Molecular detected of heat shock protein70 gene in Layer hens (Lohmann breed)

Hassan Nima Habib¹, Alfred S. Karomy¹, Qutaiba J. Gheni¹ and Wessam Monther Mohammed Saleh²

¹ Department of Animal Production, College of Agriculture, University of Basrah, Iraq
² Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Basrah, Iraq

Corresponding Author Email: hassan.nima@uobasrah.edu.iq

Abstract
The polymorphisms of the hsp70 gene have been associated with diverse resistance of heat stress in hens. The aim of the current study was to explore the genetic variation of the hsp70 gene in Layer hens that bred in Iraq. One hundred-fifty Lohmann breed hens aged 12 months were used in this study. Blood samples were collected during the period from 1st September to 31st December 2018 and examined for detection the polymorphism of hsp70 gene. We have detected four main polymorphisms groups in the coding region of hsp70 gene among these layer hens. A significant association between the silent and the missense mutations with the polymorphisms of hsp70 gene in Layer hens was found. There was a high homology of the hsp70 gene sequences that obtained from our local layer hens with the related sequences obtained from different hottest and coldest areas. In conclusion, this study demonstrates that the different mutations (silent and missense) in the coding region of the hsp70 gene of these local Layer hens predict improve birds'ability to the tolerance of stress conditions, and highlights the need of further investigations.

Keywords: HSPs, hsp70 gene, Polymorphism, Layer hens, Stress condition
Introduction

Heat stress is one of the most environmental conditions that generally adversely influence the poultry production. Layer hens industry is particularly impacted by heat stress as it often lead to reduced feed intake, low egg weight, reduced eggshell quality, poor growth rate and could increase mortality causing significant economic losses [1-4]. Thus, it is very influential in the hottest countries including Iraq in which the temperature raises two to seven folds higher than the global rates [5].

Despite the hens has protective mechanisms against various stress conditions; heat shock proteins (HSPs), a group of proteins proliferating due to stress conditions such as high environmental temperature [6], are fully effective for protecting and repairing mechanism of cells and tissues during stress [7]. However, HSP70 is the main member of HSPs family that plays a crucial role in heat tolerance [8], which can be used as a potential biomarker for dry and heat tolerance [9-11], as a higher bioavailability of its peptides in all variants extracellular [12]. Genetically, the gene in charge of HSP (hsp70 gene) does not contain introns in the coding region [13], therefore, the hsp70 gene considered as a high-conservation molecular [8]. As far as possible, there are known many nucleotide polymorphisms in the coding region of the hsp70 gene in goats [14], Japanese quail [15], duck [16], hens [17-18], cattle [19-20], sheep [21] and buffalo [22]. The polymorphisms of the hsp70 gene have been linked with the different impedance of heat stress in hens [17,23-24]. Furthermore, hsp70 gene polymorphisms act as genetic markers associated with heat stress tolerance, this may allow for direct selection of genes [17]. The polymorphisms of the hsp70 gene are also linked with gene expression [18] as they can affect more than one phenotypic trait [25]. To the best of our knowledge, no single study has been attempted evaluating the polymorphism of the hsp70 gene in Layer hens in Iraq. Therefore, the current study was aimed to detect the polymorphism of hsp70 gene in Layer hens that bred in Iraq.

Materials and methods

Animals and experimental design

One hundred fifty Lohmann breed hens aged 50 weeks old (at close stage of egg production) were used in this study. All hens were kept in the
Poultry Field of the College of Agriculture, University of Basrah, Iraq. The study was conducted during the period from 1st September to 31st December 2018. All hens were bred under the same conditions and fed with standard recommended diet. Blood samples were collected from hens then subjected for molecular analysis of hsp70 gene as following.

Samples and DNA extraction
Ten milliliters of blood was collected from the thigh from all hens, aseptically in EDTA tubes and the samples of blood were kept frozen at -20°C till the DNA extraction process. DNA extraction was done by using the DNeasy blood and tissue kit, obtained from Qiagen®, following the methods of Aryani et al., (2019) [26].

PCR amplification
The amplification reaction was performed in 25μl, consisted of 1μl DNA template (75ng), 1μL (10μM) forward primer, 1μL (10μM) reverse primer, 9.5μL water (free nuclease) and 12.5μL of 2 X PCR master mix. The primer (Table 1) was used according to the design of Gan et al., (2015) [27], while the PCR conditions were denaturation at 94°C (3 min) followed by 32 cycles of denaturation at 94°C for 30 seconds, 62°C for 30 seconds, annealing at 72°C for 45 seconds, and a final elongation step at 72°C for 6 minutes. Then to reveal the PCR product, 1.5% ethidium bromide 0.5 μg/ml stained agarose gel was used.

| Primer  | Sequence                                      |
|---------|-----------------------------------------------|
| hsp70- F | 5'-CGATCTGGCTGCAATCTACG-3'                   |
| hsp70- R | 5'-AT TTCCAGAAGCTGCACTTTG-3'                 |

The analysis of sequences
The resulting sequences were subjected to the nucleotide BLAST analysis on NCBI website, and compared with Hens 70 kd heat shock protein complete cds as a reference gene in GenBank (accession number J02579). The multiple sequence alignment (MSA) carried out on website http://www.ebi.ac.uk/Tools/msa/clustalo/ [28]. Further analysis was done by applying “Geneious Prime 2019.0.4” software in order to determine the expected mutations in amino acids [29].
Three-dimensional structure of protein
To detect the 3D structure of the protein, the Swiss model has been used [30].

The analysis of phylogenetic tree
Mega–x version 10.0.5 [31] software was applied to conduct the analysis of the phylogenetic tree. The resulting sequences were matched with the upper 10 outcomes of the Gallus gallus hsp70 gene in BLAST, which included each of accession numbers (J02579 USA, AY143691 Brazil, AY143692 Brazil, AY143693 Brazil, MH422506 Iran, MH422507 Iran, MH422508 Iran, EU747335 China, AY288299 China, and NM_001006685 China).

Results
The product size of PCR in the current study was 2692bp (Figure 1). However, four different polymorphism groups were obtained when compared to the reference gene (accession number J02579) in the GenBank. They were submitted to DDBJ, EMBL and GenBank, which available under accession numbers as follows: LC498496 (37 hens), LC503772 (23 hens), LC503773, (41 hens) and LC503774 (49 hens).

Multiple sequence alignment analysis (Figure 2) showed development of four groups, this was expressed in the numbers of silent mutations as well as the missense mutation that occurred due to new amino acid coding (Figure 3). Nevertheless, thirty-three different mutations occurred in all of the resulting groups, some of these mutations occurred only in one group, while some of them occurred in more than one group; the mutations were summarized as follows:

1. LC498496
The positions 710 (G>A), 832 (G>C), 1013 (T>A), 1280 (T>G), 1430 (C>G), 1694 (T>G) and 1935 (A>T) are missense mutations that happened as a result of change of amino acids from glycine to glutamic, alanine to proline, phenylalanine to tyrosine, leucine to arginine, serine to cysteine, leucine to arginine, and glutamine to histidine respectively. Whereas the positions 804 (A>G), 1413 (G>A) and 2484 (G>A) are silent mutations.
2. LC503772
The positions 149 (G>C), 710 (G>A), 821(G>T), 1013 (T>A), 1430 (C>G), 2613 (A>T), 2633 (A>C) and 2660 (A>T) are missense mutations that happened as a result of change of amino acids from arginine to proline, glycine to glutamic acid, cysteine to phenylalanine, phenylalanine to tyrosine, serine to cysteine, lysine to asparagine, glutamic acid to alanine and isoleucine to asparagine respectively. While the positions 804 (A>G) and 855 (A>C) are silent mutations.

3. LC503773
The positions 54 (A>T), 947 (T>A), 1508 (G>C), 1531 (A>T), and 2356 (T>A) are missense mutations that happened as a result of change of amino acids from lysine to asparagine, leucine to histidine, cysteine to serine, serine to cysteine, and phenylalanine to isoleucine respectively. While the position 600 (A>G) is a missense mutation that happened as an outcome of coding tryptophan, new amino acid. The positions 1341(T>G), 1488 (T>C) and 1845 (C>A) are silent mutations.

4. LC503774
The positions 219 (A>C), 947 (T>A), 1508 (G>C), 1580 (T>C), 1696 (A>G), 1904 (A>C), 2338 (C>G) and 2358 (T>A) are missense mutations that happened as a result of change of amino acids from leucine to phenylalanine, leucine to histidine, cysteine to serine, valine to alanine, arginine to glycine, asparagine to threonine, histidine to aspartic acid and phenylalanine to leucine respectively. While the positions 669 (T>A) and 1341(T>G) are silent mutations.

The 3D structure of the protein was predicted for all the resulting sequences (Figure 4). Despite the great structural similarities, there were not completely identical, taking into consideration the selection of the highest quality molds for all sequences.

The optimal tree with the sum of branch length = 9.43103611 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Consistently, phylogenetic tree analysis (Figure 5) showed that the group
LC498496 was in the same clade with the reference gene (accession number J02579), while the LC503772 group shared them origin with the same clade, the group LC503773, and LC03774 were in the same clade. Interestingly, the resulting sequences shared the same origin with all accession numbers of hsp70 gene in Layer hens with high homology.

Discussion
The gene expression of hsp70 gene of layer hens in the current study was higher as previously shown by Morimoto et al., (1986) [32]. Through this higher expression of hsp70 gene, the sequences that obtained in this study did not fully coincide with any of other related sequences. However, our findings correspond well with the previously found results [27,33-34], which describe the nucleotide polymorphisms in the coding region of the hsp70 gene in hens. Our findings suggest that the changes in the codon region of the hsp70 gene might be explained by a mismatch that resulted from silent and missense mutations that can mainly develop this gene [27]. Furthermore, the diversity of the hsp70 gene is directly related to the different production traits of poultry [35-36], thus, the mutations can generally enhance the function of gene [13]. Even though the silent mutations do not usually affect protein function [37], however, it may be evidence of tolerating different stress conditions [38]. However, the silent mutation impacts the related proteins by altering the transcription process and the accuracy and the efficiency of binding to mRNA [39]; therefore, its effect can reach the stability of the encoded protein [40].

As expected for missense mutations, the incidences of which were high, their influence is highly dependent on their locations [41] and on the composition and the function of resulting amino acids [42]. Therefore, so not all of these mutations cause significant changes in protein, the amino acid can be changed with another amino acid that has similar chemical properties in which the protein function remains normal and unchanged. Contrariwise, the protein can be turned into non-functional if there is a significant difference in the properties of amino acids [43]. The polymorphisms resulting from missense mutations that mentioned in previous studies are strongly linked to tolerate the stressful conditions and have better productive characteristics [44]. Another aspect is that the missense mutation may have caused a change in protein structure [45],
this change can impact the protein function [46], and thus, the hsp70 modeling is mainly useful by giving a clear perception about its function [47].

The present data suggests that there was a potential relationship between the genetic diversity in the hsp70 gene and the expression of gene that well corresponds with the previously reported results [24,48], where the levels of gene expression differ between polymorphisms of hsp70 gene. By this way, the election of the most productive breeds is being fully effective, as high gene expression was positively associated with resistance to stress conditions, especially high temperature [16].

Interestingly when analyzing the sequences of hsp70 gene in local Layer hens, all the resulting sequences were closer to the reference gene (accession number J02579) that obtained from a coldest area (USA). However, hsp70 gene can be completely stimulated even with sharp elevation or decline of the environmental temperature [22]. Moreover, there were high homology with different hsp70 genes that obtained from Brazil, China, and Iran. This finding is clearly indicates that the hsp70 gene well preserved in Gallus gallus, these differences were also previously reported by [33], [26] and [49].

**Conclusion**
The present data provide the first insight into genetic variation of the hsp70 gene of Layer hens (Lohmann breed) that bred in Iraq, and the first evidence that the different mutations (silent and missense) in the coding region of the hsp70 gene of these local Layer hens predict improve birds’ability to the tolerance of stress conditions. Our study records four polymorphisms in the coding region of the hsp70 gene in a limited number of local Layer hens. Further deep studies may explore the relationship between the polymorphisms of the hsp70 gene and the productive characteristics of laying hens.

**Acknowledgments**
We gratefully thank the staff of the poultry field, college of agriculture, University of Basrah for their helping and supporting.

**Funding**
The current study was funded by the authors themselves.

Conflict of Interest
We declare that there are no conflicts of interest.

References
1. Ajakaiye J J, Pérez B A, and Mollineda T A, Effects of high temperature on production in layer chickens supplemented with vitamins C and E. Revista MVZ Córdoba 2011; 16 (1): 2283-2291.
2. Kilic I and Simsek E, The effects of heat stress on egg production and quality of laying hens. Journal of Animal and Veterinary Advances 2013; 12 (1): 42-47.
3. Lara LJ and Rostagno MH, Impact of heat stress on poultry production. Animals 2013; 3 (2): 356-369.
4. Melesse A, Maak S, Schmidt R, and Von Lengerken G, Effect of long-term heat stress on some performance traits and plasma enzyme activities in Naked-neck chickens and their F1 crosses with commercial layer breeds. Livestock Science 2011; 141 (2-3): 227-231.
5. Salman SA, Shahid S, Ismail T, Chung E-S, and Al-Abadi AM, Long-term trends in daily temperature extremes in Iraq. Atmospheric research 2017; 198: 97-107.
6. Staib JL, Quindry JC, French JP, Criswell DS, and Powers SK, Increased temperature, not cardiac load, activates heat shock transcription factor 1 and heat shock protein 72 expression in the heart. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 2007; 292 (1): R432-R439.
7. Zhang Y-Q and Sarge KD, Celastrol inhibits polyglutamine aggregation and toxicity though induction of the heat shock response. Journal of molecular medicine 2007; 85 (12): 1421-1428.
8. Zuiderweg ER, Bertelsen EB, Rousaki A, Mayer MP, Gestwicki JE, and Ahmad A, Allostery in the Hsp70 chaperone proteins, in Molecular Chaperones. Springer; 2012. pp. 99-153.
9. Dang W, Xu N, Zhang W, Gao J, Fan H, and Lu H, Differential regulation of Hsp70 expression in six lizard species under normal and high environmental temperatures. Pakistan J. Zool 2018; 50: 1043-1051.
10. Hassan F-u, Nawaz A, Rehman MS, Ali MA, Dilshad SM, and Yang C, Prospects of HSP70 as a genetic marker for thermo-tolerance and immuno-modulation in animals under climate change scenario. Animal Nutrition 2019; 5 (4): 340-350.
11. Moraa G, Oyier P, Maina S, Makanda M, Ndiema E, Alakonya A, Ngeiywa K, Lichoti J, and Ommeh S. Genetic background and hsp70 gene polymorphisms for heat tolerance in indigenous chickens of kenya. in Scientific Conference Proceedings. 2016.

12. Ginting R and Basyuni M. Bioinformatics Identification of HSP70 in Chicken (Gallus gallus domesticus). in Proceedings of the International Conference on Natural Resources and Technology (ICONART). 2019.

13. Wang Q, Wang F, Liu L, Li Q, Liu R, Zheng M, Cui H, Wen J, and Zhao G, Genetic Mutation Analysis of High and Low IgY Chickens by Capture Sequencing. Animals 2019; 9 (5): 272.

14. Gaviol HCT, Gasparino E, Prioli AJ, and Soares MAM, Genetic evaluation of the HSP70 protein in the Japanese quail (Coturnix japonica). Genetics and Molecular Research 2008; 7 (1): 133-139.

15. Gade N, Mahapatra R, Sonawane A, Singh V, Doreswamy R, and Saini M, Molecular characterization of heat shock protein 70-1 gene of goat (Capra hircus). Molecular biology international 2010; 2010.

16. Xia M, Gan J, Luo Q, Zhang X, and Yang G, Identification of duck HSP70 gene, polymorphism analysis and tissue expression under control and heat stress conditions. British poultry science 2013; 54 (5): 562-566.

17. Chen Z, Zhang W, Gan J, Kong L, Zhang X, Zhang D, and Luo Q, Genetic effect of an A/G polymorphism in the HSP70 gene on thermotolerance in chicken. Genet. Mol. Res 2016; 15.

18. Zhen F-S, Du H-L, Xu H-P, Luo Q-B, and Zhang X-Q, Tissue and allelic-specific expression of hsp70 gene in chickens: basal and heat-stress-induced mRNA level quantified with real-time reverse transcriptase polymerase chain reaction. British poultry science 2006; 47 (4): 449-455.

19. Habib H, Hassan A, and Khudaier B, Molecular detection of polymorphism of heat shock protein 70 (hsp70) in the semen of Iraqi Holstein bulls. Asian J Anim Sci 2017; 11: 132-139.

20. Mariana E, Sumantri C, Astuti D, Anggraeni A, and Gunawan A. Association of HSP70 gene with milk yield and milk quality of Friesian Holstein in Indonesia. in IOP Conference Series: Earth and Environmental Science. 2020. IOP Publishing.

21. Habib HN, Khudaier BY, and Hassan AF, Molecular Detection of Polymorphism of Heat Shock Protein 70 (hsp70) in the Semen of Arabi Rams. Basrah Journal of Veterinary Research 2018; 17 (3).

22. Habib HN and Saleh WMM, The Role of Heat Shock Proteins 70 (HSP70) in Farm Animals Adaptation, A Review Paper, in The
23. Duangjinda M, Tunim S, Duangdaen C, and Boonkum W, HSP70 genotypes and heat tolerance of commercial and native chickens reared in hot and humid conditions. Brazilian Journal of Poultry Science 2017; 19 (1): 7-18.

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

25. Deb R, Mukhopadhyay CS, Sengar GS, da Cruz AS, Silva DC, Pinto IP, Minasi LB, Costa EOA, and da Cruz AD, Genetic markers for improving farm animals, in Genomics and Biotechnological Advances in Veterinary, Poultry, and Fisheries. Elsevier; 2020. pp. 107-129.

26. Aryani A, Solihin D, Sumantri C, Afnan R, and Sartika T, Genetic Diversity of the Structure of HSP70 Gene in Kampung Unggul Balitbangtan (KUB), Walik, and Kate Walik Chickens. Tropical Animal Science Journal 2019; 42 (3): 180-188.

27. Gan J, Jiang L, Kong L, Zhang X, and Luo Q, Analysis of genetic diversity of the heat shock protein 70 gene on the basis of abundant sequence polymorphisms in chicken breeds. Genet. Mol. Res 2015; 14: 1538-1545.

28. McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N, Cowley AP, and Lopez R, Analysis tool web services from the EMBL-EBI. Nucleic acids research 2013; 41 (W1): W597-W600.

29. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, and Duran C, Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 2012; 28 (12): 1647-1649.

30. Waterhouse A, Bertoni M, Bienert S, Studer G, Tauriello G, Gumienny R, Heer FT, de Beer TAP, Rempfer C, and Bordoli L, SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic acids research 2018; 46 (W1): W296-W303.

31. Kumar S, Stecher G, Li M, Knyaz C, and Tamura K, MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution 2018; 35 (6): 1547-1549.

32. Morimoto R, Hunt C, Huang S, Berg KL, and Banerji S, Organization, nucleotide sequence, and transcription of the chicken HSP70 gene. Journal of Biological Chemistry 1986; 261 (27): 12692-12699.
33. Najafi M, Rouhi M, and Mokhtari R, Genetic analysis of a novel polymorphism in coding region of HSP70 gene and its association with some productive and reproductive traits in Mazandaran native breeder hens. Journal of Genet Disord and Genet Med 2019; 2 (1): 1-5.

34. Sheraiba N, Hemed S, and Mahboub H, HSP70 And HSP90β Genes Polymorphism And Its Association With Thermotolerance In Fayoumi And Leghorn Chicken Breeds. Journal of Current Veterinary Research 2019; 1 (2): 56-62.

35. Abdolalizadeh N, Noshary A, and Eila N, Identification of single nucleotide polymorphisms of Hsp70 gene in a commercial broiler strain. Research Opinions in Animal and Veterinary Sciences 2015; 5 (6): 265-269.

36. Kang S, Lin C, Cheng Y, Lin D, Huang T, Hung K, and Liang H, Genetic parameters for body weight and egg production traits in Taiwan native chicken homozygous for the heat shock protein 70 gene. Asian Journal of Agriculture and Biology 2018; 6 (3): 396-402.

37. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, and Walter P, Molecular biology of the cell: Reference edition. Garland Science: New York; 2007. pp. 264.

38. Duangduen C, Duangjinda M, Katawatin S, and Aengwanich W, Effects of heat stress on growth performance and physiological response in Thai indigenous chickens (Chee) and broilers. Warasan Sattawaphaet 2007.

39. Komar AA, Silent SNPs: impact on gene function and phenotype. Pharmacogenomics 2007; 8 (8): 1075–1080.

40. Karakostis K, Vadivel Gnanasundram S, López I, Thermou A, Wang L, Nylander K, Olivares-Illana V, and Fåhraeus R, A single synonymous mutation determines the phosphorylation and stability of the nascent protein. Journal of molecular cell biology 2019; 11 (3): 187-199.

41. Ming D, Chen R, and Huang H, Amino-Acid Network Clique Analysis of Protein Mutation Non-Additive Effects: A Case Study of Lysozyme. International journal of molecular sciences 2018; 19 (5): 1427.

42. Hormoz S, Amino acid composition of proteins reduces deleterious impact of mutations. Scientific reports 2013; 3: 2919.

43. Chou JY and Mansfield BC, Mutations in the glucose-6-phosphatase- α (G6PC) gene that cause type Ia glycogen storage disease. Human mutation 2008; 29 (7): 921-930.

44. Liang H-M, Lin D-Y, Hsuuw Y-D, Huang T-P, Chang H-L, Lin C-Y, Wu H-H, and Hung K-H, Association of heat shock protein 70
gene polymorphisms with acute thermal tolerance, growth, and egg production traits of native chickens in Taiwan. Archives Animal Breeding 2016; 59 (2): 173-181.

45. Han B, Yuan Y, Liang R, Li Y, Liu L, and Sun D, Genetic effects of LPIN1 polymorphisms on milk production traits in dairy cattle. Genes 2019; 10 (4): 265.

46. Khan RH, Siddiqi MK, and Salahuddin P, Protein structure and function. Basic Biochemistry 2017: 1-39.

47. Mishra S and Gomase V, Computational comparative homology based 3d-structure modelling of the hsp70 protein from gwd. J Health Med Informat 2016; 7 (233): 2.

48. Gong WJ and Golic KG, Loss of Hsp70 in Drosophila is pleiotropic, with effects on thermotolerance, recovery from heat shock and neurodegeneration. Genetics 2006; 172 (1): 275-286.

49. Kennedy GM, Diversity, Genetic Background and Hsp70 Gene Functional Polymorphisms for Heat Tolerance in Indigenous Chickens in Kenya. IBR, JKUAT. 2016.

Figure 1: Gel electrophoresis of the PCR amplification to hsp70 gene, M: 10kb DNA ladder, 1-7: DNA templates
Figure 2: The multiple sequence alignment (MSA) of hsp70 gen in Layer hens
Figure 3: The changes of amino acids in the *hsp70*. J02579: reference gene. LC498496, LC503772, LC503773 and LC503774 are the results sequences of *hsp70* in Layer hens.
Figure 4: The expected 3D structure of the proteins of hsp70 gene in Layer hens

Figure 5: Phylogenetic tree of hsp70 gene in Layer hens