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RESEARCH ARTICLE

Absolute Quantification of Heat Shock Protein 70 Gene in Jamunapari Goat Breed

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Abstract

Environmental stresses, particularly heat stress can reduce livestock productivity. Effect of heat stress needs to be narrowed down for maintaining animal health status and performance. Domestic animals undergo different kinds of stress e.g. physical, nutritional, chemical and thermal stress which includes both heat and cold stress. High environmental temperature is the major concern in tropical and arid areas and very low environmental temperature in temperate areas. Heat stress is a major problem in the era of climatic change. Goats contribute to about 4% in the national economy through wool, meat, milk, skin and manure. Goats undergo thermal stress largely on exposure to high temperature. The cells in turn mount a strong physiological response to heat stress which is reflected in the expression of heat shock proteins (HSPs).In goats Hsp70 is considered to be the leading member of HSP family consisting of solely inducible and constitutive proteins. HSPs are molecular chaperones, which are involved in housekeeping functions in the cell. Jamunapari breed of goat have a great potential for meat as well as milk production. In the present study, analysis of the Hsp70 gene expression in Jamunapari goat breed by using absolute quantification of Real time PCR indicates variation in the Hsp70 gene expression among different tissues in summer and winter seasons.

Keywords: Animal productivity, Goat, Heat stress, Heat shock protein 70, Real-Time PCR

Introduction

All organisms are occasionally or regularly exposed to environmental conditions that challenge the physiological functioning of the cells. When this effect becomes severe enough it can be considered as stressful and will require counter measures in order to maintain cellular homeostasis. Goats are very important farm animals in India. One of the major problems facing the goat is heat stress (Alam *et al,* 2011). The thermal environment is a major factor that can negatively affect goat performance. Increased body temperature and respiratory rate are the most important signs for heat stress in goats. Usman *et al,* (2013) reported that there is a network of genes which are directly related to the heat stress regulation and therefore, marker assisted selection can be an effective approach. On exposure to high temperature, cells mount a strong physiological response, including the heat stress response and the expression of heat shock proteins (HSPs) (Sorensen, 2010). HSPs are molecular chaperones, which are involved in "house-keeping" functions in the cell. Nikbin *et al,* (2014) explained that Heat Shock Protein 70 (Hsp70) is produced by the hsp70 gene and includes a family of HSPs which range in size from 68 to 73 kDa. The hsp70 gene is encoded by a single exon. The size of the open reading frame of this gene is 1926 bp and its protein consists 641 amino acids (Gade *et al,* 2010). Its functions include the prevention of aggregation of damaged proteins, folding and unfolding of proteins, transportation and general handling of peptides and proteins and involvement in the degradation of misfolded or aggregated proteins. Due to the generality of the stress genes and responses among organisms and stress types, the heat shock genes have been suggested as candidates for a major role in the protection of cells during or after thermal stress and thus a key component of adaptation to environmental conditions as well as biological markers for exposure to stressful conditions. The expression profiling of hsp70 gene in Jamunapari goats to heat stress in different climatic conditions was undertaken with this view in mind.

Materials and Method

Animals & Tissue Sampling

Total 40 different tissue samples were collected from Jamunapari goat breed in summer and winter season &

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stored at -20°C.Within a season i.e. summer or winter, tissue samples from all the animals of a breed were collected on three consecutive days and the environmental conditions on the date of collection were recorded and considered during data analysis.

Measurement of the severity of heat & cold stress

Ambient temperature and relative humidity (RH) of day animal were recorded at 12:00 noon during the month of May-June (summer) and January-February (winter). The temperature-humidity index (THI) was calculated as according to Mader *et al*, 2006.

THI=0.8 x ambient temperature + [(% relative humidity ÷ 100) x (ambient temperature – 14.4)] + 46.4

The classification for the heat stress are as follows:

≤ 74: Normal, 74<THI<79: Alert, 79d≤THI<84: Danger and THI≥84: Emergency.

Total RNA extraction & Quality determination

Total RNA was extracted using Trizol reagent (Sigma Aldrich) following standard protocol and the quality was determined by using nanodrop spectrophotometer.

cDNA Synthesis

Constant amount of 1ì g of total RNA was reverse transcribed using ProtoScript First Strand cDNA synthesis kit (BioLab) with the following master mix: 2ì l (1ì g) RNA+ 4ì l nuclease free water, 2ì l (5ì M) oligo(dT)n primer, 10ì l of 1x M-MuLV Reaction mix (50mM Tris-Acetate pH 8.3, 75mM KOAc, 3.1 mM $Mg(OAc)_2$ & 0.5mM dNTPs each and 2ì l of 1x M-MuLV Enzyme Mix (0.5 unit/ì l M-MuLV Reverse transcriptase & 1 unit/ì l Murine RNase Inhibitor).

Primers

Primers of Hsp70 were designed on a GenBank reference sequence (Accession No. NM001114192.2) from NCBI as shown in Table 1.

Table 1 : Primers Description

S. No	Primers	Primers sequences (5′-3′)	Annealing temperature (°C)	Products (bp)
1.	Hsp70 (F)	TCC TCA GTC TGA TGG CTC CAG TT	62	297
2.	Hsp70 (R)	GCT TGA GGT GGT TGG TCC ATC TT		

Quantitative RT-PCR analysis

The complimentary DNAs (cDNA) were used in quantitative RT-PCR (qRT-PCR) reactions. The qRT-PCR for each cDNA and the heat shock protein 70 gene expression reaction was performed in duplicate using q. Eva Green mix. The PCR templates containing 25ng reverse transcribed total RNA, was added to 0.5ì l forward primer (250nM), 0.5ì l reverse primer (250nM) and 4 ì l of 5x q. Eva Green mix, to a final volume of 20 ì l and were subjected to general real-time PCR protocol for hsp70 gene under study. The following general real-time PCR protocol was employed for all investigated factors: denaturation for 5 min at 95°C, 45 cycles of a three segmented amplification and quantification program-denaturation for 10 sec at 95°C, annealing for 10 sec at the primer specific temperature (62°C for hsp70 gene), elongation for 15 sec at 72°C, a melting step by slow heating from 5 sec to 95°C with a rate of 4.4°C/sec and continuous fluorescence measurement and a final cooling down to 40°C. After the run had ended, the cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the "Eva Green (with melting curve)" method of the real-time machine.

Statistical Analysis

The statistical significance of differences in mRNA expressions of the examined factors was assessed by ANOVA. Differences were considered significant if $p<0.05$.

Results and Discussion

Climatic conditions

The average climatic parameters recorded during the experimental periods are presented in Fig. 1.

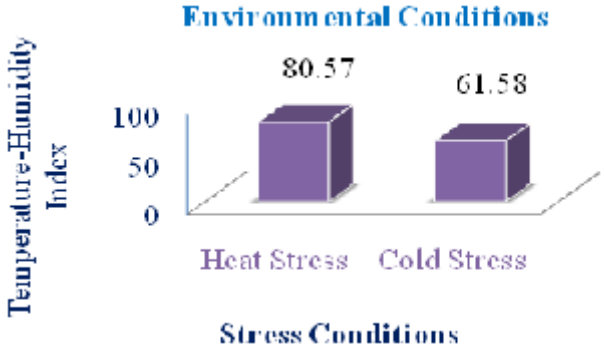

Fig.1. Temperature Humidity Index (THI) of the environment during the experiment

During summer, THI was above 79, representing heat stress, whereas during winter, THI was nearly 60, indicating cold stress.

Integrity and purity of RNA

The RNA was dissolved in DEPC-treated water and the purity of RNA was verified by Optical density (O.D.) absorption ratio at 260/280nm was between 1.8 to 2.0 was pure RNA.The quality and integrity of the total RNA was checked using denaturing 1% agarose gel electrophoresis and visualization under UV light. Two intact bands of 28S & 18S with smearing indicated good quality and intactness of RNA as shown in Fig. 2.

Fluorescent quantitative RT-PCR analysis

Standard curves of Hsp70 are shown in Fig. 3. All cDNA samples were evaluated by fluorescent quantitative RT-PCR and "S" amplification curve was obtained shown in Fig. 4.

The size of the amplicon was 297bp. hsp70 gene was identified by agarose gel electrophoresis as shown in Fig. 5.

Gene expression analysis of mRNA for HSP70 under different climatic conditions

The expression of hsp70 gene shows temperature sensitivity & seasonal variation. mRNA expression of hsp70 genes varied in different tissues of Jamunapari goat breed in summer and winter seasons and showed a specific response to heat stress. In Jamunapari goats, the mRNA expression of Hsp70 gene was higher in liver tissue as compared to kidney, heart & spleen tissues (Table 2 and Fig. 6). The level of expression obtained for different tissues: **Liver>Kidney>Heart>Spleen**.

Table 2. Expression values of Hsp 70 mRNA during different seasons

S.NO	TISSUES	SUMMER	WINTER	COMFORT
1.	LIVER	2.075E+12	1.282E+12	4.145E+11
2.	HEART	7.616E+11	3.375E+11	4.829E+11
3.	SPLEEN	4.101E+11	2.839E+11	3.795E+11
4.	KIDNEY	1.196E+12	7.956E+11	4.113E+11

In Jamunapari goats, the mRNA expression between combinations of the four tissues was statistically significant ($p<0.05$) in summer. However during winter, the mRNA expression between combinations of all the tissues was significant ($p<0.05$) but insignificant ($p>0.05$) between heart-kidney tissues (Table 3).

Table 3. Tissue specific expression analysis of hsp70 gene by absolute quantification in Jamunapari goat breed in different seasons at ($p<0.05$) level

S. No	Target Name	Summer	Winter
1.	Liver-Heart	Significant	Significant
2.	Liver-Spleen	Significant	Significant
3.	Liver-Kidney	Significant	Significant
4.	Heart-Spleen	Significant	Significant
5.	Heart-Kidney	Significant	Insignificant
6.	Spleen-Kidney	Significant	Significant

Among all the HSPs, Hsp70 is the most temperature sensitive and is positively correlated with thermotolerance. It is found in cytosol and nucleus and plays an important role in the folding of proteins and refolding of misfolded proteins. According to our finding that hsp70 mRNA in the liver was significantly higher than in other tissues ($p<0.05$). Besides the hsp70 gene expression was found to be higher in the summer season as compared to the winter season. Our findings are in accordance with the previous studies that show a significant increase in expression of Hsp70 following heat stress. Various other studies show an increase in expression of Hsp70 in kidney of goats (Zulkifli *et al,* 2010), in myocardium (Gray *et al,* 2000), in lung cells (Fargnoli *et al,* 1990) and in hepatocytes and liver (Hall *et al,* 2000). Hamzaoui *et al,* 2012 explained that heat-stressed lactating goats and non-lactating ewes were able to maintain similar blood glucose levels as compared to thermo-neutral animals with no change in blood insulin concentration. Neverthless, blood glucose significantly decreased by heat stress in dairy cows in accordance with greater blood insulin (Rhoads *et al,* 2009; Baumgard and Rhoads, 2013). Liver and kidney probably play an important role to maintain blood glucose under heat stress because they produce glucose through gluconeogenesis and release it under various conditions (Gerich *et al,* 2001). Therefore, there is a need to study the change in expression level of hsp70 gene especially in liver and kidney.

Conclusion

HSPs are involved in intra and extracellular responses to stress and have the potential to be developed into a key biomarker in ecological and evolutionary research for detecting natural adaptation and exposure to stress in natural populations. The above results indicate that elevation of temperature dramatically increased the expression of hsp70 and reduction in temperature inhibited the over expression of hsp70 in a short period. In conclusion this area of research gives us a good initiative for the investigations that are needed to increase our knowledge on adaptation of farm animals to natural stressful conditions.

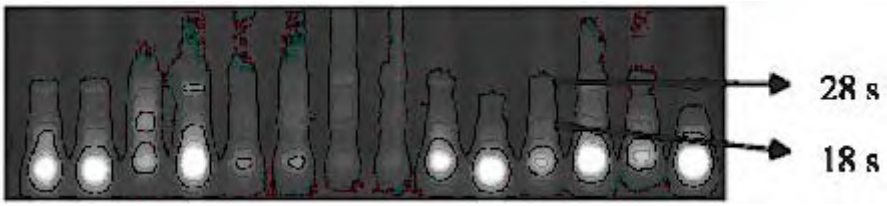

Fig.2. Purity and Integrity of total RNA as checked by 1% Agarose gel electrophoresis

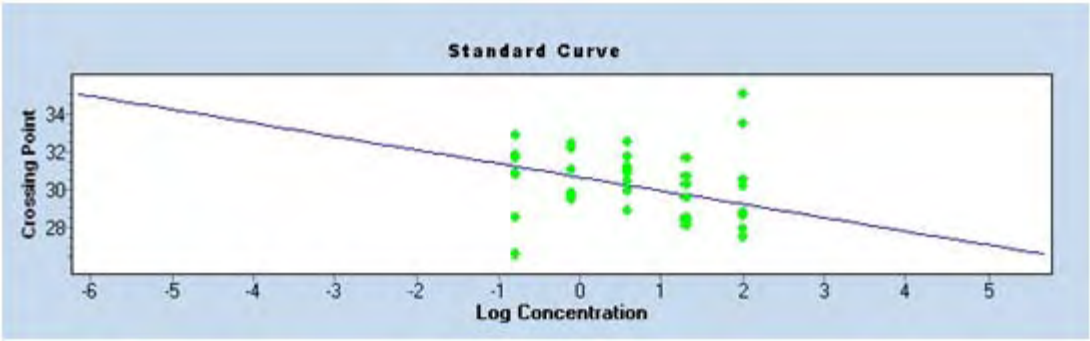

Fig.3. Standard curve of hsp70 gene in summer and winter seasons

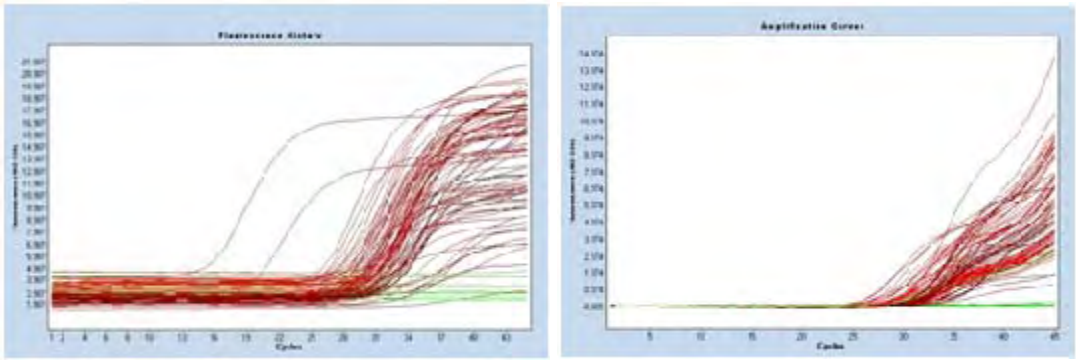

Fig.4. Amplification curve of hsp70 gene in different seasons (a) Summer (b) Winter

Fig.5. PCR amplification of hsp70 gene in Jamunapari goat breed
Lanes 1-3: PCR products, Lane 4: 100 bp marker

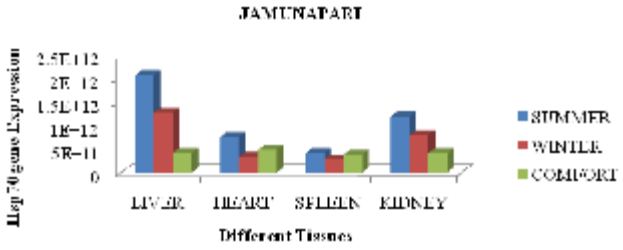

Fig.6. Expression profile of Hsp 70 mRNA during different seasons

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