSP70-1* partial cDNA sequence of Beetal goat

The percentage identity of Beetal goat *HSP70-1* cDNA sequence with CDS (coding sequences) of other species was taken from BLAST. The percentage identity of Beetal goat *HSP70-1* cDNA sequence with other species is shown in **Table 4.1**. The comparison demonstrated 99.08, 97.66, 97.93, 98.10, 94.73, 95.43, 93.78, 94.22 and 91.39 percent homology with sheep, cattle, yak, buffalo, horse, camel, pigs, human and mouse, respectively which indicates close evolutionary relationship and high sequence homology among the species.

Table 4.1: Percentage identity of Beetal goat *HSP70-1* cDNA sequence with other species

Species	Percent identity
Goat (*Capra hircus*)	100 %
Sheep (*Ovis aries*)	99.08 %
Buffalo (*Bubalus bubalis*)	98.10 %
Yak (*Bos grunniens*)	97.93 %
Cattle (*Bos taurus*)	97.66 %
Camel (*Camelus dromedarius*)	95.43 %
Horse (*Equus caballus*)	94.73 %
Human (*Homo sapiens*)	94.22 %
Pig (*Sus scrofa*)	93.78 %
Mouse (*Mus musculus*)	91.39 %

The percentage identity of translated protein (deduced amino acid) of Beetal HSP70-1 with other species was taken from BLAST **(Table 4.2)**. Inferred amino acid sequence of 613 residues of Beetal goat *HSP70-1* gene was 100, 98.99, 98.83, 98.4, 98.83, 98.49, 98.49, 97.32, 90.45 and 97.99 percent similar to goat, buffalo, sheep, yak, cattle, horse, camel, pig, mouse and human, respectively.

Table 4.2: Percentage identity of translated protein (deduced amino acid) of Beetal HSP70-1 with other species

Species	Percent identity
Goat (*Capra hircus*)	100 %
Sheep (*Ovis aries*)	98.83 %
Buffalo (*Bubalus bubalis*)	98.99 %
Yak (*Bos grunniens*)	98.99 %
Cattle (*Bos taurus*)	98.83 %
Camel (*Camelus dromedarius*)	98.49 %
Horse (*Equus caballus*)	98.49 %
Human (*Homo sapiens*)	97.99 %
Pig (*Sus scrofa*)	97.32 %
Mouse (*Mus musculus*)	90.45 %

4.7 Sequence analysis

The obtained sequence of *HSP70-1* gene of Beetal goat was analysed by MEGA X software with other reported CDS (Coding sequences) of *HSP70-1* in different species of livestock. The *HSP70-1* sequences of other species were downloaded from NCBI (National Center for Biotechnology Information).

4.7.1 Multiple Alignment of Beetal *HSP70-1* gene with coding sequence of different species by ClustalW method.

The nucleotide sequence was aligned and compared with respective sequences of other species and presented in **Table 4.3**. All *HSP70-1* cDNA sequences of different domestic species viz. goat, sheep, cattle, yak, buffalo, horse, camel, pig, chicken, mouse and human were aligned using ClustalW method which reveals the nucleotide substitutions.

Table 4.3: Multiple alignment report of Beetal goat *HSP70-1* gene partial CDS with that of other species

```
hsp_70.1_beetal_partial_cds      ------------------------------------------------TACTCCTGCGTGGGGGTGTTCCAGCACGGCAAGG
KC790104.1_yak_CDS               ----ATGGCGAAAAACATGGCTATCGGCATCGACCTGGGCACCACC...........A.....................
M69100.1_Pig_CDS                 ----ATGGCGAAGAGCGTGGCCATCGGCATCGACCTGGGCACCACG.....G.....................
LC495678.1_Ovis_aries_CDS        ----ATGGCGAAAAACATGGCTATCGGCATCGACCTGGGCACCACC.....................
M35021.1_Mouse_CDS               ----ATGGCCAAGAACACGGCGATCGGCATCGACCTGGGCACCACC.....G........C.....................
M11717.1_Human_CDS               ----ATGGCCAAAGCCGCGGCAGTCGGCATCGACCTGGGCACCACC.....................A.....
NM_001256923.1_horse_CDS         ----ATGGCTAAGAGCACGGCCATCGGCATCGACCTGGGCACCACC.....G.....................
NM_001285703.1_Goat_CDS          ----ATGGCGAAAAACATGGCTATCGGCACCGACCTGGGCACCACC.....................
AY143691.1_chicken_CDS           ATGTCTGGCAAAGGGC-CGGCCATCGGCATCGATCTGGGCACCACG..T..T........T..C........T.....A.
KC616314.1_Camelus_dromedarius_c  ----ATGGCGAAAAACACGGCCATCGGCATCGACCTGGGCACCACC.....T.....................
MH814760.1_Bubalus_bubalis_breed  ----ATGGCGAAAAACATGGCTATCGGCATCGACCTGGGCACCACC...........A.....................
AY662497.1_Bos_taurus_cds        ----ATGGCGAAAAACATGGCTATCGGCATCGACCTGGGCACCACC...........A.....................

hsp_70.1_beetal_partial_cds      TGGAGATCATCGCCAACGACCAGGGAAACCGCACTACCCCCAGCTACGTGGCTTTCACCGATACCGAGCGGCTCATCGGC
KC790104.1_yak_CDS               .....................C........C.................C.....................
M69100.1_Pig_CDS                 .....................C........C..........CA.....C.....G..C...........G......
LC495678.1_Ovis_aries_CDS        .....................C........C.................C.....................
M35021.1_Mouse_CDS               .....................C........G.................C........C........C........G
M11717.1_Human_CDS               .....................C........C.................C.....G..C.................G
NM_001256923.1_horse_CDS         .....................C........C.................C.....G..C...........G.....G
NM_001285703.1_Goat_CDS          .....................C........C.................C.....G..C...........G.....G
AY143691.1_chicken_CDS           ..........T.............G........C..A........T.....C...........A.....C........G
KC616314.1_Camelus_dromedarius_c  .....................C........A.................C.....G..C.....................
MH814760.1_Bubalus_bubalis_breed  .....................C........C.................C.....................
AY662497.1_Bos_taurus_cds        .....................C........C.................C.....................G

hsp_70.1_beetal_partial_cds      GATGCAGCCAAGAACCAGGTGGCGCTGAACCCGCAGAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGG
KC790104.1_yak_CDS               .....G.....................................G.....................................
M69100.1_Pig_CDS                 .....G.....................................G.....T...........C........G...........
LC495678.1_Ovis_aries_CDS        .....................................................................................
M35021.1_Mouse_CDS               ..C..C.....................................................................................
M11717.1_Human_CDS               .....G.....................................T...........C.....................
NM_001256923.1_horse_CDS         .....G.....................................T.....................................
NM_001285703.1_Goat_CDS          .....................................................................................
AY143691.1_chicken_CDS           .....T...........A.....AA.......CACC......A.C..T..T..C.....T..C.............AT.A
KC616314.1_Camelus_dromedarius_c  .....G.....................................G.....................................
MH814760.1_Bubalus_bubalis_breed  .....G.....................................G.....................................
AY662497.1_Bos_taurus_cds        .....G.......G.....................G.................C.....................
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```
hsp_70.1_beetal_partial_cds      CGACCCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTTCCGCGTGATCAACGACGGAGACAAGCCTAAAGTGCAGGTGA
KC790104.1_yak_CDS               A.....................................C.....................G..........
M69100.1_Pig_CDS                 ...............AGGC.................C.....G.............G........C..G..........
LC495678.1_Ovis_aries_CDS        ..................................................................
M35021.1_Mouse_CDS               ...TG.............C.................C....AG...G.G........C........C..G..........
M11717.1_Human_CDS               .....................................AG.....................C..G..........
NM_001256923.1_horse_CDS         ...........................................................G........C..G..........
NM_001285703.1_Goat_CDS          ..................................................................
AY143691.1_chicken_CDS           T.....CACA.........................C.....T...G.G.....G..T.G......C..G.........G
KC616314.1_Camelus_dromedarius_c  .....................................C.....................T.....G..G..........
MH814760.1_Bubalus_bubalis_breed  ....................................................................G..........
AY662497.1_Bos_taurus_cds        A.....................................C.....................G..........

hsp_70.1_beetal_partial_cds      GCTACAAGGGGGAGACCAAGGCGTTCTACCCAGAGGAGATCTCGTCGATGGTGCTGACCAAGATGAAAGAGATCGCCGAG
KC790104.1_yak_CDS               .......A.....................G.....................................G..........
M69100.1_Pig_CDS                 ..........C........A.GC........C.....................................G..........
LC495678.1_Ovis_aries_CDS        ..................................................................
M35021.1_Mouse_CDS               A.........C....G.CG.T......T...G.............C...........G........G........T...
M11717.1_Human_CDS               .......................A........C.............C.....................G..........
NM_001256923.1_horse_CDS         ...............................G...........T..C...........G........G..........
NM_001285703.1_Goat_CDS          ..................................................................
AY143691.1_chicken_CDS           AG........T....TG...A.C....T.............AGC..T.........C...........G.....T..T...
KC616314.1_Camelus_dromedarius_c  ..............................C.....................................A.....G..........
MH814760.1_Bubalus_bubalis_breed  ..............................G.....................................G........G...
AY662497.1_Bos_taurus_cds        .......A.....................G.....................................G..........

hsp_70.1_beetal_partial_cds      GCGTACCTGGGCCACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGGCAGGCCACCAA
KC790104.1_yak_CDS               ..................................................................
M69100.1_Pig_CDS                 .GC.................G.............G.....................................
LC495678.1_Ovis_aries_CDS        ..................................................................
M35021.1_Mouse_CDS               ..................................G.....C.................T..............
M11717.1_Human_CDS               ............T.....................................................C..........
NM_001256923.1_horse_CDS         ............T.....................................................C..........
NM_001285703.1_Goat_CDS          ..................................................................
AY143691.1_chicken_CDS           ..C..T.....AA.AAA...ACAG..T..T..T.....A.....C..T.............C.....C..........
KC616314.1_Camelus_dromedarius_c  ..T.....................................A.....................C..........
MH814760.1_Bubalus_bubalis_breed  ..................................................................
AY662497.1_Bos_taurus_cds        ..................................................................
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hsp_70.1_beetal_partial_cds      GGACGCGGGGGTGATCGCGGGGCTGAACGTGCTGATGATCATCGACGAGCCCACGGCCGCCGCCATCGCCTACGGCCTGG
KC790104.1_yak_CDS               ..................................G.......A.....................................
M69100.1_Pig_CDS                 ...T.................C............CG.......A.............G..G........T.........
LC495678.1_Ovis_aries_CDS        ..................................G.......A.....................................
M35021.1_Mouse_CDS               .........C........C..T..A.........CG.......A.............G.................G....
M11717.1_Human_CDS               ...T.....T.............C.........CG.......A.....................................
NM_001256923.1_horse_CDS         .........C.........................G.......A.............................G....
NM_001285703.1_Goat_CDS          .....................................................................................
AY143691.1_chicken_CDS           A..T..T..CACC...A.T..C..T......A..CGT..T...A.T........A..A..T..T..T.....T...T...
KC616314.1_Camelus_dromedarius_c  ...T..............................CG.......A.......G..C.........................
MH814760.1_Bubalus_bubalis_breed  ..................................G.......A.....................................
AY662497.1_Bos_taurus_cds        ..................................G.......A.....................................

hsp_70.1_beetal_partial_cds      ACCGGACGGGCAAG------GGGGAGCGCAACGTGCTCATCTTTGACCTGGGCGGGGGCACGTTCGACGTGTCCATTCTG
KC790104.1_yak_CDS               ..A..........------.............................T.....A.......................C...
M69100.1_Pig_CDS                 ..A..........------.................G.....C.............................A..C...
LC495678.1_Ovis_aries_CDS        ..........------.....................................................C...
M35021.1_Mouse_CDS               .......C.....------..C.....................C........G..C.....................C...
M11717.1_Human_CDS               ..A.A.........------.............C..G.......................C..............C...
NM_001256923.1_horse_CDS         ..A..........------.............................T.............C..............C...
NM_001285703.1_Goat_CDS          ..........------.....................................................C...
AY143691.1_chicken_CDS           .TAA..AA..T.CCCGGGCT..A...AAG..T..............T....A........T..T..T........C..T
KC616314.1_Camelus_dromedarius_c  ..A....C.....------.........................C........T..................A.....C...
MH814760.1_Bubalus_bubalis_breed  ..A..........------.............................T.............................C...
AY662497.1_Bos_taurus_cds        ..A..........------.............................T.....A.......................C...

hsp_70.1_beetal_partial_cds      ACGATCGACGACGGCATCTTCAAGGTGAAGGCCACGGCCGGGGACACGCACCTGGGCGGGGAGGACTTCGACAACAGGCT
KC790104.1_yak_CDS               ......................G.....................................................................
M69100.1_Pig_CDS                 ......................G.................G.....................C.....................
LC495678.1_Ovis_aries_CDS        ......................G.....................................................................
M35021.1_Mouse_CDS               ......................G.................G..C..............A..................C....
M11717.1_Human_CDS               ......................G.........................C.........T...........T...........
NM_001256923.1_horse_CDS         ......................G...................A.....C.........T...........T...........
NM_001285703.1_Goat_CDS          .....................................................................................
AY143691.1_chicken_CDS           ..C..T..G..T........TG........T....A..T........C........T...........T......C.AA.
KC616314.1_Camelus_dromedarius_c  ......................G.................................T...........T...........
MH814760.1_Bubalus_bubalis_breed  ......................G.....................................................................
AY662497.1_Bos_taurus_cds        ......................G.....................................................................
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hsp_70.1_beetal_partial_cds      GGTGAACCACTTCGTGGAGGAGTTCAAGAGGAAGCACAAGAAGGACATCAGCCAGAACAAGCGGGCCGTGAGGCGGATGC
KC790104.1_yak_CDS               ............................................................................C...
M69100.1_Pig_CDS                 ..........................................TA..................G........CC.C.
LC495678.1_Ovis_aries_CDS        ............................................................................C...
M35021.1_Mouse_CDS               .....G.....................................................C..G...C.....C...
M11717.1_Human_CDS               ............................A..A.....................................A.....C...
NM_001256923.1_horse_CDS         ............................A.........................................A.....C...
NM_001285703.1_Goat_CDS          ............................................................................
AY143691.1_chicken_CDS           ...A.....T..T..A..A.........C.T.........CGT.....TGCTGGC..T.....A..A........TC...
KC616314.1_Camelus_dromedarius_c  ...........T.....................................................................C...
MH814760.1_Bubalus_bubalis_breed  ............................................................................C...
AY662497.1_Bos_taurus_cds        ............................................................................C...

hsp_70.1_beetal_partial_cds      GCACGGCATGCGAGCGGGCCAAGAGGACCTTGTCGTCCAGCACCCAGGCCAGCCTGGAGATCGACTCCCTGTTCGAGGGC
KC790104.1_yak_CDS               ....C.....................A.....................................................
M69100.1_Pig_CDS                 .A..C..C...........T.....A...C.............G.....................................
LC495678.1_Ovis_aries_CDS        .......G.....................................................................
M35021.1_Mouse_CDS               .......G..T...A.............GC.....................................T...........
M11717.1_Human_CDS               ....C..C......A..............C.........................................T......
NM_001256923.1_horse_CDS         ....C..C......A..............C.....................................................
NM_001285703.1_Goat_CDS          .....................................................................................
AY143691.1_chicken_CDS           .T..A..T..T...A....G...C.T..TC..AGC..TTC...G..A......A.T.....T.........C..T......
KC616314.1_Camelus_dromedarius_c  .......C......A..............C.....................................................
MH814760.1_Bubalus_bubalis_breed  .......G.....................................................................
AY662497.1_Bos_taurus_cds        ....C.....................A.....................................................

hsp_70.1_beetal_partial_cds      ATCGACCTCTACACGTCCATCACCAGGGCACGGTTCGAGGAGCTGTGCTCCGACCTGTTCCGGAGCACCCTGGAGCCCGT
KC790104.1_yak_CDS               ......T.....................G.....................................................
M69100.1_Pig_CDS                 ......T.................C....G..C.................G...........C..............G..
LC495678.1_Ovis_aries_CDS        ......T.....................................................................G..
M35021.1_Mouse_CDS               ......T.......A........GC....G........A...........G...........CG....G...........
M11717.1_Human_CDS               ......T.....................GA.....................................A.............
NM_001256923.1_horse_CDS         ......T...................A..GA...................G...........C.................
NM_001285703.1_Goat_CDS          .....................................................................................
AY143691.1_chicken_CDS           ..T...T.......C........TC.T.....C..T.....A..CAATG.T..T..T.....TG.T...........A..
KC616314.1_Camelus_dromedarius_c  ......T.....................G.....................G.............................
MH814760.1_Bubalus_bubalis_breed  ......T.....................G.....................................................
AY662497.1_Bos_taurus_cds        ......T.....................G.....................................................
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hsp_70.1_beetal_partial_cds      GGAGAAGGCTCTACGCGACGCCAAGCTGGACAAGGCCCAGATCCACGACCTGGTCCTGGTGGGGGGCTCCACCCGCATCC
KC790104.1_yak_CDS               .........G.....................G.....................................
M69100.1_Pig_CDS                 ............G........G.............................G..............G..G.......
LC495678.1_Ovis_aries_CDS        ................................................................................
M35021.1_Mouse_CDS               .........C..G...........A.........G.................G........C.....G..G.......
M11717.1_Human_CDS               ............G.............................T..................C...................
NM_001256923.1_horse_CDS         .........C..G........G.....................................C.................
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           .........C..G..T..T........T..T....G.........G..GA.T..G..T..............T..T..T.
KC616314.1_Camelus_dromedarius_c  ............G.....................................................C..............
MH814760.1_Bubalus_bubalis_breed  ..................................G.............................................
AY662497.1_Bos_taurus_cds        .........G.....................G.....................................

hsp_70.1_beetal_partial_cds      CCAAAGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTCAACAAGAGCATCAACCCGGACGAGGCGGTGGCA
KC790104.1_yak_CDS               ....G.....................................................C..............G
M69100.1_Pig_CDS                 ....G.....................................................................G
LC495678.1_Ovis_aries_CDS        ....G.....................................................................
M35021.1_Mouse_CDS               ....G.....................................G...............................C
M11717.1_Human_CDS               ....G.....................................G.................C........T....GC
NM_001256923.1_horse_CDS         .G..G.....................................................C..T..A........C
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           .T..GA.C.....T.......A..T........T..CAAA..G..G..............T..A..T..A..T..T..T
KC616314.1_Camelus_dromedarius_c  ....G....G.....................................G..................C........A.....C
MH814760.1_Bubalus_bubalis_breed  ....G.....................................................C..............G
AY662497.1_Bos_taurus_cds        ....G.....................................................C..............G

hsp_70.1_beetal_partial_cds      TACGGGGCGGCGGTGCAGGCGGCCATCCTGATGGGGGACAAGTCGGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGC
KC790104.1_yak_CDS               ..............................................................T.............
M69100.1_Pig_CDS                 ..T.............................C...............................................
LC495678.1_Ovis_aries_CDS        ................................................................................
M35021.1_Mouse_CDS               ................................................................................
M11717.1_Human_CDS               .........................................C......................................
NM_001256923.1_horse_CDS         ..........T.....................................T.............................
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           ..T..T..C..T.....A..A..T.....C.....A........T..A..T.....A..T.....C...T....T..CA.
KC616314.1_Camelus_dromedarius_c  ..................A.........................T..............................
MH814760.1_Bubalus_bubalis_breed  ................................................................................
AY662497.1_Bos_taurus_cds        ..............................................................T.............
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hsp_70.1_beetal_partial_cds      TCCCCTGTCGCTGGGACTGGAGACGGCCGGAGGCGTGATGACTGCCCTGATCAAGCGCAACTCCACCATCCCCACGAAGC
KC790104.1_yak_CDS               .........................................C.....................................
M69100.1_Pig_CDS                 G..G..........G.............C...........G..G.....................C....
LC495678.1_Ovis_aries_CDS        .........................................C.....................................
M35021.1_Mouse_CDS               G..G..........C........T..G..C...........G..G..C.....................C....
M11717.1_Human_CDS               ..............G.....................................................C....
NM_001256923.1_horse_CDS         ..............G...........T..G.....................................C....
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           C........C.....CA.C.....A..T..T..A...........T..C........T...A.......T.....C..A.
KC616314.1_Camelus_dromedarius_c  ..............G.............G...........C..T.....................C....
MH814760.1_Bubalus_bubalis_breed  .........................................C.....................................
AY662497.1_Bos_taurus_cds        .........................................C.....................................

hsp_70.1_beetal_partial_cds      AGACGCAGATCTTCACCACCTACTCGGACAACCAGCCGGGCGTGCTGATCCAGGTGTACGAGGGCGAGAGGGCCATGACT
KC790104.1_yak_CDS               ...............................................................................G
M69100.1_Pig_CDS                 ...................G...........................................................G
LC495678.1_Ovis_aries_CDS        ................................................................................
M35021.1_Mouse_CDS               .........C.........................C..G.....................................G
M11717.1_Human_CDS               ........................C........A..C..G.....................................G
NM_001256923.1_horse_CDS         ...................G.................C..G..............A.....................G
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           .A..A....C..............A.........AGCA.T..C..CG..........T..A..T........T.....A
KC616314.1_Camelus_dromedarius_c  ..............G...............................................................G
MH814760.1_Bubalus_bubalis_breed  ...............................................................................G
AY662497.1_Bos_taurus_cds        ...............................................................................G

hsp_70.1_beetal_partial_cds      CGGGACAACAACCTGCTGGGGCGCTTCGAGCTGAGCGGCATCCCGCCGGCCCCGCGGGGGGTGCCCCAGATCGAGGTGAC
KC790104.1_yak_CDS               ................................................................................
M69100.1_Pig_CDS                 ..........................................................C.....................
LC495678.1_Ovis_aries_CDS        ................................................................................
M35021.1_Mouse_CDS               ..C.....................................................G..CA....C.....G.............
M11717.1_Human_CDS               AAA........T...T.............................T........AG.---C...................
NM_001256923.1_horse_CDS         ..................................T........A........CA....A.....................
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           AA.........T.......CAAG..T..C..A.CA........C.....A..C..T..A..T..T...........C..
KC616314.1_Camelus_dromedarius_c  ..........................................A........CA....A.....................
MH814760.1_Bubalus_bubalis_breed  ................................................................................
AY662497.1_Bos_taurus_cds        ............................T...................................................
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hsp_70.1_beetal_partial_cds      CTTCGACATCGACGCCAATGGCATCCTGAACGTCACGGCCACGGACAAGAGCACGGGCAAGGCCAACAAGATCACCATCA
KC790104.1_yak_CDS               ................................................................................
M69100.1_Pig_CDS                 ................C..A.............G.....................................
LC495678.1_Ovis_aries_CDS        ................................................................................
M35021.1_Mouse_CDS               ................C.......................C...........C....................
M11717.1_Human_CDS               ...........T.....C.....................................C....................
NM_001256923.1_horse_CDS         ...T.....................................C.............................
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           T..T.....A..T..T.....T............GT..TGT.........T..A..G....AG........A.......
KC616314.1_Camelus_dromedarius_c ..............................................A.................................
MH814760.1_Bubalus_bubalis_breed ................................................................................
AY662497.1_Bos_taurus_cds        ................................................................................

hsp_70.1_beetal_partial_cds      CCAACGACAAGGGCCGGCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAGGAGGCGGAGAAGTACAAGGCAGAGGACGAG
KC790104.1_yak_CDS               ..........................................................A...........G.........
M69100.1_Pig_CDS                 ..............C.........................................T...........A..G.....T...
LC495678.1_Ovis_aries_CDS        ..........................................................A.....................
M35021.1_Mouse_CDS               ..............C.........................................C...CGC........C.........
M11717.1_Human_CDS               ..............C.....................................................A..G.........
NM_001256923.1_horse_CDS         ..............C.....................................................A..C.........
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           ....T........T..C..T.....A..T..T..T..C..T.....A..A..A..A.....A.....A........T..A
KC616314.1_Camelus_dromedarius_c ..........................................................................A..C.........
MH814760.1_Bubalus_bubalis_breed ..........................................................A.....................
AY662497.1_Bos_taurus_cds        ..........................................................A...........G.........

hsp_70.1_beetal_partial_cds      GTCCAGCGCGAGAGGGTGTCTGCCAAGAACGCGCTGGAGTCGTACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGG
KC790104.1_yak_CDS               ................................................................................
M69100.1_Pig_CDS                 A...............TGG...T.........T.............T.................T..............
LC495678.1_Ovis_aries_CDS        ................................................................................
M35021.1_Mouse_CDS               ..G........C......G.C.............C.....C..T.............................C.....
M11717.1_Human_CDS               ..G.................A...........C.........C................................
NM_001256923.1_horse_CDS         ............................T..C.............G.................A.............
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           .C.A.CA.A..T......GGA.........T.C..T........TA.T.A..........CAGA.A............AA
KC616314.1_Camelus_dromedarius_c ....................C...........C........T.....................................
MH814760.1_Bubalus_bubalis_breed ........................................T.......................A.....A........
AY662497.1_Bos_taurus_cds        .........................................A......................................
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hsp_70.1_beetal_partial_cds      GCTGAAGGGCAAGATCAGCGAGGCGGACAAGAAGGTGGTGCTGGACAAGTGCCAGGAGGTGATTTCCTGGCTGGACGCCA
KC790104.1_yak_CDS               ..................................AA..............................................
M69100.1_Pig_CDS                 ...T..............................AA.............T..............................
LC495678.1_Ovis_aries_CDS        ..................................AA..............................................
M35021.1_Mouse_CDS               T..C.........C.........T.........AA.........................C..C............T...
M11717.1_Human_CDS               ...C.....................C.........AA.............T..A.....C..C..G.............
NM_001256923.1_horse_CDS         ...C..............................AA............A.............................
NM_001285703.1_Goat_CDS          ..................................AA..............................................
AY143691.1_chicken_CDS           A........A........T..CCA.......C..AAA.....C.....................CAGT.....T...CGA.
KC616314.1_Camelus_dromedarius_c  ...C.....................C.........AAA.............................G...T.........
MH814760.1_Bubalus_bubalis_breed  ..................................AA..................................T....
AY662497.1_Bos_taurus_cds        ..................................AA..............................................

hsp_70.1_beetal_partial_cds      ACACCTTGGCGGAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAGCAGGTGTGTAACCCCATCATCAGCAGACTG
KC790104.1_yak_CDS               ................................................................................
M69100.1_Pig_CDS                 ....GC....C.....................................................................G.....
LC495678.1_Ovis_aries_CDS        ................................................................................
M35021.1_Mouse_CDS               ....GC....C..C.....G.....C.T.......C..G............G......C.G............TG.G...
M11717.1_Human_CDS               ..........C.....................................................................G.....
NM_001256923.1_horse_CDS         ....T.....C..A..............................T.....................T.......CTG.....
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           ..CAGA....A.....A..A....A..........CA...A.........A.AC.C..C.....G..TG...CA.A....
KC616314.1_Camelus_dromedarius_c  ....GC....C..............................A.....................................TG.....
MH814760.1_Bubalus_bubalis_breed  ................................................................................
AY662497.1_Bos_taurus_cds        ................................................................................

hsp_70.1_beetal_partial_cds      TACCAGGGGGCGGGCGGCCCCGGGGCAGGCGGCTTTGGGGCTCAGGCCCCT------------------------------
KC790104.1_yak_CDS               ............................T...................G....---AAAGGGGGCTCTGGGTCTGGCCCCAC
M69100.1_Pig_CDS                 ..............T.........C.G........C.......CA.AT.TC---AAAGGGGGCTCTGGGTCTGGCCCCAC
LC495678.1_Ovis_aries_CDS        ............................T.......................---AAAGGGGGCTCTGGGTCTGGCCCCAC
M35021.1_Mouse_CDS               .........T.....T.CT..T.....T..G.....C.....C.....G..GCCGAAAGGAGCCTCTGGCTCAGGACCCAC
M11717.1_Human_CDS               .........T..C..T..T......C.T..G.....C..........GT..C---AAGGGAGGGTCTGGGTCAGGCCCCAC
NM_001256923.1_horse_CDS         ..............T...........T..T..................T..C---AAGGGTGGCTCTGGGTCTGGCCCCAC
NM_001285703.1_Goat_CDS          ..........................................................---AAAGGGGGCTCTGGGTCTGGCCCCAC
AY143691.1_chicken_CDS           ........A..T..A..AG.T........T....CC..-------------------------------TGGCCCAAC
KC616314.1_Camelus_dromedarius_c  ..............T...........C..T...................C---AAAGGGGGCTCTGGGTCTGGCCCCAC
MH814760.1_Bubalus_bubalis_breed  ........................T.........C..............---AAAGGGGGCTCTGGGTCTGGCCCCAC
AY662497.1_Bos_taurus_cds        ............................T...................G....---AAAGGGGGCTCTGGGTCTGGCCCCAC

hsp_70.1_beetal_partial_cds      ----------------------
KC790104.1_yak_CDS               CATTGAGGAGGTGGATTA---G
M69100.1_Pig_CDS                 CATCGAGGAGGTGGAT------
LC495678.1_Ovis_aries_CDS        CATTGAGGAGGTGGATTA---G
M35021.1_Mouse_CDS               CATCGAGGAGGTGGATTA---G
M11717.1_Human_CDS               CATTGAGGAGGTAGATTA---G
NM_001256923.1_horse_CDS         CATTGAGGAGGTGGATTA---G
NM_001285703.1_Goat_CDS          CATTGAGGAGGTGGATTA---G
AY143691.1_chicken_CDS           CATTGAAGAAGTAGATTAA---
KC616314.1_Camelus_dromedarius_c  CATTGAGGAGGTAGATTA---G
MH814760.1_Bubalus_bubalis_breed  CATTGAGGAGGTGGATTA---G
AY662497.1_Bos_taurus_cds        CATTGAGGAGGTGGATTA---G
```

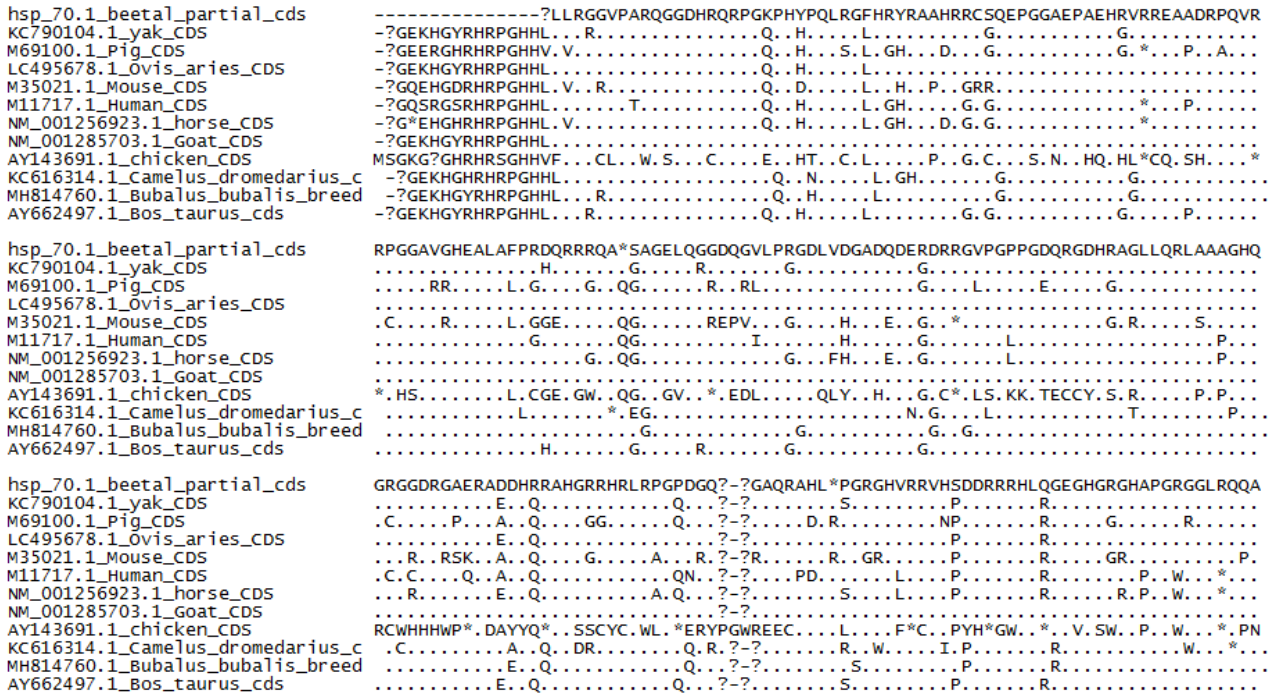
There are 43 changes in cDNA sequence of Beetal *HSP70-1* gene when compared with *Bos taurus* and 35 changes in the nucleotide sequence of *HSP70-1* gene when compared with *Bubalus bubalis*.

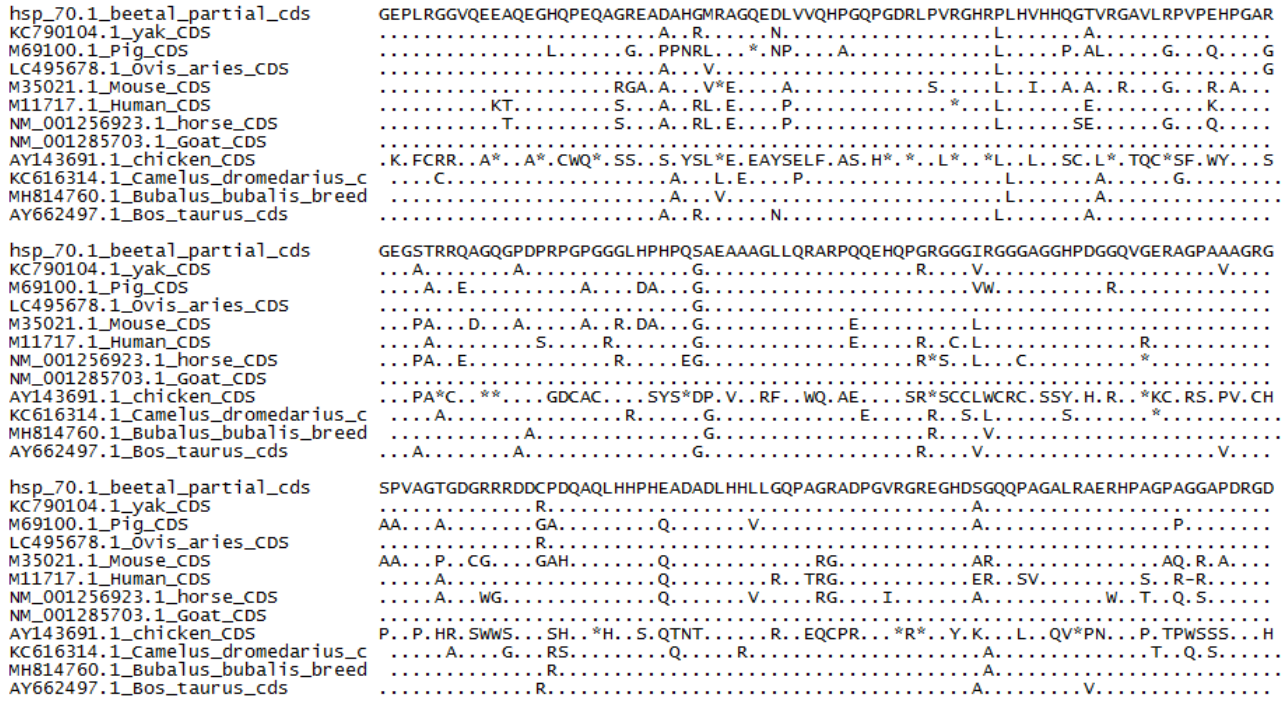
The alignment report of inferred amino acid sequence of *HSP70* gene in goat, cattle, buffalo, yak, pig, horse, camel, mouse, chicken and human is presented in **Table 4.4**. Comparison of Beetal HSP70-1 amino acid sequence with *Bubalus bubalis* showed 34 changes in amino acid sequence and 38 changes when compared to *Bos taurus*.

4.7.2 Estimating Evolutionary Distances Using Pairwise Distance

The pair wise distance between sequences aligned with ClustalW method was estimated by MEGA X software **(Table 4.5)** by computing the proportion of nucleotide differences between each pair of sequences. The pairwise distance estimates the evolutionary divergence between sequences. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al*., 2004). Maximum divergence from Beetal partial cDNA was observed with chicken CDS with value of 0.2821967867 and minimum divergence was observed with sheep with value of 0.0093044618.

Table 4.4: Multiple alignment report of deduced amino acid sequence of Beetal goat *HSP70-1* gene with that of other species




hsp_70.1_beetal_partial_cds GEPLRGGVQEEAQEGHQPEQAGREADAHGMRAGQEDLVVQHPGQPGDRLPVRGHRPLHVHHQGTVRGAVLRPVPEHPGAR
KC790104.1_yak_CDS .....A..R.....N.....L.....A.....
M69100.1_Pig_CDS .....L.....G..PPNRL...*.NP....A.....L.....P.AL.....G...Q....G
LC495678.1_Ovis_aries_CDS .....A...V.....L.....G
M35021.1_Mouse_CDS .....RGA.A...V*E....A.....S.....L..I..A.A..R...G...R.A...
M11717.1_Human_CDS .....KT.....S...A..RL.E....P.....*...L.....E.....K.....
NM_001256923.1_horse_CDS .....T.....S...A..RL.E....P.....L.....SE.....G...Q.....
NM_001285703.1_Goat_CDS .....
AY143691.1_chicken_CDS .K.FCRR..A*..A*.CWQ*.SS..S.YSL*E.EAYSELF.AS.H*.*..L*..*L..L..SC.L*.TQC*SF.WY...S
KC616314.1_Camelus_dromedarius_c ....C.....A...L.E....P.....L.....A.....G.....
MH814760.1_Bubalus_bubalis_breed .....A...V.....L.....A.....
AY662497.1_Bos_taurus_cds .....A..R.....N.....L.....A.....
hsp_70.1_beetal_partial_cds GEGSTRRQAGQGPDPRPGPGGGLHPHPQSAEAAAGLLQRARPQQEHQPGRGGGIRGGGAGGHPDGGQVGERAGPAAAGRG
KC790104.1_yak_CDS ...A.....A.....G.....R....V.....V....
M69100.1_Pig_CDS ....A..E.....A....DA...G.....VW.....R.....
LC495678.1_Ovis_aries_CDS .....G.....
M35021.1_Mouse_CDS ...PA...D...A.....A..R.DA...G.....E.....L.....
M11717.1_Human_CDS ....A.....S.....R.....G.....E.....R..C.L.....R.....
NM_001256923.1_horse_CDS ...PA..E.....R.....EG.....R*S..L...C.....*.....
NM_001285703.1_Goat_CDS .....
AY143691.1_chicken_CDS ...PA*C..**....GDCAC....SYS*DP.V..RF..WQ.AE....SR*SCCLWCRC.SSY.H.R..*KC.RS.PV.CH
KC616314.1_Camelus_dromedarius_c ....A.....R.....G.....E.....R..S.L.....S.....*.....
MH814760.1_Bubalus_bubalis_breed .....A.....G.....R....V.....
AY662497.1_Bos_taurus_cds ...A.....A.....G.....R....V.....V....
hsp_70.1_beetal_partial_cds SPVAGTGDGRRRDDCPDQAQLHHPHEADADLHHLLGQPAGRADPGVRGREGHDSGQQPAGALRAERHPAGPAGGAPDRGD
KC790104.1_yak_CDS .....R.....A.....
M69100.1_Pig_CDS AA...A.....GA.....Q.....V.....A.....P.....
LC495678.1_Ovis_aries_CDS .....R.....
M35021.1_Mouse_CDS AA...P..CG....GAH.....Q.....RG.....AR.....AQ.R.A....
M11717.1_Human_CDS .....A.....Q.....R..TRG.....ER..SV.....S..R-R.....
NM_001256923.1_horse_CDS .....A...WG.....Q.....V.....RG....I.....A.....W..T..Q.S.....
NM_001285703.1_Goat_CDS .....
AY143691.1_chicken_CDS P..P.HR.SWWS...SH..*H..S.QTNT.....R..EQCPR...*R*..Y.K...L..QV*PN...P.TPWSSS...H
KC616314.1_Camelus_dromedarius_c .....A....G...RS.....Q.....R.....A.....T..Q.S.....
MH814760.1_Bubalus_bubalis_breed .....R.....A.....
AY662497.1_Bos_taurus_cds .....R.....A.....V.....

```
hsp_70.1_beetal_partial_cds      LRHRRQWHPERHGHGQEHGQGQQDHHHQRQGPAEQGGDRAHGAGGGEVQGRGRGPAREGVCQERAGVVRLQHEERRGG*G
KC790104.1_yak_CDS               ..............................................K...G.............................
M69100.1_Pig_CDS                 ......RN.....D.................P............*...SG.*D.....W.*..S....F..........
LC495678.1_Ovis_aries_CDS        ..............................................R.................................
M35021.1_Mouse_CDS               ......R.......R...R.............P............RAL......A..Q.GR....R.LC.........R.
M11717.1_Human_CDS               ....C.R...........R.............P...............SG...A.....S...P..L............
NM_001256923.1_horse_CDS         .*............R.................P...............S.............CP....V.....S....
NM_001285703.1_Goat_CDS          ................................................................................
AY143691.1_chicken_CDS           F*..C*.Y...QCC...YRE.E..N...*..SP*.R*Y*PY.TRSR.I.S..*SQQ.*.GS..LP*..Y....ADS...E
KC616314.1_Camelus_dromedarius_c  ................K.................................S..........R...P..L.............
MH814760.1_Bubalus_bubalis_breed  ..............................................K.....................L.......S.R..
AY662497.1_Bos_taurus_cds        ..............................................K...G.................I............

hsp_70.1_beetal_partial_cds      AEGQDQRGGQEGGAGQVPGGDFLAGRQHLGGEGRV*AQEEGAGAGV*PHHQQTVPGGGRPRGRRLWGSGP?---------
KC790104.1_yak_CDS               ...........E.....................................................W.......??RGLWVWPH
M69100.1_Pig_CDS                 .*.........E.....S..........A.R...................R......W..AG..R..RS??RGLWVWPH
LC495678.1_Ovis_aries_CDS        ...........E.....................................................W.......??RGLWVWPH
M35021.1_Mouse_CDS               SQ..A...*..E........HL...L..A.RQ.G.R..AG......Q....WA...C.CSW.WG.R.P.AAERSLWLRTH
M11717.1_Human_CDS               .Q......R..E.....SR.HLV.......R.....................R....CRWS.AWG.R...S??GRVWVRPH
NM_001256923.1_horse_CDS         .Q.........E....M...........F.RK.........V......Y.HW......W...WW.....S??GWLWVWPH
NM_001285703.1_Goat_CDS          ................................................................??RGLWVWPH
AY143691.1_chicken_CDS           T..K..*P..AES.R......Q..*PKPD.R.R.....A.R..ETLQ.DCHK....SW.SW..W.R-----------WPN
KC616314.1_Camelus_dromedarius_c  .Q......R..ES.........VV....A.R.........R.........W......W....W......??RGLWVWPH
MH814760.1_Bubalus_bubalis_breed  ...........E.............C.....................................W..R....??RGLWVWPH
AY662497.1_Bos_taurus_cds        ...........E.....................................................W.......??RGLWVWPH

hsp_70.1_beetal_partial_cds      -------
KC790104.1_yak_CDS               H*GGGL-
M69100.1_Pig_CDS                 HRGGG?-
LC495678.1_Ovis_aries_CDS        H*GGGL-
M35021.1_Mouse_CDS               HRGGGL-
M11717.1_Human_CDS               H*GGRL-
NM_001256923.1_horse_CDS         H*GGGL-
NM_001285703.1_Goat_CDS          H*GGGL-
AY143691.1_chicken_CDS           H*RSRL?
KC616314.1_Camelus_dromedarius_c  H*GGRL-
MH814760.1_Bubalus_bubalis_breed  H*GGGL-
AY662497.1_Bos_taurus_cds        H*GGGL-
;
end;
```

Table 4.5: Estimates of evolutionary divergence between sequences / pairwise distance

[1] #hsp_70.1_beetal_partial_cds
[2] #KC790104.1_yak_CDS
[3] #M69100.1_Pig_CDS
[4] #LC495678.1_Ovis_aries_CDS
[5] #M35021.1_Mouse_CDS
[6] #M11717.1_Human_CDS
[7] #NM_001256923.1_horse_CDS
[8] #NM_001285703.1_Goat_CDS
[9] #AY143691.1_chicken_CDS
[10] #KC616314.1_Camelus_dromedarius_cds
[11] #MH814760.1_Bubalus_bubalis_breed_Murrah_complete_cds
[12] #AY662497.1_Bos_taurus_cds

[	1	2	3	4	5	6	7	8	9	10	11	12]
[1]												
[2]	0.0209942790											
[3]	0.0699515618	0.0632828812										
[4]	0.0093044618	0.0141822463	0.0627346002									
[5]	0.0935406732	0.0920434587	0.0982090227	0.0878583208								
[6]	0.0609564165	0.0592189529	0.0792332223	0.0592515670	0.0939510639							
[7]	0.0554496610	0.0506312854	0.0724498457	0.0511698541	0.0889273925	0.0529605109						
[8]	0.0000000000	0.0205617620	0.0707290613	0.0094054370	0.0955667486	0.0654929230	0.0573213230					
[9]	0.2821967867	0.2787296119	0.2792469904	0.2723273607	0.2829734551	0.2714852282	0.2637341097	0.2806324148				
[10]	0.0478150561	0.0428044819	0.0678709112	0.0417035970	0.0884677779	0.0557433951	0.0466947692	0.0477718428	0.2684225030			
[11]	0.0193014669	0.0088882730	0.0638790905	0.0115262777	0.0884361830	0.0586455269	0.0494728652	0.0189469597	0.2771028376	0.0405574079		
[12]	0.0238060089	0.0026012930	0.0649866082	0.0168417191	0.0932164310	0.0591932086	0.0523003044	0.0232438360	0.2800613318	0.0450162679	0.0109965911	

4.7.3 Nucleotide and Amino Acid Compositions

The nucleotide composition as calculated by MEGA X was presented in **Table 4.6** and amino acid composition in **Table 4.7**. GC content of the sequences was high at about 60% approximately. The translated protein of Beetal HSP70-1 partial cDNA sequence (1839 bp) has molecular weight of 67469.53 Daltons with 613 amino acids out of which 88 are strongly acidic (-) amino acids (D,E), 80 strongly basic (+) amino acids (K,R), 214 hydrophobic amino acids (A,I,L,F,W,V) and 144 polar amino acids (N,C,Q,S,T,Y).

Table 4.6: Nucleotide composition (in percentage) of *HSP70-1* gene CDS sequence of different species

Species	T (U)	C	A	G	Total
HSP70-1 Beetal partial cds	13.8	29.3	22.5	34.4	1839
KC616314.1 Camelus dromedarius cds	13.9	29.5	22.5	34.1	1926
AY143691.1 chicken CDS	20.3	24.2	27.8	27.8	1905
NM 001256923.1 Horse cds	14.5	29.0	22.4	34.0	1926
NM 001285703.1 Goat CDS	13.9	29.2	22.5	34.4	1926
M69100.1 Pig CDS	13.8	28.7	22.0	35.5	1923
AY662497.1 Bos taurus cds	13.8	29.1	22.4	34.7	1926
LC495678.1 Ovis aries cds	13.8	29.3	22.4	34.5	1926
MH814760.1 Bubalis bubalis Murrah complete cds	13.8	29.1	22.3	34.7	1926
M11717.1 Human cds	14.1	29.8	22.8	33.3	1923
M35021.1 Mouse CDS	13.4	30.5	21.3	34.7	1929
KC790104.1 Yak CDS	13.8	29.1	22.4	34.7	1926
Avg.	14.4	28.9	22.8	33.9	1916.8

Table 4.7: Composition (in percentage) of deduced amino acid sequences of *HSP70-1* gene of different species

Species	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
MH814760.1 Buffalo cds	10.90	0.47	5.37	6.16	0.32	22.27	8.85	0.00	0.32	6.00	0.00	0.00	8.21	9.48	14.69	0.79	0.47	4.74	0.63	0.32	633
Hsp70.1 Beetal partial cds	10.73	0.50	5.94	6.11	0.50	21.62	8.09	0.17	0.17	5.28	0.17	0.00	8.75	9.57	15.18	1.32	0.66	4.62	0.33	0.33	606
NM_001256923.1 horse cds	9.71	0.80	5.41	6.53	0.64	21.18	9.24	0.16	0.16	5.73	0.16	0.00	8.92	9.55	13.06	1.59	0.48	4.62	1.91	0.16	628
KC790104.1 yak cds	10.90	0.32	5.06	6.16	0.32	22.12	9.00	0.00	0.32	5.85	0.16	0.16	8.21	9.48	15.01	0.47	0.47	4.90	0.79	0.32	633
M69100.1 pig cds	11.61	0.48	5.88	6.20	0.32	22.73	7.79	0.00	0.00	5.72	0.00	0.64	8.59	9.22	14.31	1.11	0.16	4.45	0.79	0.00	629
AY662497.1 Bos taurus cds	10.58	0.32	5.06	6.16	0.32	22.27	9.00	0.16	0.32	5.85	0.16	0.16	8.37	9.48	14.85	0.47	0.47	4.90	0.79	0.32	633
NM 001285703.1 Goat cds	10.27	0.47	5.69	6.00	0.47	21.80	8.69	0.16	0.32	5.53	0.16	0.00	8.69	9.16	15.01	1.26	0.63	4.58	0.63	0.47	633
LC495678.1 Ovis aries cds	10.43	0.32	5.37	6.32	0.32	21.80	8.85	0.16	0.16	5.85	0.00	0.00	8.69	9.32	15.01	0.95	0.63	4.74	0.79	0.32	633
KC616314.1 Camelus dromedarius cds	10.28	0.47	5.38	6.17	0.16	20.73	8.70	0.16	0.32	6.17	0.00	0.32	8.86	9.49	15.03	1.42	0.47	4.43	1.42	0.00	632
AY143691.1 Chicken cds	6.98	5.79	3.92	5.96	0.19	12.10	9.54	0.17	1.53	6.98	0.17	1.02	8.35	8.69	8.35	7.50	1.87	3.07	3.58	3.24	587
M11717.1 Human cds	9.21	1.11	5.08	5.87	0.16	20.00	9.05	0.16	0.32	6.35	0.00	0.16	8.57	10.00	15.87	2.22	0.63	4.60	0.63	0.00	630
M35021.1 Mouse cds	13.01	0.78	5.02	5.49	0.00	20.53	9.09	0.16	0.16	6.43	0.00	0.00	7.37	9.40	16.61	0.94	0.16	4.23	0.63	0.00	638
Avg	10.41	0.96	5.27	6.09	0.39	20.81	8.82	0.12	0.33	5.97	0.08	0.20	8.46	9.41	14.45	1.64	0.59	4.50	1.06	0.44	626.3

4.7.4 Phylogenetic tree analysis

The phylogenetic tree was constructed by MEGA X software at nucleotide level as shown in **Figure 4.1.** The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Branch length represents the number of changes that have occurred in that branch. Goat and sheep *HSP70-1* gene form a same cluster and shows identical lineage. Cattle, buffalo and yak along with sheep and goat might have evolved from same common ancestor. Human and horse form same cluster, however, bootstrap value is low with only 55%. Pig, mouse and chicken sequences show dissimilarities suggesting different ancestry.

4.7.5 Z test for Test of Neutrality

Z test was conducted in order to test whether positive selection was operating on *HSP70-1* gene or not. It was done by comparing the relative abundance of synonymous and nonsynonymous substitutions that have occurred in the gene sequences. Analyses was conducted using the Nei-Gojobori method (Nei and Gojobori, 1986).

The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) (below diagonal) is shown in the Codon-based Test of Neutrality **(Table 4.8)**. The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) in favor of the alternative hypothesis ($d_N > d_S$) (below diagonal) is show in Codon-based Test of Positive Selection **(Table 4.9)**. The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) in favor of the alternative hypothesis ($d_N < d_S$) (below diagonal) is shown in Codon-based Test of Purifying Selection **(Table 4.10)**. d_S and d_N are the numbers of synonymous and nonsynonymous substitutions per site, respectively.

The above test was conducted on MEGA X software and the alternate hypotheses (a) $d_N \neq d_S$ (test of neutrality) and (c) $d_N < d_S$ (purifying selection) are accepted and so the

selection is of purifying type. There is no positive selection. It means that the number of non synonymous nucleotide changes are low as compared to synonymous changes.

Purifying selection refers to selection against nonsynonymous substitutions at the DNA level. In this case, the evolutionary distance based on synonymous substitutions is expected to be greater than the distance based on nonsynonymous substitutions. At the DNA sequence level, positive selection refers to selection in favor of nonsynonymous substitutions. In this case, the evolutionary distance based on nonsynonymous substitutions is expected to be greater than synonymous substitutions.

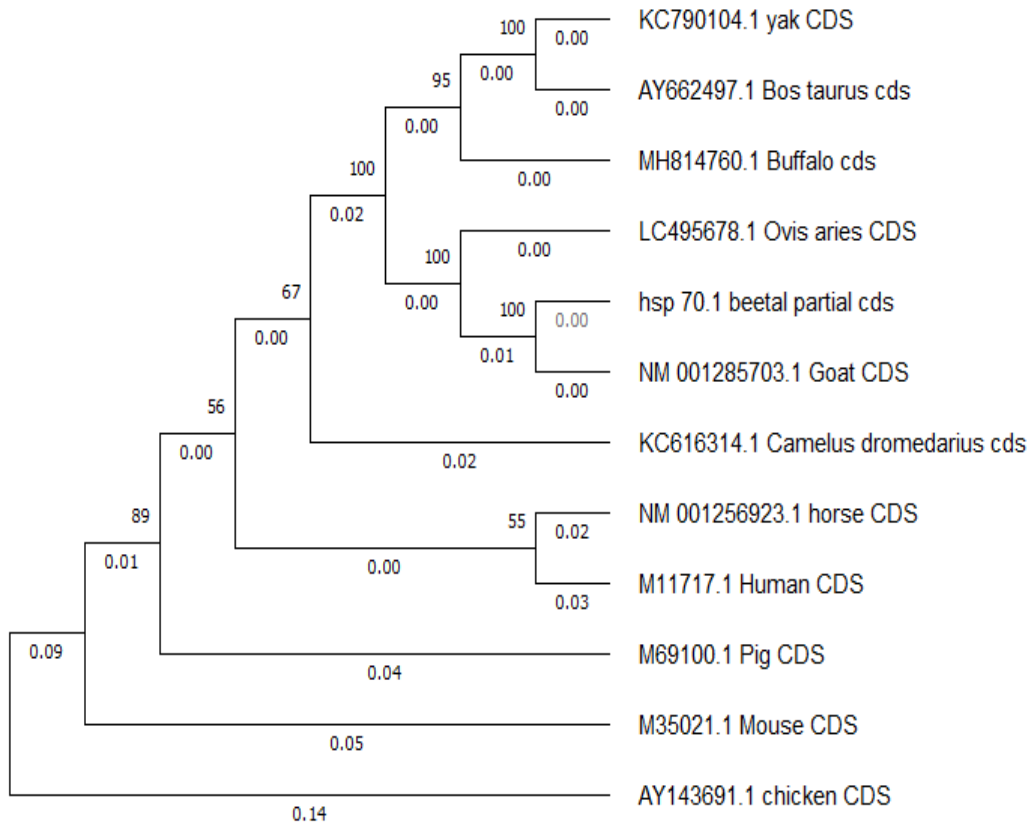


Figure 4.1: Phylogenetic tree constructed upon the alignment of *HSP70-1* nucleotide sequences.

Table 4.8: Codon-based Test of Neutrality

[1] #MH814760.1_Buffalo_cds
[2] #hsp_70.1_beetal_partial_cds
[3] #NM_001256923.1_horse_CDS
[4] #KC790104.1_yak_CDS
[5] #M69100.1_Pig_CDS
[6] #AY662497.1_Bos_taurus_cds
[7] #NM_001285703.1_Goat_CDS
[8] #LC495678.1_Ovis_aries_CDS
[9] #KC616314.1_Camelus_dromedarius_cds
[10] #AY143691.1_chicken_CDS
[11] #M11717.1_Human_CDS
[12] #M35021.1_Mouse_CDS

[	1	2	3	4	5	6	7	8	9	10	11	12]
[1]												
[2]	0.0000022877											
[3]	0.0000000000	0.0000000000										
[4]	0.0001198226	0.0000011836	0.0000000000									
[5]	0.0000000000	0.0000000000	0.0000000000	0.0000000000								
[6]	0.0000380173	0.0000002376	0.0000000000	0.0702989036	0.0000000000							
[7]	0.0000031950	1.0000000000	0.0000000000	0.0000016526	0.0000000000	0.0000003334						
[8]	0.0000045117	0.0178973344	0.0000000000	0.0000009343	0.0000000000	0.0000001959	0.0238343593					
[9]	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000				
[10]	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000			
[11]	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000		
[12]	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	

The probability of rejecting the null hypothesis of strict-neutrality (dN = dS) (below diagonal) is shown.

Values of P less than 0.05 are considered significant at the 5% level.

Table 4.9: Codon-based Test of Positive Selection

[1] #MH814760.1_Buffalo_cds
[2] #hsp_70.1_beetal_partial_cds
[3] #NM_001256923.1_horse_CDS
[4] #KC790104.1_yak_CDS
[5] #M69100.1_Pig_CDS
[6] #AY662497.1_Bos_taurus_cds
[7] #NM_001285703.1_Goat_CDS
[8] #LC495678.1_Ovis_aries_CDS
[9] #KC616314.1_Camelus_dromedarius_cds
[10] #AY143691.1_chicken_CDS
[11] #M11717.1_Human_CDS
[12] #M35021.1_Mouse_CDS

[	1	2	3	4	5	6	7	8	9	10	11	12]
[1]												
[2]	1.00											
[3]	1.00	1.00										
[4]	1.00	1.00	1.00									
[5]	1.00	1.00	1.00	1.00								
[6]	1.00	1.00	1.00	1.00	1.00							
[7]	1.00	1.00	1.00	1.00	1.00	1.00						
[8]	1.00	1.00	1.00	1.00	1.00	1.00	1.00					
[9]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00				
[10]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00			
[11]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
[12]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	

The probability of rejecting the null hypothesis of strict-neutrality (dN = dS) in favor of the alternative hypothesis (dN > dS) (below diagonal) is shown.
Values of P less than 0.05 are considered significant at the 5% level.

Table 4.10: Codon-based Test of Purifying Selection

[1] #MH814760.1_Buffalo_cds
[2] #hsp_70.1_beetal_partial_cds
[3] #NM_001256923.1_horse_CDS
[4] #KC790104.1_yak_CDS
[5] #M69100.1_Pig_CDS
[6] #AY662497.1_Bos_taurus_cds
[7] #NM_001285703.1_Goat_CDS
[8] #LC495678.1_Ovis_aries_CDS
[9] #KC616314.1_Camelus_dromedarius_cds
[10] #AY143691.1_chicken_CDS
[11] #M11717.1_Human_CDS
[12] #M35021.1_Mouse_CDS

[	1	2	3	4	5	6	7	8	9	10	11	12]
[1]												
[2]	0.0000011439											
[3]	0.0000000000	0.0000000000										
[4]	0.0000599113	0.0000005918	0.0000000000									
[5]	0.0000000000	0.0000000000	0.0000000000	0.0000000000								
[6]	0.0000190086	0.0000001188	0.0000000000	0.0351494518	0.0000000000							
[7]	0.0000015975	1.0000000000	0.0000000000	0.0000008263	0.0000000000	0.0000001667						
[8]	0.0000022559	0.0089486672	0.0000000000	0.0000004672	0.0000000000	0.0000000980	0.0119171796					
[9]	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000				
[10]	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000			
[11]	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000		
[12]	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	0.0000000000	

The probability of rejecting the null hypothesis of strict-neutrality (dN = dS) in favor of the alternative hypothesis (dN < dS) (below diagonal) is shown.
Values of P less than 0.05 are considered significant at 5% level.

CHAPTER - V

DISCUSSION

Stress represents the reaction of the body to stimuli that disturbs the normal physiological equilibrium or homeostasis, often with detrimental effects (David *et al.*, 1990). 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). In day-to-day life, animals and humans undergo various stress conditions. Stress could be physical, nutritional, chemical, psychological and environmental. High environmental temperature is the vital concern in tropical and arid areas (Silannikove, 1992) that challenges the animal's capability to maintain energy, water, hormonal, thermal, and mineral balance. Heat stress results from the animal's inability to dissipate heat to maintain homeothermy and is further intensified by high humidity, thermal radiation, low air movement and high metabolic heat (Morison, 1983).

The heat shock response is known to occur in all organisms including animal, plant and bacteria. It is a rapid but transient reprogramming of cellular activities to make certain survival during the period of stress, to protect essential cell components against damage due to heat and permit a rapid continuation of normal cellular activities during the period of recovery.

All organisms respond to heat by inducing the synthesis of a group of proteins called the heat-shock proteins or HSPs. The response is the most highly conserved genetic system, existing in every organism (Lindquist and Craig, 1988) and present in both prokaryotic and eukaryotic cells (Kiang and Tsokos, 1998). Certain features of the response vary from organism to organism. The proteins are named, however, because they were first identified on the basis of their increased synthesis following exposure to elevated temperatures (Ritossa, 1962). Expression of most HSPs is induced by heat and other stresses including hypoxia, nutrient deprivation, oxygen radicals, metaboic disruption, viral infection (Morimoto and Milarski, 1990) and transformation. The HSP

have major physiological role in protein homeostasis also (Ellis *et al*., 1991; Mathew and Morimoto, 1998).

The acquisition of thermal tolerance is related to increased levels of the HSP70 protein. The exposure of individuals to hyperthermia leads to quick and transient responses at transcriptional and translational levels, and is considered to be the mechanism responsible for cell survival during the stress period (Burdon, 1986). Among the HSPs, HSP70 is the one that shows the highest levels under stressful situations. HSP70 exist in both constitutively expressed and inducible forms that are activated by stressful stimuli (Morimoto, 1998; Jolly and Morimoto, 2000). The 70-kDa HSP assists the folding of other proteins. It binds to nascent peptide chains on ribosomes, protecting the hydrophobic surface that would normally be exposed to solvent, thus preventing aberrant folding or aggregation, until the whole peptide chain is synthesized and proper folding occurs (Gaviol *et al*., 2008). The HSP70 is the most phylogenetically conserved family (Hunt and Morimoto, 1985; Morimoto *et al*., 1986).

5.1 Isolation of genomic DNA and its preliminary analysis

Under sterile conditions, 5 ml of venous blood was collected from Beetal goat. DNA was isolated from blood samples as per the protocol of genomic DNA blood mini kit (Nucleo-pore). The isolated samples were stored at 4°C. Concentration was calculated spectrophotometrically by taking optical density (OD) at 260 nm. A total of 50-60 µg of DNA was obtained from the 200 µl of the blood.

5.2 Primers

For conducting the PCR reaction, specific primers for goat *HSP70-1* were designed based on available sequence of goat *HSP70-1* (Accession number: FJ975769) gene using Primer3 software available at NCBI.com. The optimum concentration of each primer was determined by setting up PCR trials. The optimum level of primers per 25µl reaction volume was found to be 25µM. When primers were used at less than optimum level, lower yield of desired product was obtained. On the other hand, primers at a concentration more than optimum resulted in primer dimers and non-specific amplification.

5.3 PCR standardization

PCR was carried out in a final volume of 25 µl. Amplification was performed in Eppendorf thermal cycler PCR using optimized conditions as follows: one cycle of initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min followed by final extension at 72°C for 10 min.

5.4 Polymerase chain reaction amplification of *HSP70-1* gene in Beetal goat

In the present study partial cDNA of *HSP70-1* gene of 1917 bp of Beetal goat was amplified. The total cDNA length of goat *HSP70-1* gene is of 1926 bp as reported by Gade *et al*. (2010), Pawar *et al*. (2013) in sheep and goat, Habib *et al*. (2018) in Arabi rams.

5.5 Sequencing

The amplified PCR product was sequenced by Sangers dideoxy chain termination method with Primer Walking. The sequence obtained was of 1839 bp as approximately 40-50 bp from both end (forward and reverse) clean sequence was not obtained and was unreadable.

5.6 Sequence analysis

The obtained sequence of *HSP70-1* gene of Beetal goat was analysed by MEGA X software with other reported CDS (Coding sequence) sequences of *HSP70-1* in different species of livestock. The *HSP70-1* sequences of other species were downloaded from NCBI (National Center for Biotechnology Information). All *HSP70-1* cDNA sequences of different domestic species viz. sheep, goat, buffalo, yak, cattle, horse, pig, chicken, camel, mouse and human were aligned (multiple sequence alignment) using ClustalW method which reveals the nucleotide substitutions. The comparison of Beetal goat *HSP70-1* cDNA sequence demonstrated 99.08, 97.66, 97.93, 98.10, 94.73, 95.43, 93.78, 94.22 and 91.39 percent homology with sheep, cattle, yak, buffalo, horse, camel, pig, human and mouse, respectively which indicates close evolutionary relationship and high sequence homology among the species. Deduced amino acid sequence of 613 residues of Beetal goat *HSP70-1* gene was 100, 98.99, 98.83, 98.4, 98.83,

98.49, 98.49, 97.32, 90.45 and 97.99 percent similar to goat, buffalo, sheep, yak, cattle, horse, camel, pig, mouse and human, respectively.

Similar results was shown by Gade *et al*., 2010, the entire nucleotide sequence of goat *HSP70-1* gene shows 99.4% homology with sheep (partial), 96.3% homology with buffalo, 97.5% with yak, 97.8% with cattle, 94.4% with horse, 95.3% with pig and 94.1% with human which indicates close evolutionary relationship. The deduced amino acid sequence of *HSP70* gene of goat was 100% similar to sheep (partial), 95.9% similar to buffalo, 98.6% similar to cattle, 98% to pig, 98.4% to yak, 98.1% to horse, and 97.7% similar to human sequence.

Similarly Pawar *et al*., 2013 found that the the entire nucleotide sequence of sheep *HSP70* gene shows 98% homology with goat, cattle, yak and buffalo, 95% with pig, 96% with camel, 94% with dog and 93% with human indicating close evolutionary relationship. Among the twenty one nucleotides substitutions in DNA sequence of *HSP70* gene of goat when compared to sheep, changes at amino acid positions 9, 100, 121, 184, 263 and 569 resulted in substitutions. Inferred amino acid sequence of 641 amino acids of goat HSP70 was 98.9% to buffalo, 98.8% to cattle, 98.9% similar to sheep, 94.8% to dog, 95.7% to pig and 95.7% to human sequence. The results showed that *HSP70* nucleotide and deduced amino acid sequence is highly conserved across the species.

Fatima *et al*., 2019 in her study found that the results of homology analysis showed 99.93% and 99.35% similarity with sequences of goat and sheep (partial) respectively, which was further confirmed by constructing phylogenetic tree. The phylogenetic tree revealed that Sindh ibex, sheep and domestic goat share a common ancestor.

Pelham, 1982 earlier stated that HSP proteins are highly conserved both in regulatory sequence as well as in protein coding sequence. Madhusudan, 2007 found that *HSP70* gene sequence was conserved in buffalo. He further observed 41 changes in nucleotide sequence of buffalo when compared with cattle and reported that buffalo *HSP70* cDNA sequence has 97.8% homology with cattle *HSP70-1* DNA sequence. Deduced amino acid sequence of buffalo showed 94.4% homology with cattle HSP70-1 deduced amino acid sequence. Gutierrez and Guerriero, 1995 found that amino acid sequences of HSP70 proteins in bovine are highly conserved at amino acids 9-16 and

131-139. Morimoto *et al*., 1986 characterized *HSP70* gene in chicken and found that chicken *HSP70* cDNA sequence is 80% identical to human *HSP70* cDNA sequence and 73% identical to Drosophila *HSP70* cDNA sequence. However, chicken HSP70 deduced amino acid sequence is 80% identical to human HSP70 deduced amino acid sequence but only 71% identical to Drosophila HSP70 amino acid sequence. Kano *et al*., 2004 reported in canine, that the amino acid sequence of *HSP70* gene showed 90-95% similarity with bovine, mouse and human HSP70 proteins. Hunt and Calderwood, 1990 reported that in mouse an open reading frame of *HSP70* gene encoding 642-amino acids and 70-kDa protein with 91% nucleotide homology and 95% amino acid homology to a human *HSP70* gene. Lisowska *et al*., 1994 cloned and determined the nucleotide sequence of a gene (named *HSP70-1*) in rat cells. It contains an uninterrupted open reading frame of 1926 bp which encodes a protein of approx. 70,100 Da and the predicted amino acid sequence of its product showed 98% similarity to the mouse HSP70 protein.

5.7 Phylogenetic tree analysis

The phylogenetic tree was constructed by MEGA X software at nucleotide level. Goat and sheep *HSP70-1* gene form a same cluster and shows identical lineage. Cattle, buffalo and yak along with sheep and goat might have evolved from same common ancestor. Human and horse form same cluster, however, bootstrap value is low with only 55%. Pig, mouse and chicken sequences show dissimilarities suggesting different ancestry.

Similar result was shown by Gade *et al*., 2010, phylogenetic tree analysis showed that ruminants and monogastrics according to their closer evolutionary relationship are derived from different clusters. Cattle, sheep, goat and buffalo might have evolved from a common ancestor. Pig positioned in between and diverged early from the bovine ancestors.

In a study by Sharma *et al*., 2012, phylogenetic analysis showed that bovine, humans, rat and mice according to their closer evolutionary relationship are derived from different ancestors. Among bovines, cattle and buffalo might have evolved from a common ancestor. Pig positioned in between and diverged early from the bovine

ancestors. Buffalo *HSPA1A* gene has a separate place, more close to bovine but having different lineage.

5.8 Z test for Test of Neutrality

Z test was conducted in order to test whether positive selection was operating on *HSP70-1* gene or not. Values of *P* less than 0.05 are considered significant at the 5% level. With the above tests conducted on MEGA X software the alternate hypotheses (a) $d_N \neq d_S$ (test of neutrality) and (c) $d_N < d_S$ (purifying selection) are accepted. It means that the number of nonsynonymous nucleotide changes are low as compared to synonymous changes and hence the selection is of purifying type. There is no positive selection.

In a study by Gade *et al*., 2010, *HSP70-1* gene might have evolved by positive selection ($d_N > d_S$) at 5% level of significance as dN is substantially greater than dS.

Mathew *et al*., 2013 showed a high sequence homology among different species indicating that the *HSP70* gene is evolutionarily conserved. Synonymous substitution (d_S) in the *HSP70* gene was higher than non-synonymous substitution (d_N), suggesting that the gene is not under positive selection and that no advantageous mutations had any significant role in its evolutionary adaptation.

CHAPTER - VI

SUMMARY AND CONCLUSIONS

In day-to-day life, animals and humans undergo various stress conditions. A series of genetic, physiological, immunological, biochemical and behavioral responses have been evolved for adaptation to different environmental conditions in different animal species. Heat shock proteins play an important role in conferring resistance to cells exposed to wide range of cellular stress such as high temperatures, oxidative stress, nutritional deficiencies, ultraviolet radiations, viral infections, exposure to chemicals etc. HSP serves as molecular chaperones and are involved in unfolding of cytosolic proteins and their subsequent translocation into mitochondria and endoplasmic reticulum where they are refolded and assembled into oligomeric complexes. Cells exposed to various stresses show elevated levels of HSP and can exhibit tolerance to potential stressors. The ubiquitous HSP70 proteins are the most temperature sensitive of the all HSPs and its induction is correlated with development of thermo-tolerance. In goat, few reports are available with regard to role and importance of HSP70 during heat stress. Therefore, the present investigation was undertaken to study molecular characterization of *HSP70-1* gene in Beetal goat and analyzing genetic diversity through sequences available in databases.

Genomic DNA was isolated from whole blood of Beetal goat by DNA Sure Blood Kit (Nucleo-pore). The DNA purity was checked by using Nano-drop spectrophotometer by taking the ratio of optical density at 260nm and 280nm. Only good quality DNA that was having OD ratio ranging from 1.7 to 1.9 was used for further analysis. A pair of primers *i.e.,* forward primer with sequence 5′-CGAAAAACATGGCTATCGGC-3′ and reverse primer with sequence 5′-CCACCTCCTCAATGGTGGG-3′ was used for amplification of *HSP70-1* gene that resulted in 1917 bp PCR product. After the confirmation, one PCR product was selected and was sent for Sanger sequencing with "Primer Walking" at AgriGenome Labs Pvt. Ltd. Cochin India. A partial cDNA sequence of goat *HSP70-1* gene of 1839 bp encoding 613 amino acids was obtained. Cross species comparison of nucleotide and deduced amino acid sequences of Beetal goat were done

with cattle, buffalo, yak, goat, sheep, pig, horse, camel, mouse and human. The comparison of Beetal goat *HSP70-1* partial cDNA sequence demonstrated 99.08, 97.66, 97.93, 98.10, 94.73, 95.43, 93.78, 94.22 and 91.39 percent homology with sheep, cattle, yak, buffalo, horse, camel, pigs, human and mouse, respectively which indicates close evolutionary relationship and high sequence homology among the species. Deduced amino acid sequence of 613 residues of Beetal goat *HSP70-1* gene was 100, 98.99, 98.83, 98.4, 98.83, 98.49, 98.49, 97.32, 90.45 and 97.99 percent similar to goat, buffalo, sheep, yak, cattle, horse, camel, pig, mouse and human, respectively. The nucleotide sequence of *HSP70-1* partial cDNA of Beetal goat was aligned and compared with respective sequences of other species viz. goat, cattle, buffalo, yak, sheep, pig, horse, mouse, camel, chicken and human using ClustalW method which revealed the nucleotide substitutions. There were 43 and 35 changes in cDNA sequence of *HSP70-1* gene when compared with *Bos taurus* and *Bubalus bubalis*, respectively. Comparison of Beetal HSP70-1 amino acid sequence with *Bubalus bubalis* and *Bos taurus* showed 34 and 38 changes in amino acid sequence, respectively. The phylogenetic tree drawn by MEGA X software at nucleotide level showed conserved nature of *HSP70-1* gene. Goat and sheep *HSP70-1* gene form a same cluster and showed identical lineage. Cattle, buffalo and yak along with sheep and goat might have evolved from same common ancestor. Human and horse form same cluster, however, bootstrap value was low with only 55%. Pig, mouse and chicken sequences showed dissimilarities suggesting different ancestry. The pair wise distance between sequences aligned with ClustalW method was estimated by MEGA X software. Maximum divergence from Beetal partial cDNA was observed with chicken CDS with value of 0.2821967867 and minimum divergence was observed with sheep with value of 0.0093044618. GC content of the sequences was high at about 60% approximately. The translated protein of Beetal *HSP70-1* partial cDNA sequence (1839 bp) has molecular weight of 67469.53 Daltons with 613 amino acids. Out of which 88 are strongly acidic (-) amino acids (D,E) and 80 strongly basic (+) amino acids (K,R), 214 hydrophobic amino acids (A,I,L,F,W,V) and 144 polar amino acids (N,C,Q,S,T,Y). Z test was conducted in order to test whether positive selection was operating on *HSP70-1* gene or not and it was found that gene was undergoing purifying selection and not positive selection.

The molecular characterization of Beetal *HSP70-1* gene was helpful for deriving phylogenic relationship among different species. Thus following conclusions can be drawn from the above study

Conclusion

1. Based on genetic similarity of Beetal goat *HSP70-1* cDNA, it was found to be highly conserved among different species.
2. The *HSP70-1* gene might have evolved by purifying selection ($d_S > d_N$).
3. Based on phylogenetic analysis, sheep CDS sequence was most closed to goat and chicken was most divergent.

REFERENCES

20th Livestock Census-All India Report, 2019. Ministry of Agriculture, Department of Animal Husbandry, Dairying and Fisheries, Govt. of India, New Delhi.

Archana, P. R., Aleena, J., Pragna, P., Vidya, M. K., Niyas, A. P. A., Bagath, M. and Kurien, E. K. 2017. Role of heat shock proteins in livestock adaptation to heat stress. *J. Dairy Vet. Anim. Res.*, **5**: 1-8.

Asea, A., Rehli, M., Kabingu, E., Boch, J. A., Bare, O., Auron, P. E., Stevenson, M. A. and Calderwood, S. K. 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *Journal of Biological Chemistry*, **277**(17): 15028-34.

Bardwell, J. E. and Craig, E. A. 1984. Major heat shock gene of Drosophila and the E.coli heat inducible Dnak gene are homologous. *Proc. Natl. Acad. Sci*., **81**(3): 848-52.

Benjamin, I. J. and McMillan, D. R. 1998. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ. Res.*, **83**(2): 117–132.

Bhat, S., Kumar, P., Kashyap, N., Deshmukh, B., Dige, M. S., Bhushan, B., Chauhan, A., Kumar, A. and Singh, G. 2016. Effect of heat shock protein 70 polymorphism on thermotolerance in Tharparkar cattle. *Vet. World*, **9**(2): 113–117.

Boston, R. S., Viitanen, P. V. and Vierling, E. 1996. Molecular chaperones and protein folding in plants. *Plant Mol. Biol.*, **32**(1–2): 191–222.

Burdon, R. H. 1986. Heat shock and the heat shock proteins. *Biochemical Journal*, **240**(2): 313-24.

Cameron, P. U., Tabarias, H. A., Pulendran, B., Robinson, W. and Dawkins, R. L. 1990. "Conservation of the central MHC genome: PFGE mapping and RFLP analysis of complement, *HSP70*, and *TNF* genes in the goat," *Immunogenetics*, **31**(4): 253–264.

Carra, S., Alberti, S., Arrigo, P. A., Benesch, J. L., Benjamin, I. J., Boelens, W., Bartelt-Kirbach, B., Brundel, B. J. J. M., Buchner, J., Bukau, B., Carver, J. A., Ecroyd, H., Emanuelsson, C., Finet, S., Golenhofen, N., Goloubinoff, P., Gusev, N., Haslbeck, M., Hightower, L. E., Kampinga, H. H., Klevit, R. E., Liberek, K., Mchaourab, H. S., McMenimen, K. A., Poletti, A., Quinlan, R., Strelkov, S. V., Toth, M. E., Vierling, E. and Tanguay, R. M. 2017. The growing world of small heat shock proteins: from structure to functions. *Cell Stress Chaperones*, **22**(4): 601–611.

Christians, E., Michel, E., Adenot, P., Mezger, V., Rallu, M., Morange, M. and Renard, J. P. 1997. "Evidence for the involvement of mouse heat shock factor 1 in the atypical expression of the *HSP70-1* heat shock gene during mouse zygotic genome activation," *Molecular and Cellular Biology*, **17**(2): 778–788.

Daugaard, M., Rohde, M. and Jaattela, M. 2007. The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. *FEBS. Lett.*, **581**(19): 3702–3710.

David, K., Murgo, A. J. and Faith, R. E. 1990. Effects of stress on the immune system. *Immunol. Today*, **11**(10): 348.

Dobson and Smith, R. F. 2000. What is stress and how does it affect reproduction. *Ani. Reprduc. Sci*., **60**: 743-752.

Dodamani, S., Appannavar, M. M., Ramesha, K. P., Suranagi, M. D., Tandale, M. K. and Kartikesh, S. M. 2017. Molecular characterization of *HSP70* gene in Kenguri breed of sheep using PCR-SSCP technique. *Cell and Tissue Research*, **17**(3): 6263-6268.

Ellis, R. J. and Van der Vies, S. M. 1991. Molecular chaperones. *Annual Review of Biochemistry*, **60**: 321-347.

Elzarei, M. F., Al-Seaf, A. M., Al-Haidary, A. A., Okab, A. B., Samara, E. M. and Abdoun, K. A. 2017. Comparison of heat shock protein gene (*HSP70-1*) sequence in Aradi and Damascus goat breeds (*Capra hircus*) raised under heat stress conditions. *Experimental Biology and Agricultural Sciences*, **5**: 63-67.

Fatima, F., Nadeem, A. and Javed, M. 2019. Molecular characterization of heat shock protein (*HSP70-1*) gene of *Capra aegagrus blythi. Pakistan J. Zool.,* **51**(1): 195-203.

Feder, M. E and Hofmann, G. E. 1999. "Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology," *Annual Review of Physiology,* **61**: 243–282.

Flaherty, K. M., DeLuca-Flaherty, C. and McKay, D. B. 1990. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein, *Nature*, **346**(6285): 623-628.

Gade, N., Mahapatra, R. K., Sonawane, A., Singh, V. K., Doreswamy, R. and Saini, M. 2010. Molecular characterization of heat shock protein 70-1 (*HSP70-1*) gene of goat (*Capra hircus*). *Molecular biology international*, **10**(8): 420-429.

Gallagher, D. S., Grosz, M. D., Womack, J. E. And Skow, L. C. 1993. Chromosomal localization of *HSP70* genes in cattle. *Mamm. Genome,* **4**(7): 388–390.

Garrido, C., Gurbuxani, S., Ravagnan, L. and Kroemer, G. 2001. Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem. Biophys. Res. Commun.*, **286**(3): 433–442.

Gaviol, H. C. T., Gasparino, E., Prioli, A. J. and Soares, M. A. M. 2008. Genetic evaluation of the HSP70 protein in the Japanese quail (*Coturnix japonica*). *Genetics and Molecular Research,* **7**(1): 133-39.

Grosz, M. D., Womack, J. E. and Skow, L. C. 1992. Syntenic conservation of *HSP70* genes in cattle and humans. *Genomics,* **14**(4): 863–868.

Gupta, A. K. 2004. "Origin of agriculture and domestication of plants and animals linked to early holocene climate amelioration,"*Current Science*, **87**(1): 54–59.

Gutierrez, J. A. and Guerriero, V. Jr. 1995. Chemical modifications of a recombinant bovine stress-inducible 70 kDa heat-shock protein (HSP70) mimics HSP70 isoforms from tissues. *Biochem. J.*, **305**(1): 197-203.

Habib, H. N., Khudaier, B. Y. and Hassan, A. F. 2018. Molecular detection of polymorphism of heat shock protein 70 (*HSP70*) in the semen of Arabi rams. *Basrah Journal of Veterinary Research*, **17**(3).

Hall, D. M., Xu, L., Drake, V. J., Oberley, L. W., Oberley, T. D., Moseley, P. L. and Kregel, K. C. 2000. Aging reduces adaptive capacity and stress protein expression in the liver after heat stress. *J. Appl. Physiol.,* **89**(2): 749–759.

Hightower, L. E. 1991. Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell,* **66**(2): 191–197.

Hightower, L. E., Sadis, S. E. and Takenaka, I. M. 1994. Interactions of vertebrate hsc70 and HSP70 with unfolded proteins and peptides. In*: The Biology of Heat Shock Proteins and Molecular Chaperones*, 179 - 207.

Holmgren, R., Livak, K., Morimoto, R., Freund, R. and Meselson, M. 1979. Studies of cloned sequences from four Drosophila heat shock loci. *Cell*, **18**(4): 1359-1370.

http://dahd.nic.in/division/provisional-key-results-20th-livestock-census.

http://www.ncbi.nlm.nih.gov. National Center for Biotechnology Information, U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA.

Hunt, C. and Calderwood, S. 1990. Characterization and sequence of *HSP70* gene and its expression in mouse cell lines. *Gene*, **87**(2): 199-204.

Hunt, C. and Morimoto, R. I. 1985. Conserved features of eukaryotic *HSP70* genes revealed by comparison with the nucleotide sequence of human *HSP70*. *Proc. Nail. Acnd. Sci*. U.S.A., **82**(19): 6455-6459.

Ito, Y., Ando, A., Ando, H., Ando, J., Saijoh, Y., Inoko, H. and Fujimoto, H. 1998. Genomic structure of the spermatid-specific *HSP70* homolog gene located in the class III region of the major histocompatibility complex of mouse and man. *Journal of Biochemistry*, **124**(2): 347 – 353.

Jolly, C. and Morimoto, R. I. 2000. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *Journal of National Cancer Institute,* **92**(19)**:** 1564-72.

Kampinga, H. H. and Craig, E. A. 2010. The HSP70 chaperone machinery: J pro teins as drivers of functional specificity. *Nat. Rev. Mol. Cell Biol.*, **11**(8): 579-592.

Kampinga, H. H., Hageman, J., Vos, M. J., Kubota, H., Tanguay, R. M., Bruford, E. A., Cheetham, M. E., Chen, B. and Hightower, L. E. 2009. Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones*, **14**(1): 105–111.

Kano, R., Abe, K. and Hasegawa, A. 2004. cDNA of canine heat shock protein 70 (HSP70). *Veter. Res. Commun*., **28**(5): 395 - 405.

Kerekoppa, R. P., Rao, A., Basavaraju, M., Geetha, G. R., Krishnamurthy, L., Narasimha rao, T. V. L., Das, D. N. and Mukund, K. 2015. Molecular characterization of the *HSPA1A* gene by single strand conformation polymorphism and sequence analysis in Holstein-friesian crossbred and Deoni cattle raised in India. *Turkish Journal of Veterinary and Animal Sciences*, **39**: 128–133.

Khan, M. and Ashfaq, F. 2012. 'Dairy Goats in Pakistan: *Potential, Opportunities and Challenges, Proceedings of the 1st Asia Dairy Goat Conference*. Kuala Lumpur, Malaysia.

Kiang, J. G. and Tsokos, G. C. 1998. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol. Therapeut.*, **80**(2): 183 - 201.

Kregel, K. C. 2002. Heat shock proteins: modifying factors in physiological stress responses and acquired thermo tolerance. *J. Appl. Physiol*., **92**(5): 2177–2186.

Landry, J. and Chretien, P. 1983. Relationship between hyperthermia- induced heat-shock proteins and thermotolerance in Morris hepatoma cells. *Can. J. Biochem. Cell Biol.*, **61**(6): 428–437.

Landry, J., Bernier, D., Chretien, P., Nicole, L. M., Tanguay, R. M. and Marceau, N. 1982. Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. *Cancer Res.*, **42**(6): 2457–2461.

Legare, C., Thabet, M. and Sullivan, R. 2004. Expression of heat shock protein 70 in normal and cryptorchid human excurrent duct. *Mol. Hum. Reprod.*, **10**(3): 197-202.

Li, G. C. and Werb, Z. 1982. Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA*, **79**(10): 3218–3222.

Li, G. C., Meyer, J. L., Mak, J. Y. and Hahn, G. M. 1983. Heat-induced protection of mice against thermal death. *Cancer Res.,* **43**(12): 5758– 5760.

Lindquist, S. and Craig, E. A. 1988. The heat-shock proteins. *Annu. Rev. Genet.,* **22**: 631–677.

Lisowska, K., Krawczyk, Z., Widak, W., Wolniczek, P. and Wisniewski, J. 1994. Cloning, nucleotide sequence and expression of rat heat inducible *HSP70* gene. *Biochim. Biophys. Acta.,* **1219**(1): 64-72.

Lowe, D. G. and Moran, L. A. 1986. Molecular cloning and analysis of DNA complementary to three mouse Mr = 68,000 heat shock protein mRNAs. *J. Biol. Chem*., **261**(5): 2102-2112.1

Luengrattana, Y., Kohara, S., Hara, H., Mukoyama, H., Hanzawa, K. and Watanabe, S. 2000. "Complete nucleotide sequence of a Shiba goat (*Capra hircus*) heat shock protein 70-3 (*HSP70-3*) gene," *Animal Science Journal*, **71**(4): 371–376.

Madhusudan, C. S. 2007. Molecular characterization of *HSP70* gene in buffalo, M. S. thesis, IVRI.

Mathew, A. and Morimoto, R. I. 1998. Role of the heat-shock response in the life and death of proteins. *Annals of the New York Academy of Sciences,* **851**: 99-111.

Mathew, E. N., Brah, G. S., Sunil Kumar, B. V., Mukhopadhyay, C. S. and Dubey, P. P. 2013. Molecular cloning and characterization of the gene encoding heat shock protein 70 from the chicken (*Gallus gallus*). *Proceedings of the National Academy of Sciences, Indian Section B: Biological Sciences,* **84**(3): 573-578.

Mizzen, L. A. and Welch, W. J. 1988. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression. *J. Cell Biol.,* **106**(4): 1105–1116.

Morimoto, R. I. 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones and negative regulators. *Genes and Development*, **12**(24)**:** 3788-96.

Morimoto, R. I. and Milarski, K. L. 1990. Expression and function of vertebrate *HSP70* genes. *Stress Proteins in Biology and Medicine*, 323-359.

Morimoto, R. I., Hunt, C., Huang, S. Y., Berg, L. and Banerji, S. 1986. Organization, nucleotide sequence, and transcription of the chicken *HSP70* gene. *The J. Biol. Chem*., **261**(27): 12692 - 12699.

Morison, S. R. 1983. Ruminant heat stress: effect on production and means of alleviation. *J. Anim. Sci*., **57**(6): 1594-1600.

Moseley, P. L. 1997. Heat shock proteins and heat adaptation of the whole organism. *J. Appl. Physiol.,* **83**(5): 1413–1417.

Moseley, P. L. 2000. Exercise, stress, and the immune conversation. *Exerc. Sport Sci. Rev.,* **28**(3): 128–132.

Negri, A., Oliveri, C., Sforzini, S., Mignione, F., Viarengo, A. and Banni, M. 2013. Transcriptional response of the mussel *Mytilus galloprovincialis* (Lam.) following exposure to heat stress and copper. *PLoS One,* **8(6)**: e66802.

Nei, M. and Gojobori, T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Molecular Biology and Evolution,* **3**: 418-426.

Nunes, M., Yerle, M., Dezeure, F., Gellin, J., Chardon, P. and Vaiman, M. 1993. Isolation of four *HSP70* genes in the pig and localization on chromosomes 7 and 14. *Mamm. Genome*, **4**(5): 247-51.

Park, S. H., Bolender, N., Eisele, F., Kostova, Z., Takeuchi, J., Coffino, P. and Wolf, D. H. 2007. The cytoplasmic HSP70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin–proteasome system. *Molecular biology of the cell*, **18**(1): 153-165.

Pawar, H. N., Brah, G. S., Agrawal, R. K. and Verma, R. 2012. Molecular and immunological characterization of heat shock protein 70 (*HSP70*) gene from buffalo. *Proc. Natl. Acad. Sci., India Sect. B Biol. Sci*., http://dx.doi.org/10.1007/s 40011-012-0113-2.

Pawar, N. H., Agrawal, R. K., Ramneek and Brah, G. S. 2013. Expression, purification and characterization of recombinant heat shock protein 70 *(HSP70)* from sheep and goat species. *Int. J. Curr. Microbiol. App. Sci*., **2**(11): 440-452.

Pelham, H. R. 1982. A regulatory upstream promoter element in the Drosophila *HSP70* heat-shock gene. *Cell*, **30**(2): 517-28.

Pragna, P., Sejian, V., Soren, N. M., Bagath, M., Krishnan, G., Beena, V., Devi, P. I. and Bhatta, R. 2017. Summer season induced rhythmic alterations in metabolic activities to adapt to heat stress in three indigenous (Osmanabadi, Malabari and Salem Black) goat breeds. *Biol. Rhythm Res*., **49**(4): 551-565.

Ramunno, L., Cosenza, G., Rando, A., Pauciullo, A., Illario, R., Gallo, D., Di Bernardino, D. and Masina, P. 2005. Comparative analysis of gene of goat *CSN1S1* F and N alleles and characterization of *CSN1S1* transcript variants in mammary gland. *Gene,* **345**(2): 289 -299.

Ritossa, F. 1962. A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia*, **18**(12): 571–573.

Robertshaw, D. 1968. The pattern and control of sweating in sheep and goats. *J. Physio*., **198**(3): 531-539.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* **4**(4): 406-425.

Sambrook, J., Fritcsh, E. F. and Maniatis, T. 1989. *Molecular Cloning a Laboratory Manual,* 2nd edn., **1**, **2** and **3**. New York, Cold Spring Harbor Laboratory Press.

Sejian, V. 2013. Climate change: impact on production and reproduction, adaptation mechanisms and mitigation strategies in small ruminants: a review. *Indian J. Small Rumin.*, **19**(1): 1–21.

Sejian, V., Bhatta, R., Gaughan, J., Malik, P. K. and Naqvi, SMK, L. R. 2017. Sheep production adapting to climate change. Singapore: *Springer*-Verlag GMbH Publisher, 431–441.

Sharma, D. and Masison, D. C. 2009. HSP70 structure, function, regulation and influence on yeast prions. *Protein Pept. Lett*., **16**(6): 571-581.

Sharma, D., Martineau, C. N., Le Dall, M. T., Reidy, M., Masison, D. C. and Kabani, M. 2009. Function of SSA subfamily of *HSP70* within and across species varies widely in complementing *Saccharomyces cerevisiae* cell growth and prion propagation. *PloS one*, **4**(8): e6644.

Sharma, G., Nath, A., Prasad, S., Singhal, S., Singh, N., Gade, N., Dubey, P. and Saikumar, G. 2012. Expression and characterization of constitutive heat shock protein 70.1 (*HSPA1A*) gene in invitro produced and in vivo-derived buffalo (*Bubalus bubalis*) embryos. *Reprod. Domest. Anim*., **47**(6): 975–83.

Shiber, A. and Ravid, T. 2014. Chaperoning proteins for destruction: diverse roles of HSP70 chaperones and their co-chaperones in targeting misfolded proteins to the proteasome. *Biomolecules*, **4**(3): 704–724.

Shin, J. and Tan, J. 2016. The role of heat shock proteins and glucose regulated proteins in cancer. *The Malaysian journal of pathology*, **38**(2): 75–82.

Shkolnik, A., Borut, A. and Choshiak, J. 1972. Water economy of the Bedouin goat in G. Maloiy (ed.). *Comparative Physiology of Desert Animals*, Academic Press, New York, USA., 229-242.

Silannikove, N. 1992. Effect of water scarcity and hot environment on appetite in animals experiencing severe dehydration in ruminants- A review. *Livestock Prod. Sci*., **30**(3): 175-194.

Snutch, T. P., Heschl, M. F. P. and Baillie, D. L. 1988. The Caenorhabditis elegans *HSP70* gene family: a molecular genetic characterization. *Gene,* **64**(2): 241-255.

Srivastava, P. K. 2002. Role of heat-shock proteins in innate and adaptive immunity. *Nature Review of Immunology,* **2**(3): 185-94.

Srivastava, P. K., Menoret, A., Basu, S., Binder, J. R. and McQuade, K. L. 1998. Heat shock protein come of age: primitive functions acquire new roles in an adaptive world. *Immunity,* **8**(6): 657–665.

Stojadinovic, A., Kiang, J., Goldhill, J., Matin, D., Smallridge, R., Galloway, R. and Shea-Donohue, T. 1997. Induction of the heat shock response prevents tissue injury during acute inflammation of the rat ileum. *Crit. Care Med.,* **25**(2): 309–317.

Tamura, K., Nei, M. and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (*USA*), **101**(30): 11030-11035.

Tamura, Y., Peng, P., Liu, K., Daou, M. and Srivastava, P. K. 1997. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science,* **278**(5335): 117–120.

Tantia, M. S., Vij, P . K., Sahana, G., Jain, A. and Prasad, S. K. 2001. Beetal goats in their native tract. *AGRI.*, **31**: 65-74.

Udono, H. and Srivastava, P. K. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med.,* **178**(4): 1391–1395.

Vierling, E. 1991. The roles of heat shock proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology,* **42**(1): 579– 620.

Waters, E. R., Lee, G. and Vierling, J. 1996. Evolution, structure and function of the small heat shock proteins in plants. *Journal of Experimental Botany*, **47**(3): 325–338.

CERTIFICATE – IV

Certified that all the necessary corrections as suggested by the external evaluator and the advisory committee have been duly incorporated in the thesis entitled **"Molecular characterization of *HSP70-1* gene in Beetal goat"** submitted by **Mr. Kashif Dawood Khan**, Regd. No. **J-18-MV-518**.

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