MOLECULAR DETECTION OF POLYMORPHISM OF HEAT SHOCK PROTEIN 70 (*hsp70*) IN THE SEMEN OF ARABI RAMS

Hassan Nima Habib[*], Bassam Yasein Khudaier[**] ,Amad Falah Hassan[*]

* College of Agriculture, University of Basrah, Basra, Iraq

** College of Veterinary, Medicine University of Basrah, Basrah, Iraq

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Corresponding Author: fllan8@yahoo.com

ABSTRACT

Heat shock proteins Hsp70 proteins belong to the heat shock protein family, boost cells survival in hostile environments, involved in many cellular biological processes. The polymorphism of *hsp70* gene relates to the possibility of tolerating different stress conditions. This study was carried out during 1st April to 31th June 2016, 20 Arabi Rams, known fertility, 2 – 2.5 years old, from the Agricultural Research Station / College of Agriculture / University of Basra, Iraq, were used, Semen was collected by using the artificial vagina, after training well. DNA extracted then Polymerase chain reaction(PCR) amplified sequencing, BLAST analysis and multiple sequence alignment were carried out. The results showed two haplotypes, haplotype G1 , haplotype G٢ compared with same gene in GenBank, all mutations were silent except the mutation is site 514 was missense appeared in G1. It concluded that the Arabi rams in Iraq have new polymorphism of *hsp70* gene which can help cope with difficult environmental conditions.

INTRODUCTION

Heat shock protein (HSPs) are a highly preserved polypeptides, lodging almost in any cell, they were classified according to their molecular weights, one of their most important roles is as like molecular chaperones and are decisively Participate in maintaining protein homeostasis and cell survival (1). Hsp70 is a member of HSPs family, which are spread in all living organisms, whether eukaryotic or prokaryotic (2,3), which in addition to working as molecular chaperones, have immune functions(4,5), but its main role is folding the proteins and prevent the improper assembly of proteins (6). Hsp70 has a significant role in protecting cells against cellular stress including heat stress

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(7), by promoting cells tolerance to environmental changes and pathologic conditions (8). The work of the Hsp70 can be summarized in three main axes in all organisms, prevent of ingathering of proteins, corrected folded proteins that are exposed to misfolded and dissolve and refolding of aggregated proteins under the stress conditions (9). These functions of HSP70 as a molecular chaperone and cell protect versus heat stress, making it able to denaturing proteins (10). On the other hand many studies have indicated a relationship between the polymorphism of *hsp70* gene and heat tolerance in Holstein cow (11), chicken (12), in buffalo (13) and in sheep (14).The polymorphism of *hsp70* gene also affects reproductive performance of Farm Animal males, by reducing the various stress conditions on the animals (15,16). Therefore, the *hsp70* gene is an ideal biological marker to resist heat stress in farm animals (17). No previous study in Iraq has been conducted on the polymorphism of *hsp70* gene in the semen of Arabi Rams in Iraq, except for one study on the polymorphism of the *hsp70* gene in Iraqi Holstein Bulls by (18), therefore no information about the polymorphism of *hsp70* gene in Arabi Rams. This study aimed to identify the polymorphism of *hsp70* gene in Arabi Rams.

MATERIALS AND METHODS

Animals and semen collection

Twenty Arabi Rams, known fertility, 2 – 2.5 years old, from the Agricultural Research Station / College of Agriculture / University of Basra, Iraq, were used in the present study. During 1st April to 31th June 2016. Semen collected from Rams by using the artificial vagina method, specialized artificial vagina for sheep (after training well).

DNA extraction

DNA was extracted from semen samples using Chelex (Sigma Aldrich, USA) as described by (19). Concentration and purity of DNA was estimated by using Nanodrop system (Nano Drop thermo scientific 200, USA). 1.8 was adopted as the best purity of 260/280 ratio (20)

PCR amplification and Sequencing

Amplifications were performed according to First BASE Laboratories / Malaysia, in 25 μl reactions (Table 1), to amplify conserved region within the *hsp70* promotor ,primer was used as in table 2 , the PCR amplification cycling protocols of PCR amplification as in table 3. PCR product was detected on 1.5 % ethidium bromide stained agarose gel, wih size 1926pb approximately (Fig 1), as described (18).

PCR products Purified and sequenced in First BASE Laboratories / Malaysia . (www.base-asia.com) . Nucleotides sequence alignments, were done using Geneious version 10.1.3 software. BLAST Analysis was carried out on website (www.ncbi.nlm.nih.gov.) Multiple Sequence Alignment and compared with same gene in

Gene Bank, accession number NM_001267874.1, were   carried out on website (http://www.ebi.ac.uk/Tools/msa/clustalo/.).

(1)

First BASE Laboratories (2016)	
Components	**Amount (µl)**
Water, nuclease free	9.5
2X PCR Master Mix	12.5
Forward primer, 10 µM	1
Reverse primer, 10 µM	1
DNA template (75 ng)	1
TOTAL	25

Table PCR

Reaction Components

Table (2) primer

First BASE Laboratories (2016)			
Cycle step	Temp (° C)	Time	Number of Cycles
Initial Denaturation	95	5 min	1
Denaturation	94	30 s	
Annealing	61	30 s	30
Extension	72	2 min	
Final Extension	72	10 min	1

Table (3) Thermal Cycling Protocol of PCR

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Primer pair	Hsp70-F forward	ATGGCGAAAAACATGGCTATCGGC
	Hsp70-R reverse	CTAATCCACCTCCTCAATGGTGGGGCC
		PCR product size = 1926 bp
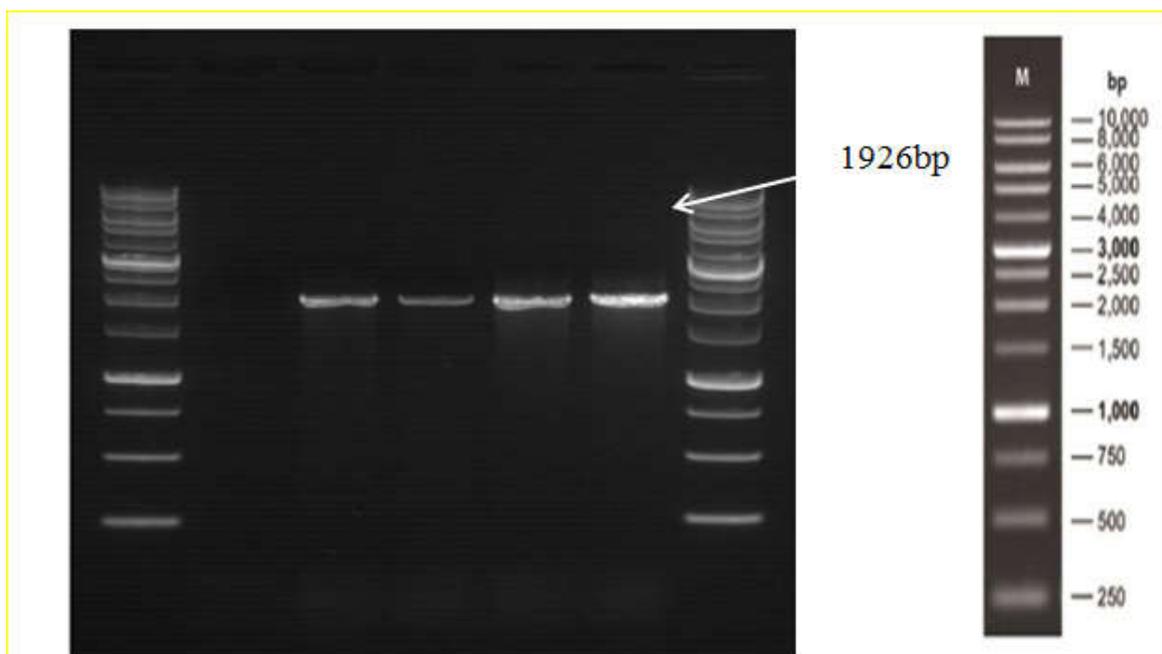
Figure 1: "-ve" is no template, control (water)

1 , 2 , 3 , 4 : DNA template *hsp70* gene in semen sheep

M : DNA Marker with size 1000

total 75 ng of DNA sample was used, in 25 ul PCR reaction. Only 3 ul of PCR product was run on 1% TAE agarose gel at 100V, 60 min. (18).

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RESULTS AND DISCUSSION

The results of nucleotides sequence analysis and the Multiple Sequence Alignment (MSA) compared with heat shock protein *hsp70* gene of sheep in the gene bank, suggest a presence two new haplotypes (Figure2) , classified as two groups :

First group : haplotype A : showed no match at 100% with other nucleotides sequence of other group or in Gene Bank heat shock protein *hsp70* gene, the nucleotide in position 1308, T was substituted by the nucleotide C (T <C), a silent mutation that did not encoding a new amino acid, 10 rams were found in this group.

Second group: haplotype B : in comparison with the first group and the same gene in Gene Bank, the nucleotide C was substituted with the nucleotide G (C <G), a missense mutation that resulted in a change in the amino acid encoding (Histidine change to Aspartic) in the position 514, a mutation not previously recorded in sheep except in Chinese sheep by (21) , registered as a patent with ID CN104745552A but did not match the results of the current study. In addition, nucleotide G was substituted by nucleotide A (G <A) in the position 1524, a silent mutation, 10 rams were found in this group.

The (MSA) of *hsp70* gene showed that the ratio of A and B haplotypes were 99.89% and 99.84% respectively with the same gene in the Gene Bank, accession number NM_001267874.1 (22), these results are consistent with Pawar *et al.,* (23) and Banerjee *et al.,* (24) in the possibility of polymorphisms of *hsp70* gene in sheep, but the results are not consistent with any previous study in the position of mutations, and may be due to the fact that the current study is the first of its kind and was not preceded by any other study.

These mutations in *hsp70* gene may refers to the development of this protein through the positive election among domestic animals (25).

On the other hand the silent mutations may effect on the role of translated protein through diverse cellular mechanisms (26), and it lead to polymorphism to the *hsp70* gene, that's associated with the semen quality (27).

As for the missense mutation that occurred in haplotype B due to the change of Histidine amino acid to the Aspartic, it may improve the quality of semen (28, 29 , 30).

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NM_001267874.1    ATGGCGAAAAACATGGCTATCGGCATCGACCTGGGCACCACCTACTCCTGCGTGGGGGTG          60
G1                ------------------------------------------TACTCCTGCGTGGGGGTG          18
G2                ------------------------------------------TACTCCTGCGTGGGGGTG          18
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NM_001267874.1    TTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCCAGC         120
G1                TTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCCAGC          78
G2                TTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCCAGC          78
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NM_001267874.1    TACGTGGCCTTCACCGATACCGAGCGGCTCATCGGCGATGCAGCCAAGAACCAGGTGGCG         180
G1                TACGTGGCCTTCACCGATACCGAGCGGCTCATCGGCGATGCAGCCAAGAACCAGGTGGCG         138
G2                TACGTGGCCTTCACCGATACCGAGCGGCTCATCGGCGATGCAGCCAAGAACCAGGTGGCG         138
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NM_001267874.1    CTGAACCCGCAGAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGAC         240
G1                CTGAACCCGCAGAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGAC         198
G2                CTGAACCCGCAGAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGAC         198
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NM_001267874.1    CCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTTCCGCGTGATCAACGACGGAGACAAG         300
G1                CCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTTCCGCGTGATCAACGACGGAGACAAG         258
G2                CCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTTCCGCGTGATCAACGACGGAGACAAG         258
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NM_001267874.1    CCTAAAGTGCAGGTGAGCTACAAGGGGGAGACCAAGGCGTTCTACCCAGAGGAGATCTCG         360
G1                CCTAAAGTGCAGGTGAGCTACAAGGGGGAGACCAAGGCGTTCTACCCAGAGGAGATCTCG         318
G2                CCTAAAGTGCAGGTGAGCTACAAGGGGGAGACCAAGGCGTTCTACCCAGAGGAGATCTCG         318
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NM_001267874.1    TCGATGGTGCTGACCAAGATGAAAGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACC         420
G1                TCGATGGTGCTGACCAAGATGAAAGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACC         378
G2                TCGATGGTGCTGACCAAGATGAAAGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACC         378
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NM_001267874.1    AACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGAC         480
G1                AACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGAC         438
G2                AACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGAC         438
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NM_001267874.1    GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCC         540
G1                GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCC         498
G2                GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCC         498
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NM_001267874.1    ATCGCCTACGGCCTGCACCGGACGGGCAAGGGGGAGCGCAACGTGCTCATCTTTGACCTG         600
G1                ATCGCCTACGGCCTGCACCGGACGGGCAAGGGGGAGCGCAACGTGCTCATCTTTGACCTG         558
G2                ATCGCCTACGGCCTGGACCGGACGGGCAAGGGGGAGCGCAACGTGCTCATCTTTGACCTG         558
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NM_001267874.1    GGCGGGGGCACGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAG         660
G1                GGCGGGGGCACGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAG         618
G2                GGCGGGGGCACGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAG         618
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NM_001267874.1    GCCACGGCCGGGGACACGCACCTGGGCGGGGAGGACTTCGACAACAGGCTGGTGAACCAC         720
G1                GCCACGGCCGGGGACACGCACCTGGGCGGGGAGGACTTCGACAACAGGCTGGTGAACCAC         678
G2                GCCACGGCCGGGGACACGCACCTGGGCGGGGAGGACTTCGACAACAGGCTGGTGAACCAC         678
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NM_001267874.1    TTCGTGGAGGAGTTCAAGAGGAAGCACAAGAAGGACATCAGCCAGAACAAGCGGGCCGTG         780
G1                TTCGTGGAGGAGTTCAAGAGGAAGCACAAGAAGGACATCAGCCAGAACAAGCGGGCCGTG         738
G2                TTCGTGGAGGAGTTCAAGAGGAAGCACAAGAAGGACATCAGCCAGAACAAGCGGGCCGTG         738
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NM_001267874.1    AGGCGGCTGCGCACGGCGTGCGAGCGGGCCAAGAGGACCTTGTCGTCCAGCACCCAGGCC         840
G1                AGGCGGCTGCGCACGGCGTGCGAGCGGGCCAAGAGGACCTTGTCGTCCAGCACCCAGGCC         798
G2                AGGCGGCTGCGCACGGCGTGCGAGCGGGCCAAGAGGACCTTGTCGTCCAGCACCCAGGCC         798
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NM 001267874.1    AGCCTGGAGATCGACTCCCTGTTCGAGGGCATCGACTTCTACACGTCCATCACCAGGGCG    900
G1                AGCCTGGAGATCGACTCCCTGTTCGAGGGCATCGACTTCTACACGTCCATCACCAGGGCA    858
G2                AGCCTGGAGATCGACTCCCTGTTCGAGGGCATCGACTTCTACACGTCCATCACCAGGGCA    858
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NM_001267874.1    CGGTTCGAGGAGCTGTGCTCCGACCTGTTCCGGAGCACCCTGGAGCCGGTGGAGAAGGCT    960
G1                CGGTTCGAGGAGCTGTGCTCCGACCTGTTCCGGAGCACCCTGGAGCCGGTGGAGAAGGCT    918
G2                CGGTTCGAGGAGCTGTGCTCCGACCTGTTCCGGAGCACCCTGGAGCCGGTGGAGAAGGCT    918
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NM_001267874.1    CTACGCGACGCCAAGCTGGACAAGGCCCAGATCCACGACCTGGTCCTGGTGGGGGGCTCC    1020
G1                CTACGCGACGCCAAGCTGGACAAGGCCCAGATCCACGACCTGGTCCTGGTGGGGGGCTCC    978
G2                CTACGCGACGCCAAGCTGGACAAGGCCCAGATCCACGACCTGGTCCTGGTGGGGGGCTCC    978
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NM 001267874.1    ACCCGCATCCCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTCAAC    1080
G1                ACCCGCATCCCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTCAAC    1038
G2                ACCCGCATCCCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTCAAC    1038
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NM 001267874.1    AAGAGCATCAACCCGGACGAGGCGGTGGCATACGGGGCGGCGGTGCAGGCGGCCATCCTG    1140
G1                AAGAGCATCAACCCGGACGAGGCGGTGGCATACGGGGCGGCGGTGCAGGCGGCCATCCTG    1098
G2                AAGAGCATCAACCCGGACGAGGCGGTGGCATACGGGGCGGCGGTGCAGGCGGCCATCCTG    1098
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NM 001267874.1    ATGGGGGACAAGTCGGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGCTCCCCTGTCG    1200
G1                ATGGGGGACAAGTCGGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGCTCCCCTGTCG    1158
G2                ATGGGGGACAAGTCGGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGCTCCCCTGTCG    1158
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NM 001267874.1    CTGGGACTGGAGACGGCCGGAGGCGTGATGACCGCCCTGATCAAGCGCAACTCCACCATC    1260
G1                CTGGGACTGGAGACGGCCGGAGGCGTGATGACCGCCCTGATCAAGCGCAACTCCACCATC    1218
G2                CTGGGACTGGAGACGGCCGGAGGCGTGATGACCGCCCTGATCAAGCGCAACTCCACCATC    1218
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NM 001267874.1    CCCACGAAGCAGACGCAGATCTTCACCACCTACTCGGACAACCAGCCGGGCGTGCTGATC    1320
G1                CCCACGAAGCAGACGCAGATCTTCACCACCTACTCGGACAACCAGCCGGGCGTGCTGATC    1278
G2                CCCACGAAGCAGACGCAGATCTTCACCACCTACTCGGACAACCAGCCGGGCGTGCTGATC    1278
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NM_001267874.1    CAGGTGTACGAGGGCGAGAGGGCCATGACTCGGGACAACAACCTGCTGGGGCGCTTCGAG    1380
G1                CAGGTGTACGAGGGCGAGAGGGCCATGACCCGGGACAACAACCTGCTGGGGCGCTTCGAG    1338
G2                CAGGTGTACGAGGGCGAGAGGGCCATGACTCGGGACAACAACCTGCTGGGGCGCTTCGAG    1338
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NM_001267874.1    CTGAGCGGCATCCCGCCGGCCCCGCGGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC    1440
G1                CTGAGCGGCATCCCGCCGGCCCCGCGGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC    1398
G2                CTGAGCGGCATCCCGCCGGCCCCGCGGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC    1398
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NM_001267874.1    GACGCCAATGGCATCCTGAACGTCACGGCCACGGACAAGAGCACGGGCAAGGCCAACAAG    1500
G1                GACGCCAATGGCATCCTGAACGTCACGGCCACGGACAAGAGCACGGGCAAGGCCAACAAG    1458
G2                GACGCCAATGGCATCCTGAACGTCACGGCCACGGACAAGAGCACGGGCAAGGCCAACAAG    1458
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NM_001267874.1    ATCACCATCACCAACGACAAGGGCCGGCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAG    1560
G1                ATCACCATCACCAACGACAAGGGCCGGCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAG    1518
G2                ATCACCATCACCAACGACAAGGGCCGGCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAG    1518
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NM 001267874.1    GAGGCGGAGAAGTACAAGGCAGAGGACGAGGTCCAGCGCGAGAGGGTGTCTGCCAAGAAC    1620
G1                GAGGCGGAGAAGTACAAGGCAGAGGACGAGGTCCAGCGCGAGAGGGTGTCTGCCAAGAAC    1578
G2                GAGGCAGAGAAGTACAAGGCAGAGGACGAGGTCCAGCGCGAGAGGGTGTCTGCCAAGAAC    1578

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NM 001267874.1    GCGCTGGAGTCGTACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGGGCTGAAGGGC    1680
G1                GCGCTGGAGTCGTACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGGGCTGAAGGGC    1638
G2                GCGCTGGAGTCGTACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGGGCTGAAGGGC    1638
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NM_001267874.1    AAGATCAGCGAGGCGGACAAGAAGAAGGTGCTGGACAAGTGCCAGGAGGTGATTTCCTGG    1740
G1                AAGATCAGCGAGGCGGACAAGAAGAAGGTGCTGGACAAGTGCCAGGAGGTGATTTCCTGG    1698
G2                AAGATCAGCGAGGCGGACAAGAAGAAGGTGCTGGACAAGTGCCAGGAGGTGATTTCCTGG    1698
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NM_001267874.1    CTGGACGCCAACACCTTGGCGGAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAG    1800
G1                CTGGACGCCAACACCTTGGCGGAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAG    1758
G2                CTGGACGCCAACACCTTGGCGGAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAG    1758
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NM_001267874.1    CAGGTGTGTAACCCCATCATCAGCAGACTGTACCAGGGGGCGGGCGGCCCCGGGGCTGGC    1860
G1                CAGGTGTGTAACCCCATCATCAGCAGACTGTACCAGGGGGCGGGCGGCCCCGGGGCTGGC    1818
G2                CAGGTGTGTAACCCCATCATCAGCAGACTGTACCAGGGGGCGGGCGGCCCCGGGGCTGGC    1818
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NM_001267874.1    GGCTTTGGGGCTCAGGCCCCTAAAGGGGGCTCTGGGTCTGGCCCCACCATTGAGGAGGTG    1920
G1                GGCTT-------------------------------------------------------    1823
G2                GGCTT-------------------------------------------------------    1823
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NM_001267874.1    GATTAG  1926
G1                ------  1823
G2                ------  1823
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G1: Haplotype A
G2: Haplotype B
NM_001267874.1 : gene of *hsp70* in GenBank

Fig. 2: Identified *hsp70* gene in Arabi rams and the same gene in GenBank CLUSTAL Omega (1.2.4) multiple sequence alignment

CONCLUSION

This study was showed two haplotypes of *hsp70* gene in the Arabi rams in Iraq, due to the occurrence of mutations in more than one location, suggesting that genotypes of *hsp*70 could have been genetically modified to adapt to environmental conditions.

ACKNOWLEDGMENTS

REFERENCES

1- **Sharma, D. and Masison, D.C. (2009**). Hsp70 structure, function, regulation and influence on yeast prions. Protein Pept. Lett.16, 571-581.

2- **Sharma, D. ; Martineau, C. N. ; Le Dall, M. T. ; Reidy, M. ; Masison, D. C. and Kabani, M. (2009).** Function of SSA subfamily of Hsp70 within and across species varies widely in complementing Saccharomyces cerevisiae cell growth and prion propagation. PloS one, *4*(8): e6644.

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3- **Kampinga, H.H. and Craig, E.A. (2010).**The HSP70 chaperone machinery: J proteins as drivers of functional specificity. Nat. Rev. Mol. Cell Biol.11(8): 579-592.

4- **Pockley, A.G. and Multhoff,G. (2008).** Cell stress proteins in extracellular fluids: friend or foe? Novartis. Found. Symp.291: 86-95.

5- **Pockley, A.G. ; Muthana, M. and Calderwood, S.K. (2008).** The dual immunoregulatory roles of stress proteins. Trends Biochem. Sci., 33:71-79.

6- **Park, S. H. ; Bolender, N. ; Eisele, F. ; Kostova, Z. ; Takeuchi, J. ; Coffino, P. and Wolf, D. H. (2007).** The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin–proteasome system. Molecular biology of the cell, 18(1): 153-165.

7- **Evdonin, A.L. ; Guzhova, I.V. ; Margulis, B.A. and Medvedeva, N.D. (2006).** Extracellular heat shock protein 70 mediates heat stress-induced epidermal growth factor receptor transactivation in A431 carcinoma cells. FEBS Lett. 580(28): 6674-6678.

8- **Njemini, R. ; Bautmans, I. ; Lambert, M. ; Demanet, C. and Mets, T. (2007).** Heat shock proteins and chemokine/cytokine secretion profile in ageing and inflammation. Mechanisms of ageing and development, 128(7): 450-454.

9- **Mayer, M.P. and Bukau, B. (2006).** Hsp70 chaperones: Cellular functions and molecular mechanism. Cell Mol Life Sci 62(6): 670-684.

10- **Bhat, S. ; Kumar, P. ; Kashyap, N. ; Deshmukh, B. ; Dige, M.S. ; Bhushan, B. ; Chauhan, A. ; Kumar, A. and Singh, G. (2016).** Effect of heat shock protein 70 polymorphism on thermotolerance in Tharparkar cattle. Veterinary World, 9(2): 113-117.

11- **Basirico, L. ; Morera, P. ; Primi, V. ; Lacetera, N. ; Nardone, A. and Bernabucci, U.(2011).** Cellular thermotolerance is associated with heat shock protein 70.1 genetic polymorphisms in Holstein lactating cows. Cell Stress and Chaperones,16(4): 441–448.

12- **Tamzil, M.H. ; Noor, R.R. ; Hardjosworo P.S. ; Manalu, W. and Sumantri, C. (2013).** Acute HS responses of three lines of chickens with different heat shock protein (hsp)-70 genotypes. Int J Poult Sci 12(5): 264-272.

13- **Gafer, J.A. ; Ghada H.A. and Rawash Z.M.(2015).** Association of Hsp70 Gene Polymorphism and Bull Semen Quality in Winter and Summer Seasons. Alexandria Journal of Veterinary Sciences,46 : 1-14. doi: 10.5455/ajvs.186038.

14- **Singh, K. M. ; Singh, S. ; Ganguly, I. ; Nachiappan, R. K. ; Ganguly, A. ; Venkataramanan, R. ... and Narula, H. K. (2017).** Association of heat stress protein 90 and 70 gene polymorphism with adaptability traits in Indian sheep (Ovis aries). Cell stress & chaperones.

15- **Rosenkrans, J.C. ; Banks, A. ; Reiter, S. and Looper, M. (2010) .** Calving traits of crossbred brahman cows are associated with heat shock protein 70genetic polymorphisms. Anim. Reprod., Sci., 119(3): 178–182.

16- **Cushman, R.A. (2013).** Physiology and Endocrinology Symposium: the current status of heat shock in early embryonic survival and reproductive efficiency. J Anim Sci., 91(3):1141-1152. doi: 10.2527/jas.2013-6231.

17- **Archana, P.R. ; Aleena, J. ; Pragna, P. ; Vidya, M.K. ; Niyas, A.P.A. ; Bagath, M. ; Krishnan, G. ; Manimaran, A. ; Beena, V. ; Kurien, E.K. ; Sejian, V. and Bhatta, R.(2017).** Role of Heat Shock Proteins in Livestock Adaptation to Heat Stress. J Dairy Vet Anim Res., 5(1): 00127. doi: 10.15406/jdvar.2017.05.00127.

18- **Habib, H.N. ; Hassan, A.F. and Khudaier, B.Y.(2017).** Molecular Detection of Polymorphism of Heat Shock Protein 70 (hsp70) in the semen of Iraqi Holstein Bulls. Asian J. Anim. Sci., 11(3): 132-139.

19- **Anju, M. ; Sonia, M. ; Balvinder, K. ; Khanna, S. and Sethi, R.K. (2010).** Evaluation of different methods of dna extraction from semen of buffalo (bubalusbubalis) bulls. Buffalo Bulletin Vol.29 No.2.

20- **Silva, E.C.B. ; Pelinca, M.A. ; Acosta, A.C. ; Silva, D.M.F. ; Gomes Filho, M.A. and Guerra, M.M.P. (2014).** Comparative study of DNA extraction methodologies from goat sperm and its effects on polymerase chain reaction analysis. Genetics and Molecular Research, 13 (3): 6070-6078.

21- **Jinshan, Z. ; Lee, J. ; Kaidong, L. ; Nan, L. ; Ming, C. (2015).** A pair of specific recognition sheep hsp70.1 gene peptide and its coding gene and application. Qingdao Institute of Animal Science and Veterinary Medicine. patents/CN104745552A.

22- **Clustal Omega. (2017).** http://www.ebi.ac.uk/ /Tools/msa/clustalo/.

23- **Pawar, N.H. ; Agrawal, R.K. ; Ramneek and Brah, G.S. (2013).** Expression, purification and characterization of recombinant Heat Shock Protein 70 (HSP70) from sheep and goat species. Int.J.Curr.Microbiol.App.Sci., 2(11): 440-452.

24- **Banerjee, D. ; Upadhyay, R.C. ; Chaudhary, U.B. ; Kumar, R. ; ingh, S.V.; Ashutosh, J. M. G. ; Polley, S. ; Mukherjee, A. ; Das, T.K. and De, S. (2014).** Seasonal variation in expression pattern of genes under HSP70 family in heatand coldadapted goats (Capra hircus). Cell Stress Chaperones, 19 (3): 401–408, doi.org/10.1007/s12192-013-0469-0.

25- **Gade, N. ; Mahapatra, R. K. ; Sonawane, A. ; Singh, V. K. ; Doreswamy, R. and Saini, M. (2010).** Molecular Characterization of Heat Shock Protein 70-1 Gene of Goat (Capra hircus). Molecular biology international, vol. 2010. 104061/2010/ 108429.

26- **Hunt, R. C. ; Simhadri, V. L. ; Iandoli, M. ; Sauna, Z. E. and Kimchi-Sarfaty, C. (2014).** Exposing synonymous mutations. Trends in Genetics, 30(7): 308-321.

27- **Nikbin, S. ; Panandam, J. M. ; Yaakub, H. ; Murugaiyah, M. and Sazili, A. Q. (2014).** Novel SNPs in heat shock protein 70 gene and their association with sperm quality traits of Boer goats and Boer crosses. Animal reproduction science, 146(3): 176-181.

28- **Sangeeta, S., Arangasamy, A., Kulkarni, S., & Selvaraju, S. (2015).** Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. Animal reproduction science, 161, 82-88.

29- **Barbato, V., Talevi, R., Braun, S., Merolla, A., Sudhakaran, S., Longobardi, S., & Gualtieri, R. (2017).** Supplementation of sperm media with zinc, D-aspartate and co-enzyme Q10 protects bull sperm against exogenous oxidative stress and improves their ability to support embryo development. Zygote, 25(2), 168-175.

30- **Habib, H.N. ; Hassan, A.F. and Khudaier, B.Y.(2017).** Association of hsp70 gene Polymorphism with some of fresh semen characters in summer and winter to Iraqi Holstein Bulls. I J S R. 78.46