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Seasonal Effect of THI on HSP70 Gene Expression Pattern in Deccani Sheep

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ABSTRACT

The region of Satara district experiences summer from March to May, with maximum temperature ranging from 35°C to 43°C and the warmest month is May (43°C) whereas, the monsoon lasts from June to September with moderate to high precipitation during which temperature ranges from 28°C to 32°C along with 80% to 90% relative humidity. The present study was designed to evaluate the effect of THI on the HSP70 gene expression pattern during summer and rainy seasons in Deccani sheep at LFC, KNP College of Veterinary Science, Shirwal, Dist. Satara (Maharashtra). The meteorological variables like temperature and relative humidity were recorded for calculating THI and whole blood samples (06 ml each) of eight non-pregnant, non-lactating and apparently healthy Deccani sheep were collected for HSP70 gene expression pattern during peak summer (month of May) and peak rainy (month of August) seasons in the year 2019. The significantly (P<0.01) higher THI was recorded during summer than rainy season. The relative mRNA expression of the HSP70 gene during the peak summer season was found 1.79 folds more as compared to peak rainy season during the present study.

Key words: Decani sheep, HSP70 gene expression pattern, Season, THI.

Deccani sheep, belonging to the native geographic region of Maharashtra andhra Pradesh, Telangana and Karnataka states of India are medium-sized, coarse wool sheep (Acharya, 1982) predominantly off-white or dark brown, dwarf in size with stumpy legs and have been used for coarse wool and mutton production. They inhabit five districts of Maharashtra, such as - Ahmednagar, Kolhapur, Satara, Sangli and Solapur and are known as Sangamneri, Kolhapuri, Lonand, Madgyal and Solapuri strains, respectively (Gokhale, 2003). The function of HSP70 as a molecular chaperone and cell protection against heat stress capable of denaturing proteins has been studied through extensive research (Bhat *et al*., 2016). It might be concluded that the biphasic expression pattern of HSPs helps to protect animals against heat stress. Moreover, it may be used as a biomarker of chronic heat stress in livestock (Bharati *et al*., 2016; Ambade, 2019). However, expression of HSP70 could be used as a valuable indicator for change in body temperature when it is more than 38.6°C and HSP70 is suggested as a reliable biomarker of chronic stress, but just in case of multiple stresses, it's not a reliable indicator of a single stressor (Gaughan *et al*., 2013).

The available literature also revealed no reports yet concerning the seasonal variation of the HSP70 gene expression pattern in sheep; hence, it is proposed to study the HSP70 gene expression pattern in Deccani sheep during peak summer and peak rainy seasons.

The experiment was carried out on eight (08) healthy, non-pregnant, non-lactating adult Deccani sheep aged above two years, having similar body weight and maintained under a semi-intensive farming system at Livestock Farm Complex (LFC), KNP College of Veterinary Science, Shirwal. Dist. Satara (Maharashtra) during March 2019 to September 2019.

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Meteorological variables

The meteorological data recorded during the present study was obtained from Indian Meteorological Department, Pune. Based on the mean temperature and relative humidity, THI was calculated by using the following formula (Mader *et al.,* 2006).

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

Where, T_{db} = Dry bulb temperature. RH = Relative humidity.

Blood sampling schedule

The Institutional Animal Ethical Committee approved the protocol of this experiment at KNP College of Veterinary

Science, Shirwal Dist. Satara. Vide Resolution No.(IAEC/15/KNPCVS/01/2019). Eight (8) whole blood samples (6 ml each) were collected aseptically by jugular vein puncture in K3 EDTA by vacutainers for the study during peak summer (month of May) and peak rainy (month of August) seasons in the year 2019.

All the safety measures were taken to minimize the effect of ribonuclease during processing and all the samples were immediately carried to the laboratory on ice for further processing after blood collection. All samples were processed within one hour of collection.

Isolation of total RNA from whole blood

It was carried out by using PureLink™ Total RNA Blood Purification Kit which was designed to purify total RNA from 50-500 µl of the fresh whole blood sample of any mammalian species, collected in the presence of anticoagulants like EDTA, heparin or citrate supplied by Invitrogen Life Technologies, Mumbai. All the components included in the kit were shipped and stored at room temperature. The kit was used for leukocyte enrichment and a non-phenol based purification system resulting in the isolation of high-quality total RNA that was suitable for cDNA synthesis and Real time quantitative PCR(qPCR).

10X Deoxyribonuclease (DNase) I Amplification Grade (200 mM Tris-HCl, PH 8.4, 20 mM $MgCl_2$, 500 mM KCl) was used to digest single and double stranded DNA to oligodeoxy-ribonucleotides containing a 5′-phosphate as supplied by Invitrogen Life Technologies, Mumbai. It was suitable for eliminating DNA during critical RNA purification procedures such as those prior to RNA-PCR amplification. DNase I Amp Grade is purified from bovine pancreas and has a specific activity of ≥10,000 U/mg.

Quantitation and evaluation of purity of RNA

Total RNA was quantified by UV Visible spectrophotometer (Systronics, 117) using the convention that one absorbance unit at 260 nm wavelength equals 40ng/µl. The absorbance in ultraviolet spectra was checked at 260 and 280 nm for RNA concentration and purity determination. The purity of RNA was judged on the 65 bases of optical density ratio at 260 and 280 nm. The ratio of readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA concerning contaminants that absorb in the UV spectrum, such as protein.

Method of cDNA synthesis

1 µg of total RNA was reverse transcribed to complementary DNA (cDNA) using Superscript™ VILO™ cDNA Synthesis Kit supplied by Invitrogen Life Technologies, Mumbai which provided the high temperature capability of SuperScript™ III Reverse Transcriptase in an optimized format for generating first-strand cDNA for use in real-time quantitative RT–PCR (qRT–PCR). This formulation provides enhanced cDNA synthesis efficiency and can be used with very low and very high amounts of input RNA (up to 2.5 µg total RNA in a 20-µL reaction), giving linear response in message abundance as measured by qPCR as per the protocol. The following protocol was optimized for generating first-strand cDNA for use in Two-step qRT-PCR.

1) The PCR tube contents were gently mixed and kept in the thermal cycler and incubated at 25°C for 10 minutes.
2) Then the PCR tubes were incubated at 42°C for 60 minutes in the thermal cycler.
3) The reaction was terminated at 85°C at 5 minutes.
4) The first strand cDNA synthesized was stored at -20°C until further use.

Primers for HSP70 gene

As the Deccani sheep sequences for the gene under study were unavailable in NCBI GENBANK or any other database, it was designed using online NCBI primer design software (Primer 3, http://bioinfo.ut.ee/primer3/) and presented in Table 1.

The relative quantification of a target gene was done by comparing the expression level of the reference gene beta-actin, as per Livak and Schmittgen (2001).

All samples were amplified with a housekeeping gene and target gene using Hi-Temp PCR Master Mix (MOLBIO™ HIMEDIA, Ltd, Mumbai) to confirm the formation of first strand cDNA and, after that, stored at -20°C for long-term storage under proper conditions. The cDNAs run on denaturing gel (1.5%) and visualized on Geldoc machine showed sharp 236 bands for Ovisariesheatshock70 and 178 bands for Betacatin.

Real time polymerase chain reaction was **e**xecuted on ABI step one using Power SYBR Green PCR Master Mix (Invitrogen Life Technologies, Mumbai).

Comparative quantitation of HSP70

The reaction mixture for the HSP70 gene separately in two replicates, both for target and housekeeping gene. The normalized target values generated the relative expression quantity and the fold change values (x) were calculated by using the formula:

$$x = 2^{-\Delta\Delta Ct}.$$

The data were analyzed using a computerized Web based Agricultural Statistics Software Package, WASP. 2.0 by applying a completely randomized design (Snedecor and Cochran, 1980).

Temperature humidity index (THI)

Mean values of the temperature humidity index (THI) during the summer and rainy seasons of the study period are presented in Table 2. The month wise mean values of THI in the experiment revealed a significantly ($P<0.01$) higher THI during summer than rainy season. THI has been widely used as an indicator of thermal stress in livestock and it involves both ambient temperature (wet /dry bulb) and relative humidity which is universally used as a heat stress index for livestock production (Vaidya *et al*., 2010). THI value of 70 or less is considered comfortable, 75-78 stressful and values greater than 78 cause extreme distress and the animals are unable to maintain the thermoregulatory mechanism or

Table 1: Primer sequences, annealing temperature (TA) and size of amplicons for each specific gene used in the gene expression.

Gene	Primer sequence	TA (°C)	Amplicon size (bp)
Ovisaries heatshock 70 kDa	F-5′AGCTGGAGCAGGTGTGTAAC 3′	54	236
	R-5′AGCTTGCATAGCTGATGGCT 3′		
Betaactin	F-5′CTCTTCCAGCCTTCCTTCCT 3′	54	178
	R-5′GGGCAGTGATCTCTTTCTGC 3′		

TA- Annealing temperature; C- Degree celsius; bp- Base pairs; F- Forward; R- Reverse.

Table 2: Season wise mean±S.E. values of temperature humidity index (THI).

Summer				Rainy	
March	April	May	July	August	September
76.16	77.87	79.47	74.47	75.47	74.47
Mean ± SE = 77.81[a]± 0.19			Mean ± SE = 74.92[b]± 0.16		

Table 3: Relative expression pattern of HSP70 gene in deccani sheep (n = 08).

Sample no.	2 Delta C_T peak summer (Month of May; THI = 79.47)	2 Delta C_T peak rainy (Month of August; THI = 75.47)
I	4.93	2.64
II	5.78	2.45
III	3.95	2.65
IV	5.94	2.94
V	3.68	2.95
VI	5.63	3.50
VII	5.84	3.69
VIII	5.22	2.55
Mean±SE	5.12 [a]±0.31	2.92 [b]±0.16

THI- Temperature humidity index; C_T - The cycle threshold.
Mean values of 2 Delta C_T (HSP) differ significantly at 1% level of significance.

the normal body temperature (Silanikove, 2000).

The home tract of Deccani sheep is western region of Maharashtra especially Solapur, Pune, Satara, Sangli and Kolhapur districts with an average temperature of 41°C and it is common to increase upto 43°C during peak summer season.

According to Taylor (1992), thermo-neutral zone of sheep is set between 12°C to 32°C which depends upon many factors like breed of the animal, age, body weight, feeding, health status and physiological state (Kolacz and Dobrzanski, 2006). The mean values of THI in the present study indicated that Deccani sheep were in severe stress during summer season than in rainy season.

Relative expression profile of HSP70 gene

The average $2^{-\Delta\Delta Ct}$ values for HSP70 gene reported in the present study are presented in Table 3. The mRNA expression of HSP70 gene was significantly (P<0.01) upregulated in the peak summer (month of May; THI = 79.47) compared to the peak rainy (month of August; THI = 75.47) season.

HSP70 gene expression analysis

The comparative analysis for HSP70 expressions associated with increased temperature in PBMCs could explain the thermoregulatory mechanism operating in the cells. The HSP70 is well-established in conferring thermo-adaptability and a high thermo tolerance level. This heat stress-induced HSP70 expression has been observed in bovine, equine, ovine and chicken lymphocytes (Guerriero and Raynes, 1990). The 70-k Da HSP70 is a protein family known for its potential role in thermotolerance. It is widely considered as a cellular thermometer, functions as a molecular chaperon and has significant roles in cellular thermotolerance, apoptosis, immune modulation and heat stress (Hassan *et al.,* 2019). The significantly (P<0.01) elevated mRNA expression of HSP70 in the present study during the peak summer season (month of May; THI = 79.47) might be an important indicator of thermal stress tolerance against hot-dry and hot-humid conditions in Deccani sheep maintained at LFC, KNP College of Veterinary Science, Shirwal, Dist. Satara (Maharashtra). This result is in accordance with the findings of Rajput *et al.,* (2016) in sheep. Further the findings of the present study corroborated with the observations quoted by Dangi *et al.,* (2012), Dangi *et al.,* (2014), Dangi *et al.,* (2015), Yadav *et al.,*(2015), Sejian *et al.,* (2015), Dangi *et al.,* (2016), Rout *et al.,* (2016), Hooper *et al.,* (2018) and Yilmiz *et al.,* (2018) in goats.

Being an indigenous breed of Western Maharashtra, Deccani sheep has well-developed defense mechanisms involving maintaining a high constitutive level of the HSP70 gene in its PBMC to protect against the extreme heat stress.

CONCLUSION

HSP70 could be used as a potential marker for selecting climate-resilient animals with superior thermo-tolerance and better immune response to enhance livestock productivity globally. In Deccani sheep, mRNA expression of the HSP70 gene during the peak summer showed 1.79 fold more than peak rainy season. It is assumed that Deccani sheep are well adapted to hot dry and hot humid climates in the Satara district of Maharashtra.

Conflict of interest: None.

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