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Expression pattern of heat shock protein genes in sheep

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ABSTRACT

Objective: To recognize the expression patterns of HSP70 and HSP90 genes in two local breeds of Sheep.
Design: Descriptive study.
Animals: Fifty ewes (25 Barki and 25 Abu Dlik).
Procedures: This investigation was carried out on fifty sheep at the northwest coast and southeast of triangle Halayeb and Shalateen during the months of March and May 2018 and 2019 (average day time temperatures: 25–35 ◦C; relative humidity: 55–65%). Total RNA was extracted using easy-RED™ Total RNA Extraction Kit. The primers for qPCR were designed on the basis of prior sequence information available at National Center for Biotechnology Information (NCBI) for the target HSP70 and 90 genes. The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) gene, housekeeping gene, was used for normalization of qPCR data. The desired genes were amplified for relative expression measurements. Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1 β, IL-6, IL-10, IL-12) were assayed.
Results: The expression levels of the HSP70 and HSP90 genes and the genes in Abu Dlik sheep were observed to be relatively up-regulated than those in Barki sheep. Abu Dlik breed exhibited an up-regulated mRNA level of Hsp70 and Hsp90 genes (1.70440938 vs 1.362954) while the Barki breed showed a down-regulated pattern (0.8550442 vs 0.4289764). In Abu Dlik ewes HSP70 gene exhibited a higher mRNA level than HSP90 mRNA.
Conclusion and clinical relevance: Gene expression patterns of HSP70 and HSP90, as well as cytokines modulations can be used as a biological marker and a reference point in sheep to identify, manipulate and cross-breed for improving the genetic potential and adaptability to tolerate harsh environmental conditions, especially heat stress.

Keywords: *HSP70*, HSP90, Real time PCR, Cytokines, Sheep.

1. INTRODUCTION

Environmental stress is a key factor driving the genome guideline, evolutionary history, and geographical distribution of organisms [1]. Worldwide normal temperatures are relied upon to increment by around 1–7 °C (2–13 °F) before the century is over. In spite of the fact that this change may not sound so huge, the issue is that even little changes in worldwide normal temperature can prompt enormous changes in the earth. Evolutionary changes in animal populations can be rapid, particularly as a consequence of human-induced environmental disturbances [1, 2]. These include the adaptation in plants, invertebrates, and vertebrates to heavy metals, adaptation to chemical and thermal emissions from factories, responses to salinity, evolutionary responses to overfishing, and adaptation to global temperature changes [3].

The selection of thermoresistant animals is an effective way to improve the productivity of livestock during high environmental temperature. Identification and exploitation of genotypes having thermo-tolerance in livestock are a major concern in the changing climate scenario which can have a great impact on livestock productivity. The environmental stress, particularly heat stress (HS) is the major concern in the livestock sector [4, 5].

Heat chock increases heat shock proteins expression in skeletal and cardiac muscles, which may enhance the tolerance to stress and increase the survival rate of the stressed cells [6]. Heat shock proteins are a family of proteins produced in all cells and tissues in response to the exposure to stressful conditions, such as HS [7]. The most important of these with respect to HS is heat shock protein70 kDa (HSP70) [6]. The HSPs have been studied extensively, especially regarding their regulation, localization, and function in the cell [3]. Stress-induced HSP accumulation is associated with thermo-tolerance, the ability to survive otherwise lethal heat stress, and later with tolerance to a variety of stresses, including ischemia [8, 9], ultraviolet irradiation [10], and cytokines such as tumor necrosis factor-α (TNF-α) [11].

Thermotolerant gene expression and elevated HSP levels are observed to be the ultimate response through which the cell sustains the impact of HS making it a full proof biomarker for the condition. The HSP is one of the cellular proteins found most abundantly under non-stress conditions also [8, 9].

One of the primary ways through which scientist across the globe are establishing the severity of HS is by expression of HSP [12, 13]. Over expression of HSPs provide protection against hyperthermia, circulatory shock, and cerebral ischemia during heat stroke which signifies the central role of HSP in cryoprotection [14]. The HSPs have a chaperonic activity ensuring the folding, unfolding and refolding of stress-denatured proteins [15]. Hydrophobic protein sequences liberated by denaturation gets bounded with the HSPs which otherwise would interact with other neighbour proteins resulting in loss of protein function.

Some research has moved on to identifying specific marker genes that underlie these genetic variants [16]. Banks of markers are emerging that can be used in the treatment of human diseases, indicating the likely vulnerability of individuals to different diseases and aiding in drug applications [17]. The mechanisms and cellular roles of Hsp70 have emerged many opportunities to design or screen for modulators of Hsp70 function [18, 19].

It is known that some local breeds have more heat tolerance compared to other breeds. Identification and exploitation of genotypes having thermo-tolerance in small ruminants are a major concern in the changing climate scenario which can have a great impact on livestock productivity. Therefore, the aim of the current study was to investigate the expression pattern of heat shock protein HSP70, and HSP90 genes in Barki and Abu Dlik sheep.

2. MATERIALS AND METHODS

2.1. Animals and blood collection

This investigation was carried out on fifty sheep at the northwest coast and southeast of triangle Halayeb and Shalateen during the months of March and May 2018 and 2019 (average day time temperatures: 25–35 ˚C; relative humidity: 55–65%). Animals of 2–2.5 years old apparently free from anatomical abnormalities and with average body weight of 30–40 kg were selected from both herds. Animals were maintained under open housing conditions with access to *ad lib.,* water, good quality green, dry feeds and supplementation of concentrate mixture. Blood samples (10 ml) were collected from animals by jugular vein, in tubes containing anticoagulant heparin and kept frozen (-20 ˚C) until analysis.

2.2. RNA Extraction

Total RNA was extracted using easy-RED™ Total RNA Extraction Kit (17063, iNtRON, Korea). Samples of 250 µl of blood were prepared in 1.5 ml microcentrifuge tube, and 750 µl of easy-REDTM Solution was added. The samples were mixed in room temperature for 15 sec by vigorous vortex and the tubes were incubated at room temperature (15 ~ 30 ℃) for 5 min. Following homogenization, insoluble material was removed from homogenate by centrifugation at 13,000 rpm for 3 min. The resulting pellet contained extracellular membranes, polysaccharides, and high molecular weight DNA, while supernatant contained RNA. An amount of 250 µl of Chloroform was added and the sample was mixed in room temperature for 15 sec by a vigorous vortex. The tubes were then incubated at room temperature for 3 min. The purpose of adding the chloroform was to separate the phenol layer from aqueous layer and eventually to isolate RNA and genomic DNA/protein. After that the tubes were centrifuged at 13,000 rpm for 3 min and a 400 µl of the upper fluid were transformed to a new 1.5 ml centrifuge tube. Equal volume (400 µl) of isopropanol (2-propanol) was added and mixed well by inverting the tube 4 ~ 5 times. The tubes were incubated for 5 min at room temperature. The tubes were centrifuged at 13,000 rpm for 3 min, carefully the supernatant was removed without disturbing the pellet. Then 1 ml of 70% ethanol was added and the solution was mixed well by inverting the tube 4 ~ 5 times. The mixture was centrifuged for 3 min at 13,000 rpm. Carefully the supernatant was discarded without disturbing the pellet. Then the remaining RNA pellet was dried. RNA pellet was dissolved by using 30 ml of RNase free water. The purity of RNA was analyzed by agarose (1.5 %) gel electrophoresis.

2. 3. The cDNA synthesis and real time PCR (qPCR)

The cDNA was prepared using The HiSen Script™RH (-) cDNA Synthesis Kit (INtron Biotecnology, Korea). The cDNA products were analyzed by agarose (1.5 %) gel electrophoresis. Relative expression level of mRNA transcripts of HSP70 and 90 genes was measured by quantitative real-time PCR (qPCR). The primers for qPCR were designed on the basis of prior sequence information available at National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/), of the target genes (HSP70 and 90) through primer3 software, http://bioinfo.ut.ee/primer3-0.4.0/, one housekeeping (Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) gene was amplified for the relative expression measurements (Table 1).

2.4. Biochemical parameters of cytokines

Interleukin-6 (IL-6), Interleukin-10 (IL-10) and Interleukin-12 (IL-12) were determined using Quantikine IL ELISA Kits [20]. Interleukin-1β (IL-1β) was determined using Quantikine IL-1β ELISA Kit [21, 22]. Kits of cytokines were purchased from Sigma (Aldrich-Sigma Company, CA, U S A).

2. 5. Statistical analysis

Statistical analysis of data was carried out by SPSS version 17 (SPSS, P.C, 2004). Un-paired *t*-tests for assessment the significance differences between variables were used. Results were considered significant only at the level of ($p < 0.05$). For evaluations of selected cytokines, the following equation was used.

$$Yij=\mu+Bi + eij$$

Where Yij is the j-th observed value of the response variable for i-th group, μ is the general mean effect, Bi is the effect of i-th breed, and eij is the error term. The multiple comparisons between different groups were done using Duncan's multiple range test (DMRT). Statistical analysis was done using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

Table 1. Primer sequences and amplicon size of each gene target analyzed in sheep by quantitative real time PCR.

Gene	Primer	Sequence (5´→3´)
HSP70	Forward	GACAAGTCGGAGAACGTGCA
	Reverse	CGTACACCTGGATCAGCAC
HSP90	Forward	ATTGACATCATCCCGAATC
	Reverse	ACACCAAACTGCCCAATCAT
GAPDH	Forward	GCAAGTTCCACGGCACAGTC
	Reverse	CCCACTTGATGTTGGCAGGA

3. RESULTS

3. 1. Gene expression patterns

The expression levels of the HSP70 and HSP90 genes in Abu Dlik sheep were observed to be relatively up-regulated than those in Barki sheep. Abu Dlik breed exhibited an up-regulated mRNA level of Hsp70 and Hsp90 genes (1.70440938 vs 1.362954) while the Barki breed showed a down-regulated pattern (0.8550442 vs 0.4289764). In Abu Dlik ewes HSP70 gene exhibited a higher mRNA level than HSP90 mRNA. (Table 2, Figure 1).

Table 2. Fold changes of HSP-70 and *HSP-90* genes in samples of local breeds of sheep.

Sample Name	Fold change *HSP-70*	Fold change *HSP-90*
Abu Dlik sheep		
Minimum	1.455405587	1.108674711
Maximum	1.829372644	1.71025861
Average	1.70440938	1.362954
Barki sheep		
Minimum	0.691039512	0.339972741
Maximum	0.962696903	0.541095273
Average	0.8550442	0.4289764

Table 3. Cytokines in plasma samples of two breeds of sheep.

Variable	Abu Dlik sheep	Barki sheep	± SE
TNF-α pg/ml	22.62^{A}	15.57^{B}	0.53**
IL-1β (Pg/ml)	46.96^{A}	29.33^{B}	0.61**
IL-6 (Pg/ml)	47.75^{A}	37.98^{B}	0.49**
IL-10 pg/ml	27.34	25.19	0.51^{NS}
IL-12 pg/ml	41.95^{A}	37.81^{B}	1.87**

NS: Not significant **Significant at (P < 0.01)

Figure 1. Gel electrophoresis of total RNA samples from the two breeds of heep.

Abu Dlik is a type of dry regions breed and is profoundly adapted to heat stress compared to Barki sheep is a type of semi-arid regions breed that is nearly less adapted to heat stress conditions. This indicates that the breed of Abu Dlik was better at managing heat pressure contrasted with the Barki breed of sheep.

3.2. Cellular cytokines alterations

Cytokine production and modulation of stress occur due to production of interleukins. Interleukins can act locally and systemically. Their action locally is to modulate cellular immune response. Systemically they change behavior, metabolism and neuroendocrine secretion. IL-6 is considered as the primary mediator of metabolic response to inflammation by inducing production of a broad array of acute phase proteins. Production of these pro inflammatory cytokines directly affects bone growth and are found to modulate the mechanism of proteins, fats and carbohydrates.

In Abu Dlik sheep highly significant increases in cytokines were induced as compared to Barki sheep. These increases may be due to the interaction between genotypes and environment which helps to tolerate desert environment. Abu Dlik sheep thus appeared more tolerant than Barki sheep to the heat stress of the desert as represented in Table 3.

4. DISCUSSION

Hsps are synthesized in response to different stressors indicating that they have an important role in the physiology and biology of stressed cells [3]. In general, HSPs protect organisms from various stressors such as heat, toxins, and

pathogens. Recently, studies on animal's responses to external stressors demonstrate that such stimuli induce highly significant changes in the expression profiles of *HSPs* [6]. It has been found that the productive parameters like milk yield, growth, reproduction and carcass traits can be negatively impacted by heat stress **[4,5]**.

In the present study, Abu Dlik sheep exhibited higher *HSP70* and *HSP90* mRNA expression level than Barki sheep and this therefore indicates that Abu Dlik sheep may have higher heat tolerant activity than Barki sheep. This finding is in agreement with that of previous reports [23]. The increase in expression levels of *HSP* may be due in part to changes in genotype in Abo Dlik breed which made it more able to acclimate with high heat stress of the desert [24]. Therefore, plasma levels of *HSP*70 and *HSP*90 genes expression may act as an ideal biological marker for assessing the impact of heat stress in sheep.

Cytokines are synthesized in the brain, the anterior lobe of the pituitary, and the adrenal gland [25]. Also, cytokine receptors have been detected at all HPA axis levels, and therefore, each level can serve as an integration point for immune and neuroendocrine signals.

The variation in level of *HSP*70 gene expression has positively correlated with thermotolerance in animals [5], whereas in livestock, the relationship between differential gene expression patterns and warm resistance has been rare. In the present investigation, it was seen that heat stress-tolerant (HST) Abo Dlik sheep showed higher *HSP*70 and *HSP90* articulation than Barki sheep during periods of the study. The qualities HSP are profoundly preserved between various species; however, the HSP gene expression is breed-explicit and species-explicit. The species-explicit distinction in *HSP70* isoforms is because of variation in warm resistance. The isoform expression may differ with regard to thermo-tolerance [6].

The results of the current study revealed that *HSP70* and *HSP90* family genes were expressed in sheep both constitutively. Upon local breeds, the expression of the genes studied was significantly higher in Abu Dlik as compared to Barki sheep. The most significant finding of this study was the increased *HSP90* expression in both herds. This suggests that *HSP70* and *HSP90* may be used as an indicator for assessing stress especially the thermal stress response of the animal. However, further studies are warranted to validate this in other genes and to qualify HSP as an indicator of stress associated response of animals under field conditions.

Stress resilience is a mind-boggling component. The mechanism of stress resistance by HSPs has not been surely known; in any case, HSPs assume significant jobs in the handling of stress denatured proteins. HST phenotypes control heat stress in a productive way showing higher *Hsp70* at the cell level. Be that as it may, it is important to connect the stress phenotype with adaptation, wellness, and production parameters during development periods [26]. Similarly, association of heat stress protein90 and 70 gene polymorphism with adaptability traits in Indian sheep (*Ovis aries*) has been documented [27].

Conclusion

Gene expression pattern of *HSP70* and *HSP90* can be used as a reference point in breeding to identify, manipulate and cross-breeding for the improvement of genetic potential and adaptability in sheep, and farm animals. The selection of thermo-resistant animals is an effective way to improve productivity during high environmental temperatures, especially with potential climate changes. Further research is required to verify our results and to determine the molecular mechanism to manage stress and adaptability at a cellular level.

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Conflict of interest statement

None of the authors has any conflict of interest to declare.

Authors´ contributions

Younis F.E designed the experiment, collected blood samples, performed the gene expression, and wrote the manuscript.

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