

Original Research Article

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Expression of Heat Shock Protein 70-1 in Peripheral Blood Mononuclear Cells of Jersey-Sahiwal Cross Breeds during Thermal Stress

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ABSTRACT

Heat Shock Protein 70 (HSP70) is one of the important molecular chaperon that can protect cells from thermal damage by involving in the maintenance of the intra cellular homeostasis primarily by controlling the process of protein folding. The indigenous breeds of cattle have been found to be more resistant to heat stress in comparison to the exotic breeds by way of expressing HSP70 as an adaptive response. Therefore, the present study was done to study the expression of HSP 70-1 in peripheral blood mononuclear cells (PBMCs) of Jersey- Sahiwal cross breeds during thermal stress. Blood samples collected from the animals were used for the separation of PBMCs by using Histopaque 1077 and RBC lysis buffer. Total RNA was isolated from the separated PBMCs using Trizol reagent. The isolated total RNA was then reverse transcribed to cDNA that was used for amplification of HSP70-1 gene using specific pair of primers. The presence of 1926 base pair band in denaturing agarose indicated the amplification of HSP70-1 gene. To conclude, our results demonstrate that HSP70-1 gene is expressed in PBMCs of Jersey- Sahiwal cross breeds and further characterization of this gene may help in elucidating its role in attributing thermo tolerance to the cross bred cattle.

Keywords

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Introduction

Thermal stress is defined as “a physiological condition when the core body temperature of a given species exceeds its range specified for normal activity, which results from a total heat load (internal production and environment) exceeding the capacity for heat dissipation and this prompts physiological and behavioral responses to reduce the strain” (Bernabucci *et al.*, 2010). The variation in

upper critical temperatures (UCT) depends upon age, breed, level of production and the degree of acclimatization (Shearer and Beede, 1990). The ability to adapt to environment is of great importance to all living things.

Cells respond to heat stress with the synthesis of heat shock proteins (HSPs) that can be transcribed and translated in high ambient temperatures. HSPs commonly referred to as molecular chaperones have been implicated in

a wide variety of cellular processes, including protection of the proteome from stress, folding and transport of newly synthesized polypeptides, activation of proteolysis of misfolded proteins and the formation and dissociation of protein complexes. HSPs are the group of proteins which get expressed in response to many environmental conditions, including ultraviolet radiation, heavy metal ions, hypoxia and toxic agents. Heat shock genes are activated when cells are exposed to stress stimuli and form heat shock proteins (Christians *et al.*, 2003).

Stressors that can induce HSP gene expression include physical and chemical insults such as radiation, including ultraviolet light and magnetic fields, compression, shearing and stretching, hypoxia, pH shift, nutrient deprivation, or exposure to reactive oxygen species, alcohols, or metals. Certain biological insults like fever, cold, infection, inflammation, diseases including cancer, cardiac diseases, and neuro degenerative disorders can also induce HSP gene expression. Broadly, cellular stress can also be triggered by treatments with anticancer drugs or antibiotics (Ciocca and Calderwood, 2005; Macario and Conwayde Macario, 2005; Garrido *et al.*, 2006; Jego *et al.*, 2010; Willis and Patterson, 2010; Macario *et al.*, 2011).

The family of heat shock proteins consists of many proteins which are classified as HSP 110, HSP 100, HSP 90, HSP70, HSP 60, HSP 40, HSP 10, and other small HSP families (park *et al.*, 2007; Gething., 1997). Among HSPs, heat shock protein 70 (HSP70) is an essential molecular chaperone of primary importance to all mammalian cells.

HSP70 gene family in bovines includes HSP70-1, HSP70-2, HSP70-3, and HSP70-4 gene. HSP70-1 is an intronless gene located on chromosome 23 of bovine (BTA 23) and has 1926 nucleotides. Heat Shock Protein-70

(HSP-70) is one of the most studied and inducible HSP families under stressful conditions which is often used as stress marker and adaptation in a variety of physiological systems (Beckham *et al.*, 2004).

HSP70 is well reported to protect cells, tissues, and organs from stress (Kiang and Tsokos, 1998) by promoting the folding of nascent polypeptides and by correcting the misfolding of denatured proteins. Heat shock-induced- HSP70 expression has also a role in the anti-apoptotic pathway (Sreedhar and Csermely, 2004). Heat shock protein 70 (HSP 70) plays a vital role by bestowing cytoprotection against diverse kinds of stresses. Therefore, HSP 70 proteins are ubiquitous, the most abundant and temperature sensitive among all the HSPs.

Climate change and adapting to climate change by means of managing the heat stress will have a profound impact on household income and food security. The temperatures expected under climate change will precipitate heat stress and are found to cause decline in productivity. By 2020, heat stress was estimated to decrease the milk production from India's dairy cows by 0.73 million litres (Upadhyay *et al.*, 2007).

Heat stress is measured against a temperature– humidity index (THI), a combined measure of temperature and humidity to denote thermal comfort (Berman, 2011). Thermal stress duration and magnitude can affect the concentrations of HSP-70 (Wang *et al.*, 2003).The *Bos indicus* breeds such as Sahiwal and Gir are considered to be less susceptible to heat stress (Sirohi and Michaelowa, 2007; Upadhyay *et al.*, 2007). Several studies have also confirmed genetic differences in thermo tolerance at the physiological and cellular levels in *Bos Indicus* and *Bos Taurus* (Lacetera *et al.*, 2006; Farooq *et al.*, 2010).

Studies have also been conducted to find out the level of expression of these HSP genes and proteins in various tissues of bovines and other species, but the work on HSP70-1 gene expression in peripheral blood mononuclear cells (PBMCs) of cross bred cattle is limited (Parmar *et al.*, 2015). Hence, the present study was initiated with the objective of studying the expression of HSP 70-1 in PBMCs of Jersey- Sahiwal cross breeds during thermal stress.

Materials and Methods

The present study was conducted at College of Veterinary Science, Proddatur, Sri Venkateswara Veterinary University. Six apparently healthy non lactating Jersey - Sahiwal crossbreds were selected for the study. Blood samples were collected aseptically from these animals in 15 ml centrifuge tubes using EDTA as an anticoagulant (1mg/ ml) by jugular vein puncture. The samples were collected during summer where in the temperature range was recorded as 38°C to 42° C, THI > 72. The collected blood samples were then immediately processed in order to isolate the intact total RNA from the PBMCs.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from whole blood using two methods- one by the use of Histopaque 1077 (Sigma) and the other by the use of RBC lysis buffer (routinely used for the isolation of DNA from the whole blood with slight modifications) (Sambrook and Russell, 2001). In the earlier method, 3ml of whole blood was carefully laid over 3ml of Histopaque 1077 taken in 15 ml centrifuge tubes. Centrifuged at 1900 rpm for 30 min at room temperature to collect the opaque interphase which is rich in PBMCs into a new centrifuge tube. Then 10 ml of Diethyl pyrocarbonate –phosphate

buffer saline (DEPC –PBS, pH 7.4) was added to the PBMCs and centrifuged at 1600 rpm for 10 min at room temperature. The pellet was again washed with 5 ml of DEPC –PBS and centrifuged at 1600 rpm for 10 min at room temperature. The supernatant was removed and the contents were transferred to a new 2 ml eppendorf tube to which 0.5 ml of DEPC –PBS was added.

The remaining 12 ml of whole blood was centrifuged at 2000 rpm for 15 min at room temperature. 300µl of buffy coat was separated carefully and added to 2ml eppendorf tube. To this, 900 µl of RBC lysis buffer was added and incubated at room temperature for 10 min. Then centrifuged at 12000 rpm for 1 min at room temperature. The above step was repeated 3-4 times till a white WBC pellet was obtained. The final WBC pellet was suspended in 0.5 ml of DEPC –PBS.

Extraction of total cellular RNA from PBMCs

The PBMCs obtained by using Histopaque 1077 as well as RBC lysis buffer, and resuspended in 0.5 ml of DEPC –PBS, were used to extract the total cellular RNA using Trizol reagent. 1ml of trizol and 200 µl of chloroform were added to the DEPC –PBS resuspended PBMCs. Mixed well and incubated for 3 min at room temperature.

Centrifuged at 14,000 rpm for 15 min at 4°C. The upper aqueous phase resulted in the phase separation was then transferred to a new eppendorf tube and 0.5ml of isopropanol was added, vortexed and placed in -20°C overnight, followed by centrifugation at 14,000 rpm for 15 min at 4°C. The obtained pellet was rinsed with ice-cold 70% ethanol and centrifuged at 7,500 rpm for 8 min at 4°C. The isolated RNA was then reconstituted in nuclease free water (Himedia).

Ascertaining the purity and concentration of RNA

The purity and concentration of total RNA isolated by both the methods was verified by measuring the optical density (OD) absorption ratio OD 260: OD 280 nm using Nanodrop spectrophotometer (Thermo scientific).

The quality and integrity of total RNA was checked using denaturing agarose gel (1.5%) electrophoresis and visualized under UV light with the help of Chemi documentation system from syngene, Biocon, USA. The isolated total RNA samples whose absorption ratio OD 260: OD 280 nm ranging between 1.8 and 2.0 were used for the cDNA synthesis.

Statistical analysis

Students paired t test (Nemeth *et al.*, 2004) was used to calculate and compare the difference in the concentration and purity of total RNA isolated from PBMCs by using Histopaque 1077 and RBC lysis buffer (in terms of p value).

RNA reverse transcription

Isolated total RNA was reverse transcribed using Hi-cDNA synthesis kit from Himedia, India. Template RNA, 5 µl; oligo dT, 1 µl and nuclease free water, 4 µl were added to a PCR tube, incubated at 65°C for 5 min in a thermal cycler and cooled immediately on ice in order to use this a template for synthesis of complementary DNA.

A reaction mixture (20 µl) was then prepared in a PCR tube using 4 µg of total RNA (adjusted to 10 µl), 4 µl of RT buffer for MMuLV, 2 µl of 10X solution for MMuLV, 1 µl of M-MuLV reverse transcriptase (RNaseH-), 2 µl of 10mM dNTP mix and 1 µl of nuclease free water. The mixture was then short spinned and incubated at 42°C for 90

min. The reaction was stopped by heating the mixture at 85°C for 5 min and chilled on ice. The integrity of the cDNA was checked using β-actin primers as housekeeping (control) gene (Puech *et al.*, 2015).

In order to amplify the full length Open Reading Frame (ORF) of HSP70-1 gene sequence, a specific primer pair was used based on the HSP70-1 mRNA sequence of cattle (Gade *et al.*, 2010). The specific sets of primers used in the experiment were synthesized from Eurofins Genomics India Pvt. Ltd., Bangalore (Table 1).

The master mix for the reaction contained 50 picomoles of each forward and reverse primers, 5 µl of template cDNA, 5 µl of 10X green Taq green buffer, 1 µl of 10mM dNTP mix and 1.25 units of green Taq green DNA polymerase. The final volume of the reaction mix was made to 50 µl with nuclease free water. Amplification conditions were as follows: an initial denaturation of 93°C for 3 minutes; 30 cycles of 93°C for 15 sec, 51°C for 30sec and 68°C for 1.54 min and a final extension step at 72°C for 10 min.

Results and Discussion

RNA extraction

The use of Histopaque 1077 (Sigma) and the use of RBC lysis buffer for the separation of PBMCs had no significant difference/influence ($p \geq 0.1$) on the purity and concentration of total RNA extracted from the cells. The details are given in table 2. Further, the integrity of the total RNA isolated from PBMCs (using both the methods) was further confirmed by using denaturing agarose gel (1.5%) electrophoresis. Two intact bands of 28S and 18S r RNA were obtained which confirmed the intactness of the total RNA isolated (Fig. 1).

Table.1 Primer sequences for HSP 70-1 and β -actin genes used in RT-PCR

Name of the gene		Sequence (5'→ 3')	Expected product size
HSP 70-1	F	ATGGCGAAAAACATGGCTATC	1926 bp
	R	CTAATCCACCTCCTCAAT	
β actin	F	TGGGCATGGAATCCTG	194 bp
	R	GGCGCGATGATCTTGAT	

Table.2 Mean (\pm SE) values of the concentration of total cellular RNA and absorption ratio OD 260nm : OD 280 nm of the total RNA isolated from PBMCs of Jersey- Sahiwal cross breeds during thermal stress

Type of the Method	Concentration of total cellular RNA (ng/ μ l)	Absorption ratio OD 260nm : OD 280 nm
Using Histopaque 1077 for the separation of PBMCs	313.8 \pm 18.3	1.8 \pm 0.03
Using RBC lysis buffer for the separation of PBMCs	297.2 \pm 22.9	1.8 \pm 0.2

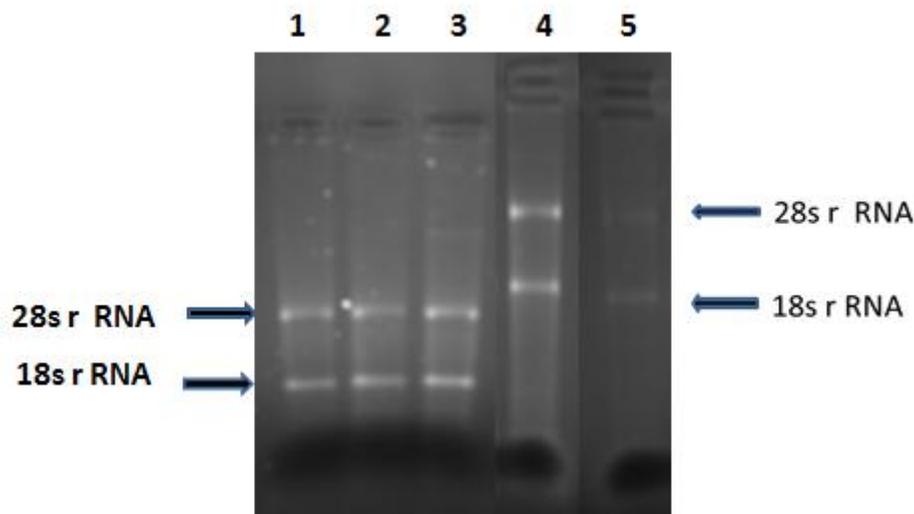


Fig.1 1% formaldehyde agarose gel electrophoresis of RNA isolated from PBMCs of Jersey-Sahiwal cross breeds during thermal stress- Lanes 1, 2 and 3 (Histopaque 1077) ; Lanes 4 & 5 (RBC lysis buffer)

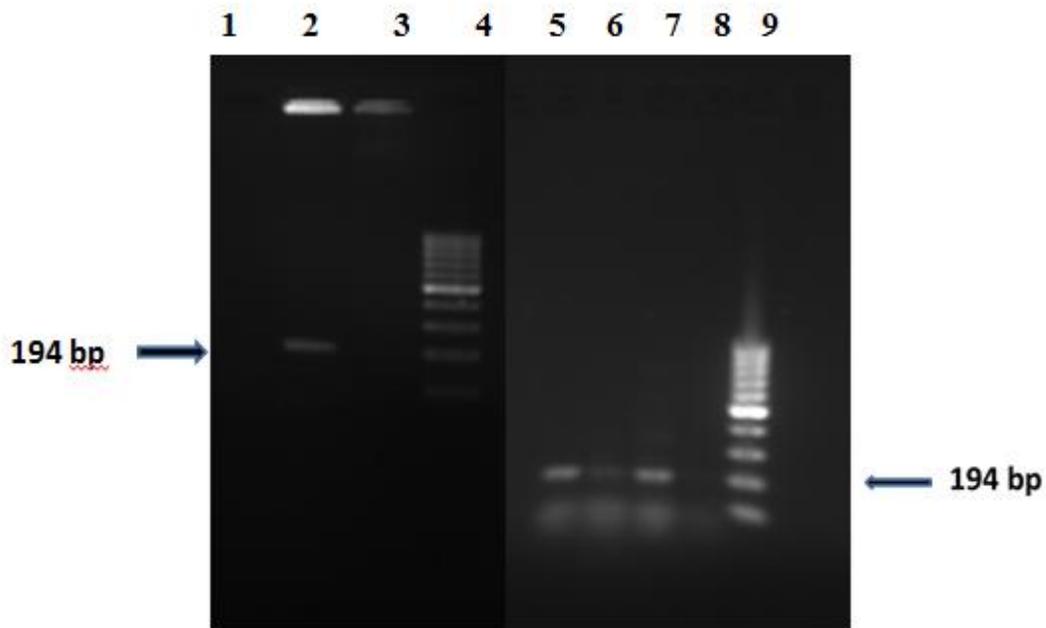


Fig.2 β - actin (house keeping gene) amplified gene fragment of 194 bp
Lane 1 & 8: Negative control, Lanes 2, 5, 6 & 7: 194 bp amplified product of β - actin gene, Lanes 4 & 9: 100bp DNA ladder

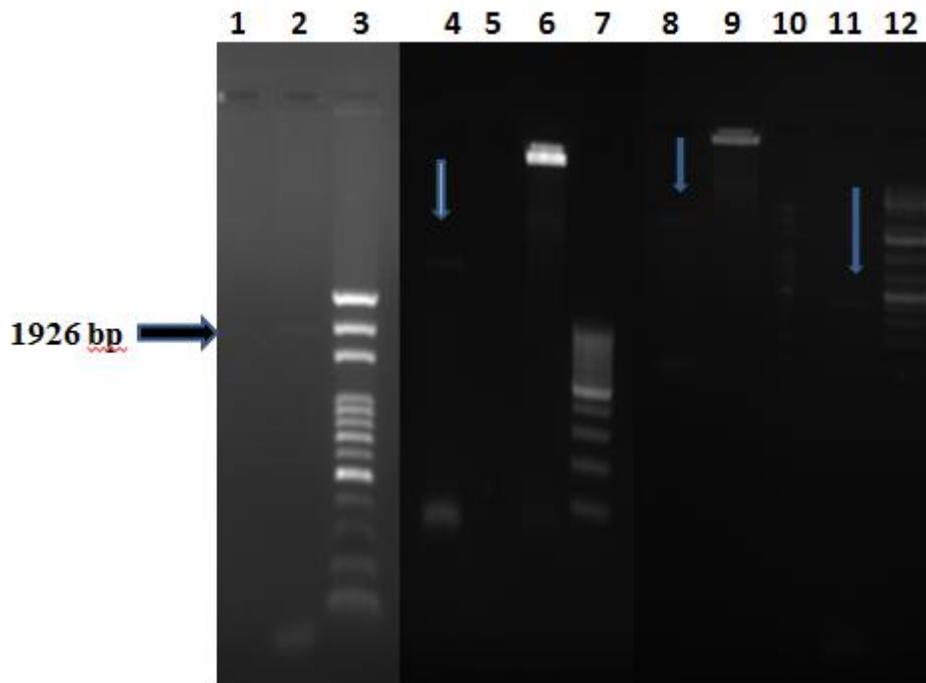


Fig.3 HSP 70-1 amplified gene product of 1926 bp by RT-PCR
Lanes 1 & 5: Negative control, Lanes 2, 4, 8 & 11: 1926bp RT-PCR amplified HSP 70-1 gene product, Lane 7: 100bp DNA ladder, Lane 3 & 10: 100 bp DNA ladder with extra 3 bands of 1500, 2000 and 3000 bp respectively, Lane 12: 1 kb plus DNA ladder

Complementary DNA synthesis

First strand of cDNA was synthesized from all the samples and its integrity was checked by PCR with the house keeping gene, β -actin gene primers which amplified 194 bp specific gene fragment (Fig. 2). PCR amplification using the set of primers for the amplification of full length HSP70-1 gene resulted in the production of 1926 bp specific PCR product that was amplified from the reverse transcribed cDNA (Fig. 3).

Adaptation to thermal stress involves the coordination of respiratory, circulatory, excretory, nervous and endocrine systems of the body. However, the adaptability response to heat stress varies between *Bos indicus*, *Bos Taurus* and between the cross bred cows (Khan *et al.*, 2018). Heat shock protein is an important biomarker produced as cellular and tissue defense mechanism whose expression gets markedly increased during heat stress (Lindquist., 1986). Among the various HSPs, HSP70 family of proteins are found to be having potential role in attributing thermo-tolerance to cows. HSP70 has also been found to be an ideal candidate genetic marker for selection of animals with better climate resilience, immune response and superior performance. The present in vivo study elucidated that there is expression of HSP70-1 in PBMNCs of Jersey- Sahiwal cross breeds during thermal stress. And, genetic selection of animals based on their thermo tolerance can become one of the possible moves towards reducing the impact of heat stress on cattle productivity by studying the expression of HSPs in PBMNCs.

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