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STUDY OF THE IMPACT THERMAL STRESS ON MOLECULAR CHARACTERIZATION OF HEAT SHOCK PROTEIN 70 GEN IN THE BLOOD OF LOCAL MALE CALVES

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Abstract

This study was undertaken in cattle field in the private sector in Al-Qurna, north of Basra Governorate, where it was conducted in the period between July 2018 to February 2019 and included the following seasons, the monthly summer season(July and August of 2018), the monthly autumn season (October and November of 2018) and the winter season is monthly (January and February of 2019). This experiment was designed to detect the effect of the THI value on genetic polymorphism of Hsp70 member A (HSPA1A) gene in the blood calves, and their relation with serum level of Hsp70 of local Holstein calves during different months and seasons. Twenty blood samples were collected monthly from the same Holstein male calves (n = 20) and revealed that : After DNA extracted from calves blood, A Polymerase Chain Reaction (PCR) for the amplification product 2956bp was carried out. Nucleotide sequence analysis was done to detect polymorphism and compared with the genotypes of the Hsp70 gene in the Gene Bank (accession number NM_203322.3.) There are two Genotypes of Hsp70 gene were obtained in calves : Group A: This genotype exposed to different mutation on different sites which include : On the site (A302 del and G504 del) nucleotide Adenine and Guanine have been deleted, and three missense mutation on positions (A329G, C359G andG506T) encoding to new amino acid, while the genetic mutation at the position (G367C and G403A) are silent (calves, n = 12). Group B: This genotype is the closest to heat shock protein Hsp70(HSPA1A) gene than to genotype A in the Holstein calves in Gene Bank. This genotype was exposed to genetic mutations at different position: On the site (G506A) one missense mutation encoding to new amino acid was recorded, while the rest mutation at sites (G367C and A504G) are silent did not encoded to new amino acid (calves, n = 8). The result of Multiple Sequences Alignment (MSA) of Hsp70 calves showed that the genotype A has identities 97%, while genotype B has identities 99% with *Bous taraus* heat shock protein 70 Member A (HSPA1A1) in Gene Bank. Significantly higher ($P \leq 0.05$) in serum level of HSP70 in genotype A than genotype B in same months and summer season than the rest months and seasons.

Key words: *Heat Stress, HSP, Polymorphism.*

Introduction

The heat stress has happen to a major issue in the time of weather change and it directly affects compliance and survivability of livestock to thermal physical attack. It has been shown that animals can yield to hyperthermia as they fail to decline the impact of heat stress load. Increased heat stress in cattle breeds and other farm animals have been linked to pitiable food intake and sluggish metabolism, thereby affecting growth, milk production and reproductive efficiency with unfavorable economic loss Several husbandry and management strategies have been in employment to help moderate effect of stress in dairy and beef animals however, this strategies is not far reaching (Kapila *et al*., 2013). These stimulate responses to environmental heat loads over thermo-neutral zones in animals through intra and extracellular signals that coordinate cellular and whole animal metabolism (Collier *et al*., 2008). The importance of selecting the most resilient and adaptive animals to climatic changes and different stress conditions arises through the selection of some molecular mechanisms in protecting the cells that protect them from the effects of heat stress and different stress conditions (Hassan, 2019), and the most important of these molecular mechanisms that body possesses are a family of heat shock proteins(HSP), especially heat shock proteins 70 (HSP70), which activation when animals exposure to heat or cold

stress that have the ability to protect and keep the cells alive when exposed to different stress conditions, by act as molecular chaperones in regulating the cellular homeostasis and folding and unfolding of damaged proteins during the stress (Kapila *et al*., 2013; Hamzah and Hasso, 2019). In addition, these family is characterized by being highly-preserved molecules (few genetic mutations) and the heat shock protein gene is inherited well by parents to children (Kesorn *et al*., 2017). So, its genetic formations can be used as an electoral name to resist stress conditions, as well as its level and a genetic expression that varies between individuals and genetic formations (Rout *et al*., 2016; Srikanth *et al*., 2017) respectively. The studied aimed to evaluate the effect of natural change in temperature humidity index (THI) value at different months and seasons and to determine the difference in hereditary formations to the study their relation with serum concentration of HSP70 in calves.

Materials and Methods

Animals and blood collection

A total twenty male calves (1-1.8) years old which exposed to different value of temperature humidity index (THI) during different months and season of years from cattle filed of the private sector in the area of Al- Quranh, north of Basrah Governorate, Iraq and the laboratories of the college of Veterinary Medicine at the University of Basrah, including the physiological laboratory, medicinal laboratory, central research unit and Bayan Group For Advance Laboratories Diagnostic were used in this study. The animals were supplied with a standardized food as well as green fodder free once a week. Salts and drinking water were constantly present in front of the animals. According to Minimum and maximum temperatures and humidity percentage values were obtained from the reports of Basrah Metrological service in Basrah, Governorate, the temperature humidity index (THI) value was calculated by (Mader *et al*., 2006).

This experiment was designed to detect the effect of the THI value on genetic polymorphism of Hsp70 gene in the blood calves, and their relation with serum level of Hsp70 of local Holstein calves during different months and seasons.

A blood samples was collected from local calves by venipuncture into two tube type of test blood: ethylene diamine tetra acetic acid (EDTA) vacationers for DNA extraction and gel tube for serum analysis and these tubes stored at (-20ºC, -4ºC) respectively until used.

DNA Extraction, PCR Protocol and PCR Sequences

The gSYNC™DNA Extraction Kit Quick Protocol (catalogue No.GS100) was used to extract the DNA from whole blood and stored at -80ºC for PCR reaction. The concentration and purity of DNA was detected by Nano-drop system (Nano Drop thermo scientific 200, USA) according to Dauphin *et al*.,(2011).

A set of specific primer was used for the fragment amplification of heat shock protein 70gene at size 295bp according to (Bahat *et al*., 2016). The amplifying of polymerase chains reaction in 20µl volume containing 12.5 µl of GO Taq Green Master Mix, 1µl for each primer, 4µl of Genomic DNA and 6.5µl of Nuclease free water. The thermal cycles program of PCR were performed as follow: initial denaturation of the DNA at 95°C for 5 minutes , second denaturing at 95°C for 30 seconds, annealing at 62°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 7 minutes all in 35 cycles. The PCR product size 295bp was detected on 2 % SYPER safe stained agarose gel Fig. 1 and the final product of PCR were sequenced on Laboratories of Macrogen Company/Korea.

Nucleotide Sequence alignment was done to detected the polymorphism and compared with the genotypes of the Hsp70 gene in the Gene Bank at accession number NM_203322.3.

Measuring the Heat Shock Protein 70 Level in Serum

Bovine heat shock protein 70 assayed by using enzyme linked immunosorbent assay (ELISA) kit manufactured by Shanghai Korain Biotech Company, China.

Statistical Analysis

Data was analyzed by using SPSS (Statistical Program for Social Sciences) program version 22.0 and presented as mean ± standard deviation (Abo-Allam, 2003). One way ANOVA was used to compare mean

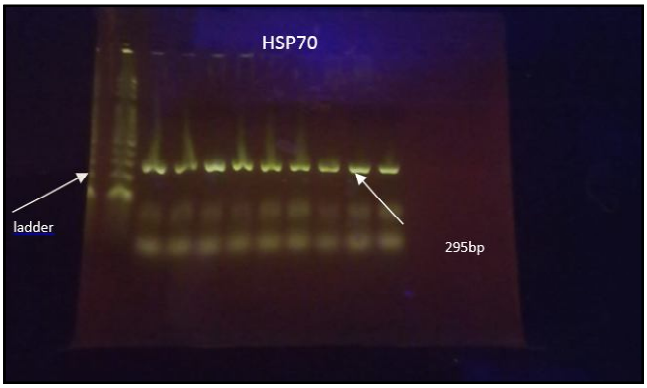

Fig. 1: PCR product of HSP70 with a band size of 295bp. The product was electrophoresis on 2% agarose at 80 volt/ cm^2 and 200mA. 1x TBE buffer for DNA Kappa ladder l00pb.

different among more variable. Blast analysis by using NCBI BLAST (NCBI 2017) (https://blast.ncbi.nlm.nih.gov/Blas) was used to compare the observed nucleotide sequences with same nucleotide of the same gene in Gene Bank.

Multiple sequence alignment analysis MSA (Clustal Omega software 1.2.4.2017), (http://www.ebi.ac.uk/Tools/msa/clustalo) was used to compare all sequences with same gene.

Results

The Maximum, Minimum Temperature, Relative Humidity and Average THI Value During Months and Seasons of Study Period

The maximum and minimum climate temperature and relative humidity percentage are obtained from the Basrah Meteorological Center is clarified in the table 1 and revealed that high value of maximum and minimum climate temperature was obtained during July and August months, while the lowest values were recorded during January month than others months. The high and low value of maximum of relative humidity percentage was recorded during January and July, but a highest and lowest value of minimum relative humidity percentage was recorded during February and July months respectively than the others months.

The mean ± SD value of monthly average of temperature and relative humidity percentage during different months were reveled in the table 2. In the present study, a highest and lowest values of average climate temperature was obtained from July and January months, while a highest and lowest values of the relative humidity percentage was obtained during February and July months.

The average value of temperature humidity index (THI) during different months and seasons of experimental study period are present in table 3. The average THI value varied among different months and seasons. The average THI value was recorded high significant ($p<0.05$) with summer season, where recorded highest value in August months compared to the rest months, while the lowest significant ($P<0.05$) was recorded in January month during winter season as compared to other months and seasons.

Table 1: Mean±SD Values of Maximum and Minimum Temperature and Relative Humidity During Study Months Period.

Min.RH(%)	Max. RH(%)	Min.Temp (ºC)	Max.Temp(ºC)	Months
7.26±2.25f	26.03±3.14e	28.40±1.13a	47.63±1.44a	July
16.93±3.90d	38.40±1.79d	27.16±1.01a	47.46±1.43a	August
11.12±2.01e	44.06±2.48c	19. 00±1.48b	35.87±1.38b	October
21.30±1.64c	69.33±4.64b	12.50±0.62 c	27.53±0.86c	November
24.29±1.67b	72.09±5.17a	5.74± 0.85e	19.90±1.39e	January
26.71±1.86a	71.21±2.79a	10.28±1.38d	21.75±1.87d	February
2.42	1.88	1.23	1.84	LSD

Mean THI value with different superscripts (a, b, c, d, e and f) in the column differ significantly ($P<0.05$). (Basrah Meteorological Center 2018-219).

Table 2: Mean±SD Values of Monthly Average Temperature and Relative Humidity During Study Months Period.

Average . RH(%)	Average. Temp(ºC)	Months
16.90±2.13d	38.16±0.79a	July
27.66±2.22c	37.31±0.94b	August
27. 59±1.68c	27.43±1.03c	October
45.33±2.32 b	20.01±0.51d	November
48.32± 3.23a	12.82±0.92f	January
48.96±1.77a	16.01±1.15e	February
2.98	0.85	LSD

Mean THI value with different superscripts (a, b, c, d, e and f) in the column differ. significantly ($P<0.05$). (Basrah Meteorological Center 2018-219).

Table3: Mean ±SD Values of Average Temperature Humidity Index (THI) During the Study Months and Seasons Period.

Months	AverageTHI	Season	AverageTHI
July	83.66±0.27b	Summer	84.44±0.86a
August	85.22±0.41a		
October	77.87±0.00c	Autumn	69.17±8.92b
November	60.48±0.00d		
January	55.19±0.00f	Winter	55.85±0.68c
February	56.52±0.00e		
LSD	1.33	LSD	13.32

Mean THI value with different superscripts (a, b, c, d, e and f)in the column differ significantly ($P<0.05$).

Detection the Polymorphism of Fragment of Heat Shock Protein 70 Genotype in Holstein Calves During Study Period

The nucleotides sequence analysis and multiple sequences alignment of DNA fragment (295bp) of the bovine Hsp70 gene position on mRNA HSPA1A1(181-475) with *Bos taurus* heat shock protein family A (Hsp70) member 1A (HSPA1A) in table 4, revealed two genetic polymorphism of Hsp70 gene (A and B) and both of them have different mutation.

Relation of Hsp70 Polymorphism With, Average THI value and Hsp70 Serum Level During months and Seasons of Study Period

The relation of Hsp70 genotype (A and B) with serum concentration of HSP70 in calves was

Table 4: Types of Heat Shock Protein Genotype, Nucleotide Substitution and Amino Acid Changes in Calves, n = 20.

Genotype	Nucleotide location	Nucleotide substation	Amino acid change	Type of substitution
Genotype	A302 Del	AAC>A-C	-----	Delet
A(n=12)	A329G	AGC>GGC	Serine >Glycine	Transition (missense)
	C359G	CTC>GTC	Leucine>Valine	Transversion (missense)
	G367C	GGG>GGC	Glycine>Glycine	Transversion (silent)
	G403A	CAG>CAA	Glycine>Glycine	Transversion (silent)
	G504 Del	GGA>G-A	-----	Delet
	G506T	GAC>TAC	Aspartate > Tyrosine	Transversion (missense)
Genotype	G367C	GGG>GGC	Glycine> Glycine	Transversion (silent)
B(n=8)	A504G	GGA>GGG	Glycine> Glycine	Transition(silent)
	G506A	GAC>AAC	Aspartate> Aspargine	Transition(missense)

The symbols A, C, G and T indicate to Adenine, cytosine, Guanine and Thiamine nucleotides, Del = delete and n = number of calves in genotype.

indicated in table (4-20). This result, that Serum concentration of Hsp70 in genotype A recorded high significant ($P<0.05$) in August months and summer season than the Genotype B than the rest months and seasons.

Discussion

It's clear that the THI value recorded significant higher at August month than others month and during summer season and autumn season compared to cold season which present in the table 3, might be due to the elevated the global temperature and humidity in this month and these season in Basrah governorate. Thus, likewise with (Trana *et al*., 2006) who revealed a significant increase of THI value at hot season than other years season.

Cincovic *et al*., (2011) showed that THI value 75 to 80 was moderate to high power of heat stress, while THI value73-77 were considered mild thermal stress and THI value 72 and underneath was considered as no warmth stress. But, (Srikandakumar *et al*., 2003) considered that THI value 78 to 89 as moderate and above 90 as extreme thermal stress. In this manner, in the current examination the average THI of during the cold season (winter) demonstrated that the creatures were under no warmth stress. While, the average THI value throughout the autumn and hot (summer) seasons respectively showed that the creatures were under moderate to high power of warm pressure at Basrah governorate.

These genetic formations was obtained in this study have been subjected to the occurrence of genetic mutations, which is often attributed to the animal's ability to adapt in an environment that differs from the environment of its original habitat for survival. The results agreed with the previous reports of several scientists such as Ciftci, (2015); Han *et al*., (2016) about the presence of multiple genetic formations of the of the heat shock protein gene. Also consistent with study by Onasanya *et al*., (2019) who observed 21 SNPs variants of HSP70 gene in different breed of Zebu cattle : one Indel A7Del *i.e.* deletion of A at base position 7 (White Fulani), 10 transversions (White Fulani: C154G and G220T, Ambala: G220T, Sokoto Gudali: T198A, Red Bororo: C154G, A78T, G106C, T198A, G220T and T254A) and 10 transitions (White Fulani: C145T and G220A, Ambala: C154T and C244T, Sokoto Gudali: C184T and Red Bororo: G157A, C157T, G196A, C244T and G199A).

Kerekoppa *et al*., (2015) who recorded 7 SNPs in Deoni (Zebu type) which including 5 transitions and 2 transversions, also recorded 5 SNPs in Holstein Friesians crossbred cattle, including 2 indels, 2 transitions and 1 transversion and indicated a high degree of genetic variability in the HSPA1Agene in the breeds. Li *et al*., (2011) observed five novel SNPs and eleven different haplotype in the HSP70-1 gene of Chinese Holstein cattle. Hence, recent researcher found association between observed SNPs in the HSPA1A gene and economically important traits like heat tolerance and reproductive performance in cattle (Rosenkrans *et al.,* 2010; Twegh *et al*., 2020).

In present data the silent mutation that occurred in both genotype did not recorded any change of the amino acid structure because each amino acid have same nucleotide form (Kimchi-Sarfaty *et al*., 2007). While, previous worker found that the synonymous mutations may impact on the role of translated protein through diverse cellular mechanisms and it lead to polymorphism to the Hsp70 gene (Hunt *et al*., 2014). These, agreement with previous authors who indicated that synonymous SNPs might be affect the relevant protein by change in

transcription and their level and function Bali and Bebok, 2015).

The SNP that occurred in hereditary morphology A and B on different site that lead to substitution one nucleotide with other nucleotide, as well known as three nucleotide that make up one amino acid (Turanov *et al*., 2009). Missense SNP are those that result in a change in the codon and amino acid sequence in the protein which led to the change the neuclotide position which led to the formation of a new amino acid (Medicine, 2012), as well as it affected on the formation of heat shock proteins 70 and agreed with study by (Yudin and Voevod, 2015) that indicated that genetic mutations in the heat shock protein 70 increase cattle resistance to various stress conditions. Additionally, previous studies indicated that the mutation produced by the amino acid Aspartic makes livestock more resistant to pathogens (Ibeagha *et al*., 2008). While, Bhat *et al*., (2016) who recorded SNP (G149T) substitution leads to a change of amino acids from Aspartate to Tyrosine in gene transcript and concluded that gene have been more resistant than other genotype in Tharparker cattle.

We reached in this study, that the Iraqi Holstein calves have new haplotypes of Hsp70 in comparison with that recorded in Gen Bank and the differences in the mutation are due to the differences between the amino acid produced before the mutation and the amino acid formed after the mutation, and the mutation that occurred positively affected the properties of the produced protein.

The serum level of heat shock protein has been varied between the different months and seasons, which revealed the combined action of meteorological, HSP70 genotype and serum level of HSP70 in the table 5. The HSP70 serum level under investigation were fundamentally higher during summer season than autumn, and winter season and same season in genotype A than genotype B. The HSP70 polymorphorism gene such as HSP70 A1A was recorded in heat stressed Holstein cattle which play very crucial role to adapted the animal in harm condition (Li *et al*., 2011; Deb *et al*., 2014). Also we recorded, The expression level of Hsp70 gene is affected by SNP location in Hsp70 gene region and different genotype. The serum level of HSP70 recorded high significant in HSPA1A genotype A than genotype B in same and in both seasons. These results agreement with Basirico *et al*., (2011) showed that association between the heat tolerance and the polymorphic pattern of HSP70-1gene. However, Xiong *et al*., (2013) discovered that a genetic mutation at various locus in HSP70 gene in Holstein breed that demonstrated contrasts in thermotolerance.

A close relation between the polymorphic pattern of HSP70 with respect the heat stress factor in dairy cattle has been indicated by (Deb *at el*., 2014). The elevated serum level of HSP70 in genotype A might indicate that the local Holstein calves have adapted significantly to resist heat stress conditions through changes in the polymorphism of the HSP70 (HSPA1A) gene. That's because the mutations in HSP70 gene could be used as marker for heat stress tolerances (Li *et al*., 2011) and assisted selection for resistant to heat stress in the breeding (Gafer *et al*., 2015). These studies support that the gene is important in thermotolerance in cattle of different breeds and suggest that cattle managed under stressful conditions such as heat due to climate change would likely have altered HSP expression. Although HSP70 is heavily induced by stress, non-stressed" cows were found to have circulating concentrations of plasma HSP70, which might indicate that it is produced in preparation of combating increased stress (Kristensen *et al*., 2004).

Our findings in this study indicate that there is high variability in the HSPA1A gene in calves , which suggests that HSPA1A in genotype A could be used as a candidate gene for identifying markers for heat tolerance.

Table 5: Effect of Thermal Stress on Value of Serum Hsp 70 (ng/dl) of Genotypes A (n = 12) and B (n = 8) In Holstein Calves During Months and Seasons of study Period, (Mean ± SD., n = 20).

Months	HSP70		Season	HSP70	
	GenotypeA	GenotypeB		Genotype A	GenotypeB
July	3.44± 0.35ab	2.61±0.43a	Summer	3.45 ±0.33a	2.65 ±0.45a
August	3.46±0.33 a	2.70±0.48a			
October	3.17±0.34 b	2.32±0.41ab	Autumn	2.90 ±0.45b	2.18±0.32b
November	2.64±0.40c	2.03±0.03b			
January	1.63±0.35d	1.21±0.49c	Winter	1.60±0.34c	1.28±0.46c
February	1.57±0.34d	1.35±0.44c			
LSD	0.29	0.58	LSD	0.54	0.47

Values express as mean ± SD., n = 20 calves, with different superscripts (a, b and c) in the column differ significantly (P<0.05).

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