



# GENETIC POLYMORPHISM IN HSP90AA1 GENE AND ASSOCIATED WITH THE HEAT TOLERANCE COEFFICIENT IN HOLSTEIN COWS AT SOUTH OF IRAQ

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## Abstract

This study was carried out in Iraq at the Taj Al-Nahrain Animal Production located in Diwaniyah province, one of the governorates of the Euphrates Middle, located south of the Iraqi capital Baghdad, about 180 kilometers away.

The study was undertaken to investigate the association of single nucleotide polymorphisms (SNPs) of HSP90AA1 gene with some of reproductive and productive traits and all so heat tolerance coefficient during different season (warm, moderate, cold) Here are the most important results obtained during this study. The analysis revealed a site of heterogeneity in the HSP90AA1 gene within the Exon3 region at site 1690. The genotype of the heterogeneity site was recorded with two genotypes: TT (Wild) and genotype TC) Heterozygous), and the heterogeneity of the nitrogen bases was determined. The results showed that the nitrogenous base of T was changed and replaced with the nitrogenous base C Within the heterogeneity site, the results show a significant difference at the level ( $P < 0.01$ ) between the genotypes. Results have indicated in terms of the effect of genotypes For the studied piece For the site of heterogeneity and its relation to some reproductive qualities and productive qualities and heat tolerance coefficient To the absence of statistically significant differences between genotypes.

**Key words:** heat shock gene, HSP90AA1, heat tolerance coefficient

## Introduction

Cattle are the main source of milk production among farm animals. The livestock sector contributes to the agricultural economy, 90% of the total production in the world. The productive qualities of economic importance, including the status of milk production Depends heavily on the environment in which the cow is created, We note that the change in the productive capacity of cows is a combination of environmental changes on the one hand, and the genetic capacity of cows on the other, because the status of milk production is a quantitative property controlled by a large number of genes, has been pointed out recently that the effects of climate Is seen as a threat to the sector as a result of global warming. (FAO, 2009; sejian, 2016). The negative effect of heat stress will become more apparent in the future if climate change continues .moreover due to the close relationship between metabolic heat generation and production levelly development in genetic programs that enhance production traits may increase an animal susceptibility to height

environment temperature (Nardone *et al.*, 2010).

Through some mathematical equations, the production and production performance of the animal can be predicted depending on their ability to withstand heat.

On this basis, the aim of this study is to:

1. Measuring the heat tolerance coefficient for each cow and its relation to the studied qualities and predicting the production and physiological performance.
2. Molecular characterization of HSP90 with a molecular weight 90 KD in dairy cows of the Holstein-Freizian strain.
3. Studying the relationship between the HSP90 gene and the production and physiological performance.

The process of progress in the modification of the environment and management of nutrition is part of the mechanisms used to reduce the impact of heat stress on the performance of the animal during the seasons, especially at high temperatures in the summer.

However, new mechanisms with a long-term strategy

must be developed To adapt to climate change, where there are differences in the tolerance to heat stress between animal species in general and species of milk cattle in particular, Which provide tools for selecting animals with good ability to withstand stressful temperatures using thermoplastic genes, Recognition of heat-tolerant animals in high-yielding breeds would be beneficial only if they were able to maintain high productivity when exposed to thermal stress conditions (Das, 2016). All objects respond to heat By stimulating the synthesis of a group of proteins called thermal shock proteins. Response is the most conservative genetic system, and it exists in every living organism that has been sought, from bacteria to eukaryotes, From plants to animals. Although some of the characteristics of the response vary from organism to another organism. Thermal shock proteins are classified among the most advanced cellular proteins across all species, The name also shows that heat shock proteins protect cells when temperatures reach a fairly high level (Shahid, 2011). Animals require the base grade to have a relatively constant body temperature, so that the biological and production activities can perform better, All animals have a convenient range of surrounding environmental temperatures called the neutral thermal zone (TNZ), The thermal neutral zone for dairy animals ranges from 16°C to 25°C, to maintain the physiological body temperature within 38.4 -39.1°C (Yousef, 1985). Temperatures above the comfort zone, high relative humidity and intense solar radiation alone or with low wind speed are major factors that increase the load or heat load and eventually cause thermal stress (Mader *et al.*, 2006). Upper Critical Temperature is the point at which heat pressure begins to influence animal performance (Shahid, 2011). Thermal shock or pressure response is a cellular adaptive response process, which helps to balance cellular under stress. Stress refers to the size of external forces Which make a difference in the state of the animal and displacement of the body system of the animal From rest to discomfort (Yousef, 1985), (The stress may be caused by climatic conditions (such as high or low temperature), Nutritional factors (Such as undernourishment, poor feed quality, lack of water sources and severe thirst) Internal factors (such as certain physiological disorders, pathogens or toxins) (Stott, 1981).

HSP90 proteins are basic molecular accomplices involved in signal transfer, cell cycle control, stress management, folding, decomposition, and protein transfer. HSP90 proteins have been found in a variety of living organisms suggesting that they are old and preserved or preserved (Bin and others, 2006). Unlike other molecular

accompaniments, Hsp90 does not work , In the folding of modern proteins only, but is linked to the substrate proteins that are close to the original protein at a later stage of folding (Young *et al.*, 2001; Lund, 2001). It has been suggested later that it works to help other proteins to shut down and fold properly, It works to stabilize proteins against heat stress, helps in protein degradation, and works to stabilize a number of proteins needed for tumor growth, which is why Hsp90 inhibitors are examined as anticancer drugs.

## Materials and methods

### Experimental animals

The study was carried out in southern Iraq In the Taj Al-Nahrain tributaries belonging to Taj Al-Nahrain, located within the administrative boundaries of Diwaniyah Governorate for the period from 1/6/2018 to 15/5/2019, on a sample of 50 cows from the Holstein-Freizian cattle imported from Germany. The birth sequences were heterogeneous.

### Measurement of physiological parameters

#### Respiratory rate:

The process of measuring respiratory rate was done by Estimate the number of movements of the loin area , Per minute for each cow twice daily in the morning and evening. Over four days of each season, This process was done in two ways by counting the naked eye, And the method of counting using a camera On the loin area of the cow's body After the recording of the video recording the number of breathing times.

#### Body temperature:

Body temperature is measured by A German-made mercury thermometer is created by inserting the thermometer into the rectum, Taking into account the position of the thermometer in a tilted way to ensure the contact of the rectangle of the wall of the rectum from the inside for accurate reading, where taking the reading after a minute this is the practical aspect of the field.

#### Measuring temperature and humidity:

Temperature and humidity were measured twice a day at 6:00 am and 2:00 pm using a German mercury-based thermometer and the Hygrothermograph, which contains two measurements, one for measuring the temperature and one for measuring the relative humidity. The two devices were compared to obtain a correct reading. Experience.

#### Thermal tolerance coefficient:

The Heat Tolerance Coefficient (HTC) was calculated according to the following formula (Benezra,

1954).

$$\text{HCT} = \text{RR}/23 + \text{TR}/38.3$$

Where HCT is represented Heat Tolerance Coefficient, RR Respiratory rate of the cow, TR Heat of the rectum For the cow, 23 normal breathing rate of the cow in normal conditions, 38.33 represents the optimal rectal temperature of cows.

#### Laboratory side:

Laboratory side: While genetic tests (laboratory part) of blood samples were carried out in order to separate the genetic material and determine the genetic structure of the animals Genotype , For Jane Heat Shock Proteins with a molecular weight of 90 kDa , And the relationship of this to the performance of production and physiological, as well as to study the proportions of the distribution of structures in the herd and repeat the resulting alleles.

#### DNA extraction:

DNA was extracted from the blood samples of the cows for the purpose of conducting the molecular examination of the studied gene (HSP90AA1) in the scientific progress laboratory located in Baghdad / Harthiya for the period from 1/12/2018 to 15/4/2019 in order to separate the genetic material and determine the genetics of the heat shock gene HSP90AA1.

#### Molecular characterization and selection of the initiator of the studied gene:

Exon3 was determined by the HSP90AA1 thermal shock gene. The studied gene was made up of 450 pb and analyzed by analyzing the polymorphism of the single nucleotide SNP in the Exon3 region of the HSP90AA1 gene located on chromosome 21. Pomp and others (1997) The first to discover the polymorphisms of single-nucleotide SNP.

#### Load of the polymerase chain reaction and electrical relay:

After the implementation of the polymerase chain reaction program and PCR regulation, the electrical relay is adopted In the template Gel Agarose To ensure the presence of PCR products.

#### Molecular Characterization to Identify the Phenotypic Diversity of HSP90AA1 Using the Technology of Determination of Neocluidite

**Table 1:** Primers sequence used in the study.

Annealing temp C°	name gene	Amplicon size (pb)	Primer Name	(Primer Seq-)
63°	HSP90AA1	450pb(826...1276)	Exon3-F	F-GCGTCATCACGTGTCATCTT
			Exon 3-R	R-CCTCCTTTGGGGTTCCAGT
				Based on gene sequences available in the genebank database) Gene bank) Sequence number AC 000178.1

#### Sequences for DNA Cutting

After the electrical transfer of the PCR product and the confirmation of the Exon3 pieces of the HSP90AA1 thermal shock gene from a DNA molecule using specialized primers for this piece, the SNP sequence was then determined by sending the polymerization reaction results and volume 20 µL to Macrogen Corporation-Korea.

#### Results and discussion

DNA was extracted and its concentration was between 50 to 75ng/µl as the first step to extract the HSP90AA1 bovine gene within PCR technology and using the diagnostic kit supplied by Promega USA and the method of work referred to in the separation of materials and methods of work. The product was then migrated from each sample, or not.

#### Single nucleotides polymorphism Exon3 heat shock gene HSP90AA1:

Huge piece of heat shock gene HSP90AA1 with PCR technology and using several US company Promega PCR The initiator of the heat shock gene Exon3 (Primers), DNA samples and control of the thermal cycle device As mentioned above, and then A 5 µL sample of each sample was carried out in 2% agarose gel with 100 volts voltage difference and at 50 mA for 70 min and paging output to ensure successful extraction and acquisition of 450bp required DNA fragments (100-1500 bp DNA Ladder Marker) As shown in Fig. 4.2,

#### The location of heterogeneity in the sequence of nitrogen bases, percentages of genotypes and allelic replication of HSP90AA1 in the Holstein Frisian cows:

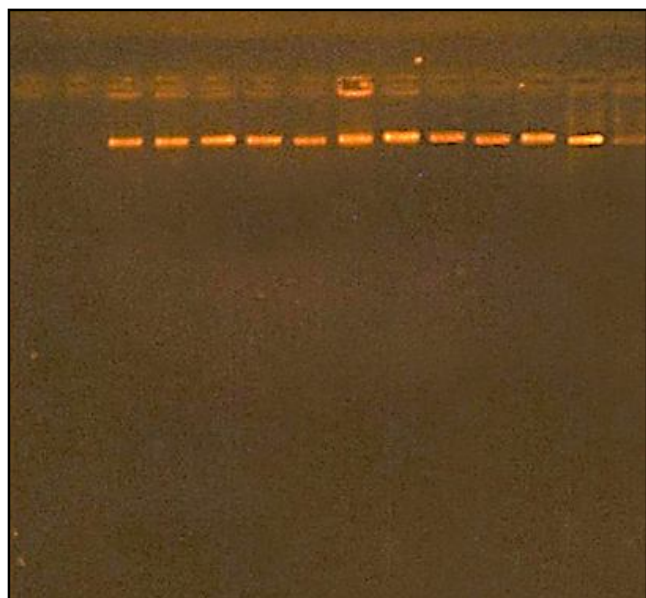
The analysis revealed a heterogeneity site in the HSP90AA1 gene within the Exon3 region, in location 1690 and carrying the symbol rs446647692 T/C, the genotype of experimental animals was determined in the studied HSP90AA1 gene using SNP technology as shown in Fig. 3. Genotype was recorded for heterogeneity site rs446647692 T/C where it contained two genotypes: TT (Wild) and Heterozygous (Heterozygous), and heterogeneity was determined for the nitrogen bases. Results showed that the nitrogen base was changed from T and substituted with the base Nitrogen C is in the

**Table 2:** Shows the program of multiplication of HSP90AA1 gene using PCR technology by temperature and number of cycles.

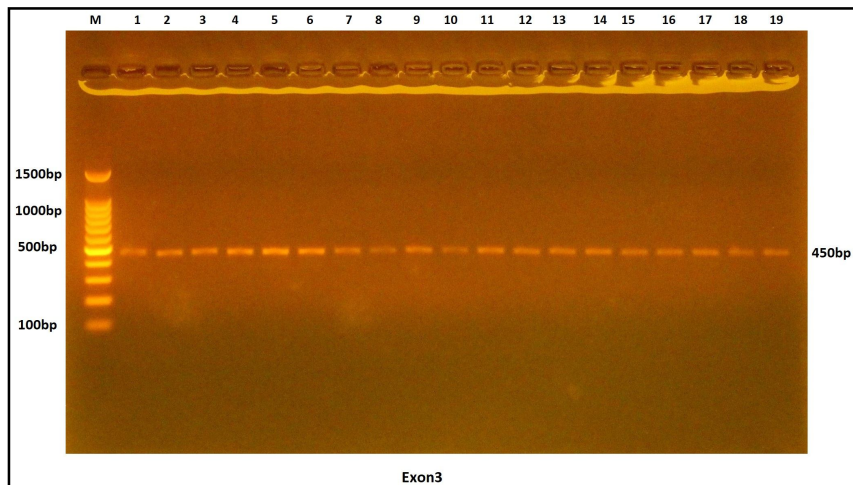
Seq	Steps	°C temperature	Time	Number of Cycle
1	Initial Denaturation	95	5minutes	5 Cycle
2	Denaturation	94	30 Second	30 Cycle
3	Annealing	63	30 Second	
4	Extension	72	2 minutes	
5	Final Extension	72	10 minutes	1 Cycle -
6	Hold	10	-	

heterogeneity of the symbol rs446647692 at 1690.

The genetic ratios of the heterogeneity site are rs446647692 Were 0.92, 0.08 and 0.0% for genotypes, TT (n = 46), TC (n = 4) and CC (n = 0) respectively, with a total frequency of 0.96 for T and 0.04 for allele C,



**Fig. 1:** DNA extraction process and migration product.



**Fig. 2:** The electrical transfer of the Exon3 PCR product to the thermocouple HSP90AA1 in the size of 450 bp in the Holstein-Freisian cows, M (Marker).

The difference in the results of the genetic composition of imported Holstein cattle from some studies and the differences between the other studies may be due to differences in the variety and suitability of environmental conditions as well as to the difference in the size of the studied sample. This difference may also be due to the fact that the current study population is not in the Hardy- Weinberg.

Through analysis of the results of my heterogeneities in the segment studied None of the variations were observed, which led to the alteration of the amino acid and was injected into the same amino acid, as these mutations were mutated mutations and were not encoded into a different amino acid.

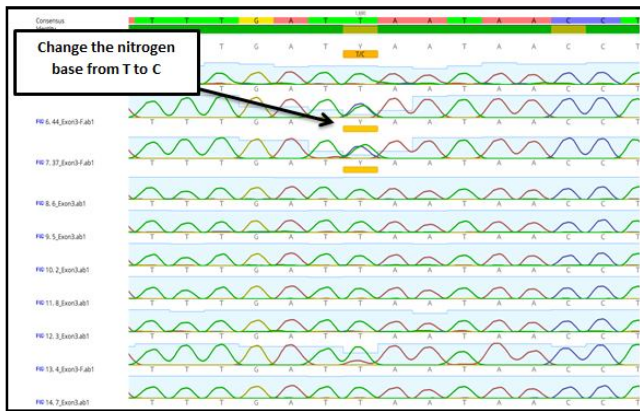
**Effect of genotype in the segment studied of rs446647692 T/C heterogeneity in the sample of the Holstein cattle and its relation to certain reproductive physiological traits:**

Table 4 shows the effect of genetic makeup On the reproductive characteristics of the Holstein-Friesian cows for heterogeneity site rs446647692 T/C The analysis shows that there are two genotypes within this site, with 46 cows carrying the TT genotype, while the cows carrying the TC gene were 4 cows of the total experimental animals. Table 4 shows no statistically significant differences between the genotypes of some phylogenetic traits including the number of vaccinations, open days (days of rest), length of period between births and length of pregnancy, but differences may be considered to be of economic benefit Livestock breeding cattle milk.

The results from Table 4 showed no significant differences between the TT genotype and the TC genotype in the number of vaccinations for heterogeneity

site rs446647692 T/C from the HSP90AA1 heat shock gene for the studied sample. The averages were respectively for each of the two genotypes TT, TC (2.304 ± 0.17), (2.00 ± 0.40).

As for the number of rest days (open days) the average value for each hereditary structure was TT, TC estimated (70.434 ± 5.89), (61.5 ± 5.87), respectively. The genetic makeup TC recorded a period of days of rest (open days) with fewer days of rest in the genotype TT and a difference of 9 days ,This difference may lead to the superiority of the TC genotype from the



**Fig. 3:** The heterogeneity of the nitrogen bases at the heterogeneity site of rs446647692 T / C of the exon3 component of the HSP90AA1 heat shock gene is determined for a sample of Holstein-Friesian cows.

TT structure at the rate of normal reproductive system recovery faster than the other structure and the differences were not statistically significant. In terms of the length of the period between the two births, TT, TC recorded an average of (344.43 ± 5.64), (337.5 ± 6.74) respectively for both structures and this also shows the superiority of the TC genotype with a difference of days less than seven days of the TT genotype. The results of this study were in terms of the lack of significance with the findings of the researcher Shahid (2011) in his study

**Table 3:** Number and percentage of heat shock gene HSP90AA1 according to the location of the first variance rs446647692 T / C in the sample of cows studied.

(Genotype)	Number of samples	(%) percentage
TT	46	0.92
TC	4	0.08
CC	0	0.00
Total	50	% 100
(χ <sup>2</sup> ) Values Kay square	---	119.92 **
Allelic repetition	17.64 89.78	
T	0.96	
C	0.04	
** (P<0.01).		

**Table 4:** Genetic structure of the first heterogeneity site rs446647692 T / C and its effect on the reproductive characteristics of a sample of Holstein Friesian cows.

Trait Structure Genetic	N	SPC	DAYA OPEN	CI	PREGN%
TT	46	2.304 ± 0.17	70.434 ± 5.89	344.43 ± 5.64	274.0 ± 0.86
TC	4	2.00 ± 0.40	61.5 ± 5.87	337.5 ± 6.74	276.0 ± 3.41

N : Number of samples . SPC\* Number of times Fertilization . CI\* Calving interval.

on the same gene for a different piece included Exon 8, 9, 10 to the absence of significant differences between the genotypes in terms of the length of the period between two births, The explanation of the simple difference in mean numbers between genotypes is that the genotype TT, Requires a number of vaccinations required for fertilization rather than genetic makeup TC In terms of speed of fertilization This gives the genetic makeup TC preference in terms of election on the basis of the lowest number of vaccinations, Which was shown to have an effect on the number of rest days (open days) and the length of the period between two births , As for the length of gestation, there were no significant differences between the two structures, with the mean TT/TC, respectively (274.0 ± 0.86), (276.0 ± 3.41), where the results were fairly similar between the two structures.

**Effect of genotype on the studied plot of rs446647692 T / C heterogeneity in the sample of the Holstein cows and their relation to certain production traits:**

Table 5 shows the effect of structure genetic On the productive characteristics of the Holstein-Friesian cows for the heterogeneity site rs446647692 T/C The results showed no statistically significant differences between the genotypes within this site within the daily milk production rate. The averages for both the TT and the TC were measured respectively (13.95 ± 0.50), (14.65 ± 1.49).

On the one hand, the average milk yield was 305 days (4247.15 ± 153.86) The TC genotype has a mean value (4470.3 ± 455.8). The TC structure is superior to the TT structure by 223.15 kg in this capacity,

The genotypes did not differ in terms of peak production, However, the milk production status during the first three months was the average value of the genetic structure TT was (1355.6 ± 61.15)kg, The average genetic structure TC for this characteristic was recorded at a value of (1395.0 ± 359.0) kg, The differences between the two structures were not statistically significant, but

the genetic makeup of the TC surpassed the structure TT by 40 kg for this attribute and this is due to a good economic difference for high-energy milk production projects.

The milk production status of the last three months has exceeded the genetic structure of TT on the genetic makeup TC with a difference of 66 kg of milk, The results of the analysis recorded mean values for both TT and TC values (1119.4 ± 46.77), (1053.7 ± 153.01), respectively. The difference between the two structures was 65 kg

**Table 5:** The genetic structure of the heterogeneity site of rs446647692 T / C of Exon3 thermal shock gene and its effect on some of the productive characteristics of a sample of Holstein-Friesian cows.

Trait Structure genetic	N	DMY (kg)	Milk Top	Milk TOP(kg)	Time to Top(day)	Milk A3(kg)	MILK B3(kg)	Persist %	LP/day
TT	46	13.95±0.50	4247.15± 153.86	511.17± 22.46	47.54± 2.81	1355.6± 61.15	1119.4± 46.77	0.84± 0.03	259.52± 9.42
TC	4	14.65±1.49	4470.3±455.8	502.5± 104.1	41.25± 2.95	1395.0± 359.0	1053.7± 153.01	0.85± 0.155	248.00± 33.90

N\* Number of samples . DMY\*Daily Milk Yield/kg . Milk Top\*Milk production rate of 305 days . Milk Top\*Milk production peak/kg . Time to top\*The period from birth to the top of the production/day . Milk A3\* Milk production 3 months first /kg . Milk B3\* Milk production last 3 months/kg . Persist\*perseverance on production . LP\*Length of production season.

for this characteristic and the differences were not statistically significant. As for the persistence of production, there were no significant differences between the two structures. However, the length of the production season for both genotypes showed the average TT and TC genotypes respectively ( $259.52 \pm 9.42$ ), ( $248.00 \pm 33.90$ ), Where the TT gene structure exceeds the TC structure by 11 days more than the TC genotype and this may increase the economic return of the project . Direct markers identified with milk production and the length of the milking season in Holstein's breeding programs can lead to faster genetic progression in order to increase milk production.

Note that the multi-aspect relationship of the Exon3 site to the HSP90AA1 Heat shock gene is studied With milk production characteristics not previously studied in the Holstein Frisian cows in the Iraqi environment and were limited in other countries to total production only.

#### **Effect of genotype on the studied plot of the first heterogeneity of rs446647692 T / C in the Holstein cow sample and its relation to thermal tolerance coefficient:**

Table 6 shows the relationship between the genetic compositions and the thermal tolerance coefficient of HTC for warm, temperate and cold seasons for the early morning period when the temperature is low as well as in the evening when the temperature is high during the day. The average value of the HTC heat tolerance coefficient for TT and TC was ( $2.59 \pm 0.02$ ), ( $2.73 \pm 0.06$ )

respectively, In the early morning in the summer when temperatures are low at the beginning of the day as there is a difference in the value of HTC between the two genotypes. As the genetic register TT coefficient of thermal tolerance less than the genetic structure TC This indicates that the rate of respiration in the cows with the TT genotype is lower than the rate of respiration in cattle with TC. In the evening, when the temperature reaches the maximum temperature in the middle of the day (summer peak) in the summer, where the average values recorded for the two genotypes TT/TC, ( $2.73 \pm 0.05$ ), ( $2.91 \pm 0.05$ ) respectively, The heat tolerance coefficient of the TT genotype is low. This indicates a low respiratory rate for the cows carrying this structure. Therefore, the decrease in HTC may indicate an improvement in the thermal tolerance characteristic, which may be useful for the genetic improvement of heat tolerances in the Friesian Holstein cattle. Iraqi airspace. As for the moderate and cold seasons of the morning and evening, there were no significant differences to the degree of heat tolerance coefficient HTC Where the mean separation rates for the two genotypes TT and TC For heterogeneity rs446647692 in the early morning ( $2.06 \pm 0.02$ ), ( $1.95 \pm 0.06$ ) respectively, In the evening, when the temperature reached its peak, the averages were for the two genotypes TT and TC ( $2.73 \pm 0.05$ ) respectively. Winter averages also recorded average morning values as they reached their value ( $1.85 \pm 0.01$ ), ( $1.87 \pm 0.04$ ) for the genotype TT and TC respectively, There were no differences as the results came close, and the values of

**Table 6:** The genotypes of the first heterogeneity site rs446647692 T / C of Exon3 heat shock gene and its effect on the thermal tolerance coefficient of a sample of Holstein Friesian cows.

Trait Structure genetic	N	HTC SUM1/AM	HTC AUT1/AM	HTC WINT1/AM	HTC SUM2/PM	HTC AUT2/PM	HTC WINT2/PM
TT	46	2.59±0.02	2.06±0.02	1.85±0.01	2.73±0.05	2.48±0.02	2.01±0.01
TC	4	2.73±0.06	1.95±0.06	1.87±0.04	2.91±0.05	2.51±0.06	2.06±0.07

N\* Number of samples . HTC SUM1/AM \* Heat Tolerance Coefficient/ Summer morning . HTC AUT1/AM\* Heat Tolerance Coefficient/ Moderate morning . HTCWINT1/AM\* Heat Tolerance Coefficient/ Winter mornings. HTC SUM2/PM\* Heat Tolerance Coefficient/ Summer evening . HTC AUT2/PM\* Heat Tolerance Coefficient/ Moderate evening . HTCWINT2/PM\* Heat Tolerance Coefficient/ Winter evening

the averages in the evening at high temperature were the averages for the genotypes TT and TC ( $2.01 \pm 0.01$ ), ( $2.06 \pm 0.07$ ), Respectively and notes the low heat tolerance coefficient HTC For the genetic structure of TT from the other genotype. This slight difference in the coefficient of simple thermal endurance in winter and moderate season may be due to differences in humidity, leading to an increase or decrease in respiratory rate. We conclude from this that the genotype TT During the summer in the early morning when the temperature is low during the day and during the evening when temperatures reach its peak record the lowest value of thermal tolerance coefficient may indicate the extent of suitability of the cows carrying this installation within the site of the first heterogeneity of temperatures and adapting to the surrounding environmental conditions.

### References

- Benezra, M.V. (1954). A new index for measures the adaptability of cattle to tropical condition. *Proc. J. Anim. Sci.*, **13**: 1015.
- Chen, B., D. Zhong and A. Monteiro (2006). Comparative genomics and evolution of the Hsp90 family of genes across all kingdoms of organisms. *BMC. Genomics*, **7**: 156-167.
- Das, R., L. Sailo, N. Verma, P. Bharti, J. Saikia, Imtiwati and R. Kumar (2016). Impact of heat stress on health and performance of dairy animals: A review, *Veterinary World*, **9(3)**: 260-268.
- FAO (2009). The state of food and agriculture. Rome, Italy, pp. 1-180.
- Sejian, V., J.B. Gaughan, R. Bhatta and S.M.K. Naqvi (2016). Impact of climate change on livestock productivity. Feedipedia-Animal Feed Resources Information System - INRA CIRAD AFZ and FAO, 1-4.
- Liu, Y.X., X. Zhou, D.Q. Li, Q.W. Cui and G.L. Wang (2010). Association of ATP1A1 gene polymorphism with heat tolerance traits in dairy cattle. *Genetics and Molecular Research*, **9**: 891-896.
- Lund, P.A. (2001). *Molecular chaperones in the cell*. Oxford University Press, Oxford.
- Mader, T.L., M.S. Davis and T. Brown-Brandl (2006). Environmental factors influencing heat stress in feedlot cattle. *J. Anim. Sci.*, **84**: 712-719.
- Shahid, A.S. (2011). Molecular Genetic Characterization of HSP90 gene in Deoni (*Bos indicus*) cattle. Dairy roduction section southern campus national dairy research institute (I.C.A.R.) Adugodi, Bangalore- 560030, India.
- Stott, G.H. (1981). What is animal stress and how is it measured. *J. Anim. Sci.*, **52**: 150-153.
- Young, J.C., I. Moarefi, F.U. Hartl (2001). Hsp90: a specialist but essential protein-folding tool. *J. Cell Biol.*, **154**: 267-273.
- Yousef, M.K. (1985). *Stress Physiology in Livestock*. Vol. 1. CRC Press, Boca Raton. 67-73.