

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

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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 G₂ 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

(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, with 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	
First BASE Laboratories (2016)			
Annealing	61	30 s	
Extension	72	2 min	
Final Extension	72	10 min	1

Table (3) Thermal Cycling Protocol of PCR

Primer pair	Hsp70-F forward	ATGGCGAAAAACATGGCTATCGGC
	Hsp70-R reverse	CTAATCCACCTCCTCAATGGTGGGGCC
		PCR product size = 1926 bp

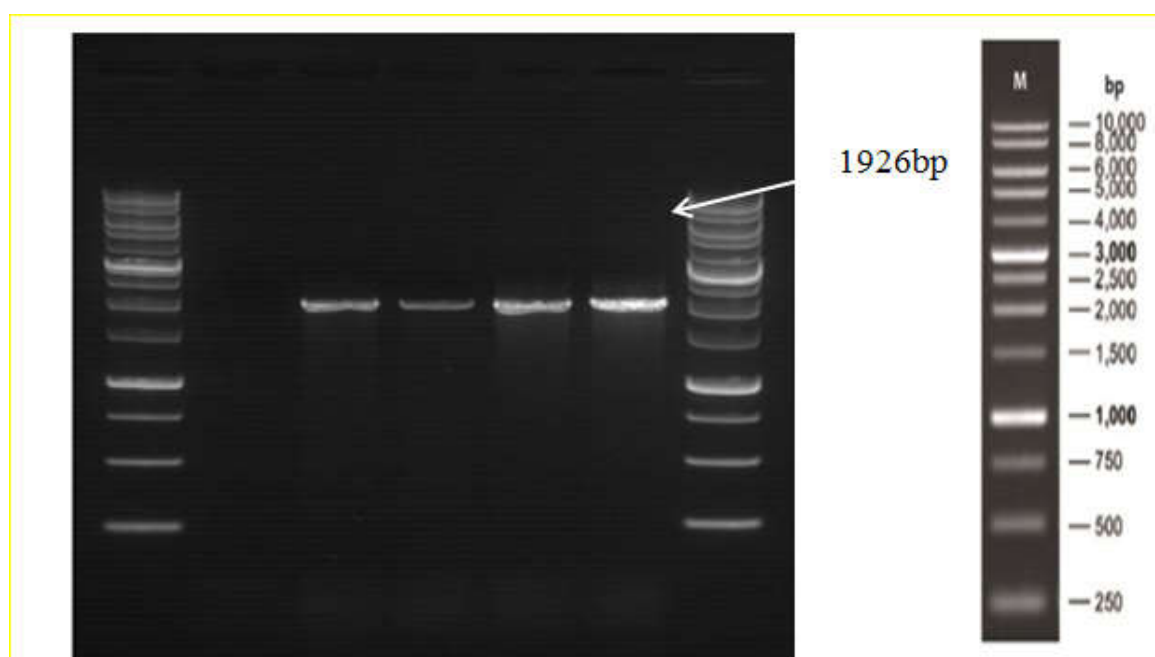


Figure 1: “-ve” is no template, control (water)

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).

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).

NM_001267874.1	ATGGCGAAAAACATGGCTATCGGCATCGACCTGGGCACCACCTACTCCTGCGTGGGGGTG	60
G1	-----TACTCCTGCGTGGGGGTG	18
G2	-----TACTCCTGCGTGGGGGTG	18

NM_001267874.1	TTCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCAGC	120
G1	TTCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCAGC	78
G2	TTCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCAGC	78

NM_001267874.1	TACGTGGCCTTACCCGATACCGAGCGGCTCATCGGCGATGCAGCCAAGAACCAGGTGGCG	180
G1	TACGTGGCCTTACCCGATACCGAGCGGCTCATCGGCGATGCAGCCAAGAACCAGGTGGCG	138
G2	TACGTGGCCTTACCCGATACCGAGCGGCTCATCGGCGATGCAGCCAAGAACCAGGTGGCG	138

NM_001267874.1	CTGAACCCGACAGAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGAC	240
G1	CTGAACCCGACAGAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGAC	198
G2	CTGAACCCGACAGAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGAC	198

NM_001267874.1	CCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTCCCGTGATCAACGACGGAGACAAG	300
G1	CCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTCCCGTGATCAACGACGGAGACAAG	258
G2	CCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTCCCGTGATCAACGACGGAGACAAG	258

NM_001267874.1	CCTAAAGTGCAGGTGAGCTACAAGGGGAGACCAAGGCGTCTACCCAGAGGAGATCTCG	360
G1	CCTAAAGTGCAGGTGAGCTACAAGGGGAGACCAAGGCGTCTACCCAGAGGAGATCTCG	318
G2	CCTAAAGTGCAGGTGAGCTACAAGGGGAGACCAAGGCGTCTACCCAGAGGAGATCTCG	318

NM_001267874.1	TCGATGGTGTGACCAAGATGAAAGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACC	420
G1	TCGATGGTGTGACCAAGATGAAAGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACC	378
G2	TCGATGGTGTGACCAAGATGAAAGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACC	378

NM_001267874.1	AACGCGGTGATCACCGTGCAGGCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGAC	480
G1	AACGCGGTGATCACCGTGCAGGCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGAC	438
G2	AACGCGGTGATCACCGTGCAGGCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGAC	438

NM_001267874.1	GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCACGGCCGCCGCC	540
G1	GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCACGGCCGCCGCC	498
G2	GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCACGGCCGCCGCC	498

NM_001267874.1	ATCGCCTACGGCCTGCACCGGACGGGCAAGGGGAGCGCAACGTGCTCATCTTTGACCTG	600
G1	ATCGCCTACGGCCTGCACCGGACGGGCAAGGGGAGCGCAACGTGCTCATCTTTGACCTG	558
G2	ATCGCCTACGGCCTGCACCGGACGGGCAAGGGGAGCGCAACGTGCTCATCTTTGACCTG	558

NM_001267874.1	GGCGGGGACAGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAG	660
G1	GGCGGGGACAGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAG	618
G2	GGCGGGGACAGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAG	618

NM_001267874.1	GCCACGGCCGGGACACGCACCTGGGCGGGGAGGACTTCGACAACAGGCTGGTGAACCAC	720
G1	GCCACGGCCGGGACACGCACCTGGGCGGGGAGGACTTCGACAACAGGCTGGTGAACCAC	678
G2	GCCACGGCCGGGACACGCACCTGGGCGGGGAGGACTTCGACAACAGGCTGGTGAACCAC	678

NM_001267874.1	TTCGTGGAGGAGTTCAAGAGGAAGCACAAGAAGGACATCAGCCAGAACAAGCGGGCCGTG	780
G1	TTCGTGGAGGAGTTCAAGAGGAAGCACAAGAAGGACATCAGCCAGAACAAGCGGGCCGTG	738
G2	TTCGTGGAGGAGTTCAAGAGGAAGCACAAGAAGGACATCAGCCAGAACAAGCGGGCCGTG	738

NM_001267874.1	AGGCGGCTGCGCACGGCGTGCAGCGGGCCAAGAGGACCTTGTGCTCCAGCACCCAGGCC	840
G1	AGGCGGCTGCGCACGGCGTGCAGCGGGCCAAGAGGACCTTGTGCTCCAGCACCCAGGCC	798
G2	AGGCGGCTGCGCACGGCGTGCAGCGGGCCAAGAGGACCTTGTGCTCCAGCACCCAGGCC	798

NM 001267874.1	AGCCTGGAGATCGACTCCCTGTTTCGAGGGCATCGACTTCTACACGTCCATCACCAGGGCG	900
G1	AGCCTGGAGATCGACTCCCTGTTTCGAGGGCATCGACTTCTACACGTCCATCACCAGGGCA	858
G2	AGCCTGGAGATCGACTCCCTGTTTCGAGGGCATCGACTTCTACACGTCCATCACCAGGGCA	858

NM_001267874.1	CGGTTTCGAGGAGCTGTGCTCCGACCTGTTCCGGAGCACCTGGAGCCGGTGGAGAAGGCT	960
G1	CGGTTTCGAGGAGCTGTGCTCCGACCTGTTCCGGAGCACCTGGAGCCGGTGGAGAAGGCT	918
G2	CGGTTTCGAGGAGCTGTGCTCCGACCTGTTCCGGAGCACCTGGAGCCGGTGGAGAAGGCT	918

NM_001267874.1	CTACGCGACGCCAAGCTGGACAAGGCCAGATCCACGACCTGGTCTGGTGGGGGCTCC	1020
G1	CTACGCGACGCCAAGCTGGACAAGGCCAGATCCACGACCTGGTCTGGTGGGGGCTCC	978
G2	CTACGCGACGCCAAGCTGGACAAGGCCAGATCCACGACCTGGTCTGGTGGGGGCTCC	978

NM 001267874.1	ACCCGCATCCCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTCAAC	1080
G1	ACCCGCATCCCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTCAAC	1038
G2	ACCCGCATCCCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTCAAC	1038

NM 001267874.1	AAGAGCATCAACCCGGACGAGGCGGTGGCATAACGGGGCGGCGGTGCAGGCGGCCATCCTG	1140
G1	AAGAGCATCAACCCGGACGAGGCGGTGGCATAACGGGGCGGCGGTGCAGGCGGCCATCCTG	1098
G2	AAGAGCATCAACCCGGACGAGGCGGTGGCATAACGGGGCGGCGGTGCAGGCGGCCATCCTG	1098

NM 001267874.1	ATGGGGGACAAGTCGGAGAACCTGCAGGACCTGCTGCTGGACGTGGCTCCCCTGTCTG	1200
G1	ATGGGGGACAAGTCGGAGAACCTGCAGGACCTGCTGCTGGACGTGGCTCCCCTGTCTG	1158
G2	ATGGGGGACAAGTCGGAGAACCTGCAGGACCTGCTGCTGGACGTGGCTCCCCTGTCTG	1158

NM 001267874.1	CTGGGACTGGAGACGGCCGGAGGCGTGATGACCGCCCTGATCAAGCGCAACTCCACCATC	1260
G1	CTGGGACTGGAGACGGCCGGAGGCGTGATGACCGCCCTGATCAAGCGCAACTCCACCATC	1218
G2	CTGGGACTGGAGACGGCCGGAGGCGTGATGACCGCCCTGATCAAGCGCAACTCCACCATC	1218

NM 001267874.1	CCCACGAAGCAGACGCAGATCTTACCACCTACTCGGACAACCAGCCGGGCGTGTGATC	1320
G1	CCCACGAAGCAGACGCAGATCTTACCACCTACTCGGACAACCAGCCGGGCGTGTGATC	1278
G2	CCCACGAAGCAGACGCAGATCTTACCACCTACTCGGACAACCAGCCGGGCGTGTGATC	1278

NM_001267874.1	CAGGTGTACGAGGGCGAGAGGGCCATGACTCGGGACAACAACCTGCTGGGGCGCTTCGAG	1380
G1	CAGGTGTACGAGGGCGAGAGGGCCATGACTCGGGACAACAACCTGCTGGGGCGCTTCGAG	1338
G2	CAGGTGTACGAGGGCGAGAGGGCCATGACTCGGGACAACAACCTGCTGGGGCGCTTCGAG	1338

NM_001267874.1	CTGAGCGGCATCCCGCCGGCCCCCGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC	1440
G1	CTGAGCGGCATCCCGCCGGCCCCCGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC	1398
G2	CTGAGCGGCATCCCGCCGGCCCCCGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC	1398

NM_001267874.1	GACGCCAATGGCATCCTGAACGTACCGCCACGGACAAGAGCACGGGCAAGGCCAACAAG	1500
G1	GACGCCAATGGCATCCTGAACGTACCGCCACGGACAAGAGCACGGGCAAGGCCAACAAG	1458
G2	GACGCCAATGGCATCCTGAACGTACCGCCACGGACAAGAGCACGGGCAAGGCCAACAAG	1458

NM_001267874.1	ATCACCATCACCACGACAAGGGCCGGCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAG	1560
G1	ATCACCATCACCACGACAAGGGCCGGCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAG	1518
G2	ATCACCATCACCACGACAAGGGCCGGCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAG	1518

NM 001267874.1	GAGGCGGAGAAGTACAAGGCAGAGGACGAGGTCCAGCGGAGAGGGTGTCTGCCAAGAAC	1620
G1	GAGGCGGAGAAGTACAAGGCAGAGGACGAGGTCCAGCGGAGAGGGTGTCTGCCAAGAAC	1578
G2	GAGGCGGAGAAGTACAAGGCAGAGGACGAGGTCCAGCGGAGAGGGTGTCTGCCAAGAAC	1578

NM 001267874.1	GCGCTGGAGTCGTACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGGGCTGAAGGGC	1680
G1	GCGCTGGAGTCGTACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGGGCTGAAGGGC	1638
G2	GCGCTGGAGTCGTACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGGGCTGAAGGGC	1638

NM_001267874.1	AAGATCAGCGAGGCGGACAAGAAGAAGGTGCTGGACAAGTGCCAGGAGGTGATTTCTCTGG	1740
G1	AAGATCAGCGAGGCGGACAAGAAGAAGGTGCTGGACAAGTGCCAGGAGGTGATTTCTCTGG	1698
G2	AAGATCAGCGAGGCGGACAAGAAGAAGGTGCTGGACAAGTGCCAGGAGGTGATTTCTCTGG *****	1698
NM_001267874.1	CTGGACGCCAACACCTTTGGCGGAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAG	1800
G1	CTGGACGCCAACACCTTTGGCGGAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAG	1758
G2	CTGGACGCCAACACCTTTGGCGGAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAG *****	1758
NM_001267874.1	CAGGTGTGTAACCCCATCATCAGCAGACTGTACCAGGGGGCGGGCGGCCCGGGGCTGGC	1860
G1	CAGGTGTGTAACCCCATCATCAGCAGACTGTACCAGGGGGCGGGCGGCCCGGGGCTGGC	1818
G2	CAGGTGTGTAACCCCATCATCAGCAGACTGTACCAGGGGGCGGGCGGCCCGGGGCTGGC *****	1818
NM_001267874.1	GGCTTTGGGGCTCAGGCCCTAAAGGGGCTCTGGGTCTGGCCCCACCATTGAGGAGGTG	1920
G1	GGCTT-----	1823
G2	GGCTT----- *****	1823
NM_001267874.1	GATTAG	1926
G1	-----	1823
G2	-----	1823

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 *hsp70* could have been genetically modified to adapt to environmental conditions.

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