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

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Key words: Molecular detection, Arabi rams, heat stress.

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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 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

(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	
Extension	72	2 min	
Final Extension	72	10 min	١

First BASE Laboratories (2016)

Table (3) Thermal Cycling Protocol of PCR

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

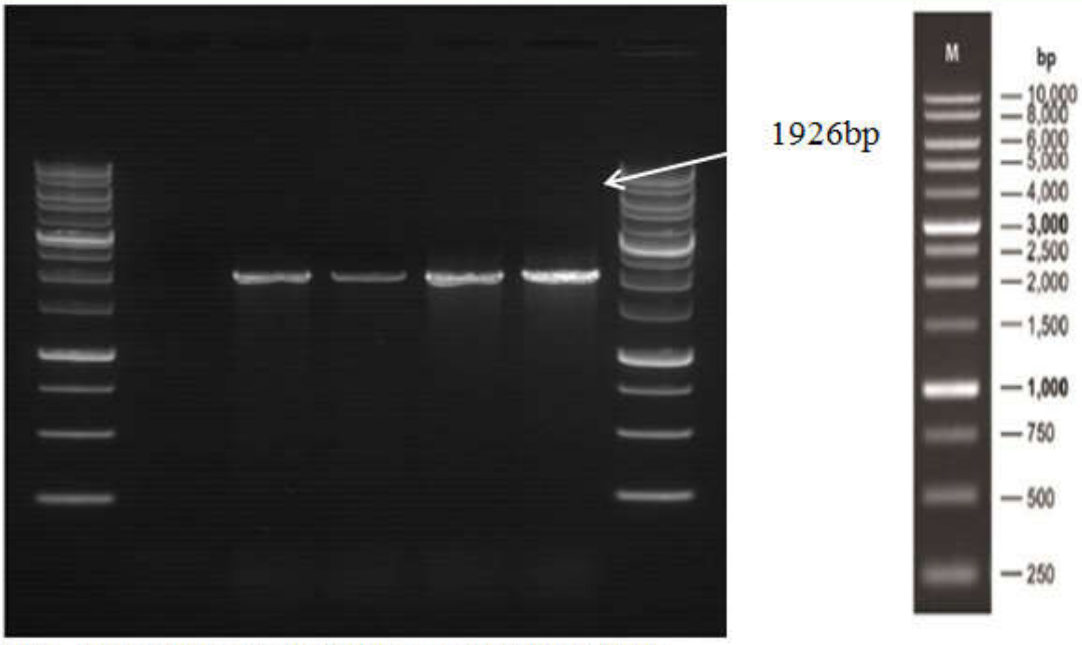

Figure 1: "-ve" is no template, control (water)
1 , 2, 3 , 4 : DNA template *hsp70* gene in semen sheep
M : DNA Marker with size 1000
total 75 ng of DNA sample was used, in 25 ul PCR reaction. Only 3 ul of PCR product was run on 1% TAE agarose gel at 100V, 60 min. (18).

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).

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

NM_001267874.1      TTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCCAGC      120
G1                  TTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCCAGC      78
G2                  TTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCCAGC      78
                    ************************************************************

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
                    ************************************************************

NM_001267874.1      GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCC      540
G1                  GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCC      498
G2                  GCGGGGGTGATCGCGGGGCTGAACGTGCTGAGGATCATCAACGAGCCCACGGCCGCCGCC      498
                    ************************************************************

NM_001267874.1      ATCGCCTACGGCCTGCACCGGACGGGCAAGGGGGAGCGCAACGTGCTCATCTTTGACCTG      600
G1                  ATCGCCTACGGCCTGCACCGGACGGGCAAGGGGGAGCGCAACGTGCTCATCTTTGACCTG      558
G2                  ATCGCCTACGGCCTGGACCGGACGGGCAAGGGGGAGCGCAACGTGCTCATCTTTGACCTG      558
                    *************** ********************************************

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
                    ************************************************************

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      CTGAGCGGCATCCCGCCGGCCCCGCGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC      1440
G1                  CTGAGCGGCATCCCGCCGGCCCCGCGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC      1398
G2                  CTGAGCGGCATCCCGCCGGCCCCGCGGGGGGTGCCCCAGATCGAGGTGACCTTCGACATC      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
                    ************************************************************

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
                    ************************************************************

NM_001267874.1      GGCTTTGGGGCTCAGGCCCCTAAAGGGGGCTCTGGGTCTGGCCCCACCATTGAGGAGGTG      1920
G1                  GGCTT------------------------------------------------------      1823
G2                  GGCTT------------------------------------------------------      1823
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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 *hsp*70 could have been genetically modified to adapt to environmental conditions.

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