MOLECULAR INVESTIGATIO OF HEAT SHOCK PROTEIN 70 (*HSP70*) EXPRESSION LEVELS IN ASPERGILLLOSIS PATIENTS

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ABSTRACT

Forty nine sputum specimens were collected from patients with aspergillosis attended to four hospitals in Baghdad. The frequent species of *Aspergillus* identified included *Aspergillus fumigatus* 23(46.9%), *Aspergillus niger* 14 (28.6%), followed by *Aspergillus flavus* 12 (24.5%). According to age group factor, the age group (50-59) years appeared to be more susceptible to infected by aspergillosis with percentage at (24.5%). The results was revealed that no significant differences between male and female with aspergillosis infection. To detect *A.fumigatus* isolates by molecular methods, the genomic DNA were extracted and amplification to detect the *aspHS* gene by the singleplex PCR method using species-specific primers for these *A.fumigatus*, to sum up 17 of isolates from 23 isolates of *A.fumigatus* which identified the previous by morphological and microscopic methods, by observing the singleplex PCR product of *aspHS* gene with ~108 bp. The total RNA of *A.fumigatus* was extracted by using TRIzol purification kit and convert to cDNA and submit for further amplification to detect the Heat Shock protein 70 genes (*Hsp70* genes) expression as virulence factor in variable temperature activation include 28 ºC, 37 ºC and 45 ºC by real time PCR. The results of *HSP70* gene expression showed the level increased at 37 ˚C but decreased when the temperature increases to 45 ˚C.

Keywords: *Aspergillus fumigatus*, *asp*HS gene, haemolysin, singleplex PCR, real time PCR

التحري الجزيئي لمستويات التعبير عن بروتين الصدمة الحرارية 70 (*HSP70*) في مرضى داء الرشاشيات

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المستخلص

تم جمع تسعة وأربعين عينة من البلغم من مرضى داء الرشاشيات اللذين حضروا إلى أربعة مستشفيات في بغداد. اشتملت الأنواع المتكررة من الرشاشيات التي تم تحديدها الى *Aspergillus fumigatus* 23(46,9%) ثم *Aspergillus niger* 14(28,6%) ويليها *Aspergillus flavus* 12(24,5%) . ظهر ان الافراد اللذين تتراوح اعمارهم 50−59 هم اكثر عرضة للاصابة بداء الراشاشيات حيث بلغت نسبتهم (24,5%) واظهرت النتائج ايضا بانه لاتوجد فروق معنوية بين الذكوروالاناث للاصابة بداء الرشاشيات . للتحري عن عزلات *A.fumigatus* بالطرق الجزيئية, تم إخضاع DNA الجينومي المستخلص من هذه العزلات الى التضخيم لغرض الكشف عن جين *aspHS* بواسطة PCR احادي البوادئ (Singleplex PCR) باستعمال البوادئ الخاصة باانواع *A. fumigatus* هذه، وقد تم تحديد 17 نموذج من أصل 23 نموذج *A. fumigatus* الذي تم تشخيصه سابقا بطرق مظهرية ومجهرية ومن خلال ملاحظة نواتج تفاعل PCR احادي البوادئ الخاص بجين *aspHS* ذات الاوزان الجزيئية ~108 زوج قاعدي. استخلاص RNA الكلي والخاص بالفطر باستعمال TRIzol purification kit وقد تم تحويله الى cDNA والذي اخضع بدوره بعد ذلك الى تضخيم اضافي لغرض التحري عن التعبير الجيني لجين الصدمة الحرارية *HSP70* كعامل ضراوة عن طريق التنشيط الحراري في درجات حرارة مختلفة شملت28 ,37و45 م° بوساطة تقنية Real time PCR. أظهرت نتائج التعبير الجيني *HSP70* أن المستوى التعبير قد زاد عند 37 درجة مئوية ولكنه انخفض عندما ترتفع درجة الحرارة إلى 45 درجة مئوية.

الكلمات المفتاحية:*Aspergillus fumigatus* , جين *aspHS*,تقنية التضخيم الاحادي PCR, الوقت الحقيقيPCR
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INTRODUCTION

Aspergillosis is an example of diseases caused by *Aspergillus spp*. The species involved with Aspergillosis are *A. fumigatus*, *A. flavus*, *A. niger* and *A. terrus*. Symptoms of aspergillosis are characterized by respiratory problems, skin disorders, poisoning and allergies. This disease can occur due to the entry of fungal spores in the air through the inhalation system. Where this fungus can be found in air, food, vegetables, soil, humus (27). *Aspergillus fumigatus* is the most common cause of infections in humans and is the most common cause of serious, invasive disease. Infections caused by *Aspergillus spp*. remain associated with high morbidity and mortality (19). It is opportunistic saprophytic mold that produces airborne spores (conidia) as people inhale, on average, hundreds of these infectious daily. So far, the immune competent hosts encounters of theses conidia and killed by cells of the pulmonary immune system. However, disease occurs when the host response is too weak. *A. fumigatus* represents a main cause of morbidity and mortality (18). The hypothesis suggested that most important genes involved in high temperature tolerance is heat shock protein (HSP) gene which controlling the production of HSPs (11). HSPs are generally present in eukaryotic and prokaryotic cells (28) and their expression levels increase under stress conditions (20). It has been shown that the expression of *HSP70* prepares a condition for fungi to adapt to new environmental situations (5). This study aimed to detection the gene expression of *HSP* 70 gene in *A. fumigatus*.

MATERIALS AND METHODS

Collection of samples

This study was carried out using 49 sputum specimens were isolated from patients with aspergillosis, this specimens were collected from National Center for Thoracic and Respiratory Diseases (NCTRD), Baghdad Teaching Hospital, Oncology Teaching hospital and Imamein Kadhimein madical. The samples were cultivated on Sabouraud′s dextrose agar with chloramphenicol **(**SDAC) The plates at 28 ºC for 7 days for molds and examined at regular intervals, then plate were sealed with Para film and stored inverted in a sealed plastic bag at 4 ºC (8).

Extraction of genomic DNA

The genomic DNA was extracted from the *A. fumigatus* isolates using a commercial wizard genomic DNA purification kit (Promega, USA) then, samples were subjected to agarose gel electrophoresis.

Primers selection

To select singleplex PCR primers that can give specific amplification DNA for the species-specific primers *aspHS* gene for detection of *A. fumigatus* were used according to Gravelat *et al*. (15) and then the general properties of these primers were checked by using Oligocalc Oligonucleotide Properties Calculator program, the name, sequence and the expected product size of these primers are listed below in Table 1.

Table1. show Name, sequence and the expected product size of *A. fumigatus* primers (15)

Fungal isolate	Name of primer	Sequence of primer	Expected product size (bp)
A. fumigatus	*aspHS* –F	5'-AGTCCACTGGGACTGTCCAT-3'	~108
	aspHS –R	5'-GCACCACCATACTTGTTCCA-3'	

Singleplex PCR master mix

Optimization of singleplex PCR master mix for amplification of *aspHS* gen**e** was accomplished after several trials; thus, the following mixtures were adopted for *A.fumigatus* (Table 2).

Table 2. Singleplex PCR master mix to detect the *aspHS* gene of *A. fumigatus* isolates

Component	Concentration	Amount(µl)
GoTaq Green Master Mix	2X	12.5
aspHS -F primer	10 µM/ µl	2
aspHS -R primer	10 µM/ µl	2
Nuclease free water	-	4.5
DNA sample	-	4
Total volume	-	25

Singleplex PCR program

Optimization of singleplex PCR program for amplification of *aspHS* gen**e** was accomplished after several trials; thus, the following programs were adopted for *A.fumigatus* (Table 3).

Table3. Singleplex PCR program to detect the *aspHS* gene of *A. fumigatus* isolates

No.	Step	Temperature	Time	No. of Cycles
1	Initial denaturation	95 °C	5 min.	1
2	Denaturation	95 °C	30 sec.	
3	Annealing	58 °C	30 sec.	30
4	Extension	72 °C	60 sec.	
5	Final extension	72 °C	7 min.	1
6	Storage	4 °C	∞	-

Detection of *HSP70* gene expression of *A. fumigatus* isolates by one-step RT-qPCR:

The RT-PCR was used to detect the *HSP70* gene expression of *A. fumigatus* isolates as follow:

Growing of *A. fumigatus*

1- One milliliter of the seven *A. fumigatus* were culture at three different temperatures including 28ºC, 37ºC and 45ºC for 7 days in SDAC (Oxoid/ England) was transferred to a 1.5 microcentrifuge tube.

2- The microcentrifuge tube was centrifuged at 14000 rpm for 3 minutes to pellet the cells and the supernatant was removed.

3- Trizol reagent (750 µl) was added to cells pellet and pipette for several times to get the homogenized sample.

Extraction of RNA

The RNA was extracted from the *A. fumigatus* isolates using a commercial TRIzol extraction kit (Invitrogen, USA) and the concentration of extracted RNA samples of *A. fumigatus* isolates were estimated by the protocol for quantitating RNA in a single tube using the Quantus fluorometer.

RT-qPCR

The RT- PCR was adopted to detect the gene expression of *HSP*70.

RT-qPCR primers

Primers selection: To select RT-PCR primers that can detect the gene expression of *HSP70* gene for the *A. fumigatus* isolates, the species-specific primers; *HSP70* for *A. fumigatus* as in (Table 4), and the *aspHS*-F and *aspHS*-R primers of the housekeeping *aspHS* gene for the *A. fumigatus*.

Table4. Name and sequence of housekeeping gene and *Hsp70* gene primers of *A. fumigatus*

Name of primer	Sequence of primer	Size
aspHS-F	5'- AGTCCACTGGGACTGTCCAT -3'	108bp
aspHS-R	5'- GCACCACCATACTTGTTCCA -3'	
HSP70-F	5'- GACCATTGAGGAGGGTATCT –3'	126bp
HSP70-R	5'- TCCTTCTTGTGCTTTCTCTTG–3'	

RT-qPCR master mix

The RT-PCR master mix to detect the gene expression of the *HSP70*-F, *HSP70*-R gene was prepared; thus, the following mixtures were adopted for *A. fumigatus* (Table 5).

Table5. RT-PCR master mix to detect the *HSP70* gene expression of *A. fumigatus* isolates

Component	Concentration	Amount (µl)
Master Mix	-	5
HSP70 -F primer	10 µM/ µl	0.5
HSP70 -R primer	10 µM/ µl	0.5
RT mix	-	0.25
MgCl2	-	0.25
Nuclease free water	-	2.5
RNA sample	-	1
Total	-	10

RT-PCR program

The RT-PCR program to detect the gene expression of the *HSP70* gene was set; thus, the following RT-PCR program was adopted for *A. fumigatus* as in Table 6.

Table6. RT-PCR program to detect the *HSP70* gene expression *A. fumigatus* isolates

Step	Temperature	Time	Cycles
Reverse Transcription	37 °C	15 minutes	
RT inactivation/Hot -start activation	95 °C	10 minutes	
Step Qpcr			
a. Denaturation	95 °C	20 seconds	
b. Annealing	63 °C	20 seconds	40
c. Extention	72 °C	30 seconds	
	72 °C	30 seconds	
Dissociation	95 °C	30 seconds	

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Least significant difference –LSD test was used to significant compare between means in this study.

RESULT AND DISCUSSION:

Conventional method

The investigation of growing fungal isolates was included characteristic features of the surface and reverse of the fungal colonies on SDAC according to Raper and Fennell (22); Ellis *et al*. (14).

Table7. The number and percentage of fungi isolated from sputum specimens

Samples	NO.	%
Aspergillus fumigatus	23	46.9
Aspergillus niger	14	28.6
Aspergillus flavus	12	24.5
	(P<0.01)	

The results of *Aspergillus* species isolation in this study showed that the first most common isolated species was *A. fumigatus* (23 isolates), the second isolated species was *A.niger* (14 isolates), the third isolated species was *A. flavus* (12 isolates). the result of this study agree with other studies (6; 13;17) they found *A.fumigatus* was the most common cause aspergillosis . Beed *et al.* (9) and Ali (4) found *A. niger* and *A. flavus* less common pathogens from *A. fumigatus*. Al-Charrakh *et al*.,(3) they found *A. flavus, A. niger,* and *A. terreus* were the most frequently isolated species followed by *A. fumigatus*, respectively, while Diba *et al.* (12) and Badiee *et al.*(7) they reserch *A. flavus* the main cause of aspergillosis then *A. niger* and finally *A. fumigatus*.

Relationship of aspergillosis with Age and Gender: The age group (50-59) years appeared to be more susceptible to infected by aspergillosis with percentage at (24.5%) followed by age group (20-29) with percentage at (20.4%), the age group (40-49) represented percentage at (16.3%), while the age group (60<) years documented about (18.4%) finally the age group less than 20 years recorded 5 with percentage (10.2 %) as show in Table8.

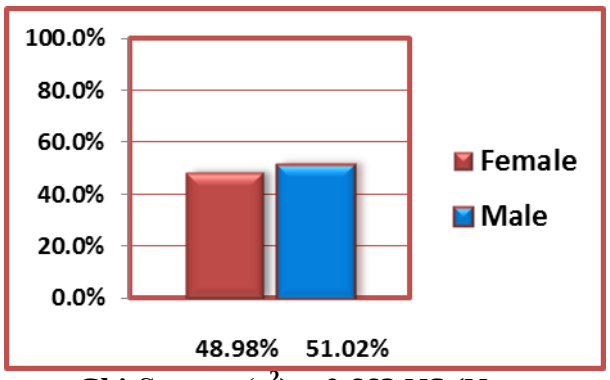
Table8. Distribution of patients with aspegillosis according to age groups and gender

Age group (years)	Female	%	Male	%	Total	%
< 20	3	12.5	2	8	5	10.2
20-29	6	25	4	16	10	20.4
30-39	3	12.5	2	8	5	10.2
40-49	5	20.8	3	12	8	16.3
50-59	2	8.4	10	40	12	24.5
≥60	5	20.8	4	16	9	18.4
Total	24	100	25	100	49	100
Chi-Square (χ^2)	---	5.307 *	---	9.255 **	---	4.982 **
** (P<0.01).						

The distribution of *Aspergillus* spp. isolated from aspergillosis, according to the age. It was found that the *Aspergillus* spp. infection increased with the age of patients. The results of higher infection levels in age between fifty and fifty nine years old. These results were in agreement to Sharma *et al.* (26); Al- Bayati *et al.* (2); Beltr *et al.* (10) revealed that aspergillosis infection the patients more than 40 years old and that the incidence of these proplems increases with age.

Gender distribution in aspergillosis

Aspergillosis disruption in male and female were equal as in Figure 1. It was showing that 25 males (51.02%) have been diagnosed with aspergillosis, on the other hand, there were 24 female cases (48.98%) have a positive diagnosis regarding the aspergillosis infection. These results were similar to Al-Charrakh *et al*. (3), reported that highest infection levels occurred in male and female patients were equal. These results no difference between the both sexes related to the distribution of infection. While disagree with Hassan *et al*. (16) mentioned the apergillosis affected males more than females. These differences came from the variety in the region, random samples and average age of the people in different countries.



Chi-Square (χ^2) = 0.662 NS (Non-Significant).

Figure1. Gender distribution in aspergillosis

DNA extraction of *A. fumigatus*

Genomic DNA was extracted from all 23 isolates of *A. fumigatus* by a commercial wizard genomic DNA purification kit (Promega, USA) and then extracted genomic DNA of *A.fumigatus* was DNA concentration and purity were measured by Quantus Florometer to detect the goodness of samples for downstream applications. The concentration of DNA ranged from 4.5 to 9 ng/ µl.

Molecular identification of of *Aspergillus fumigates* by detection *Aspergillus* hemolysin *aspHS* gene

The *aspHS* gene, as a target for the specific detection of *A. fumigatus* by PCR. This target gene encodes a haemolysin, which is over expressed in *vivo* during infection (1). The *aspHS* gene is more highly expressed in *vivo* than in *vitro* (14). Figure 2 shows that *aspHS* gene (108bp) exists in 17 of isolates from 23 of *A.fumigatus* which identified previousiy by morphological and microscopic methods.

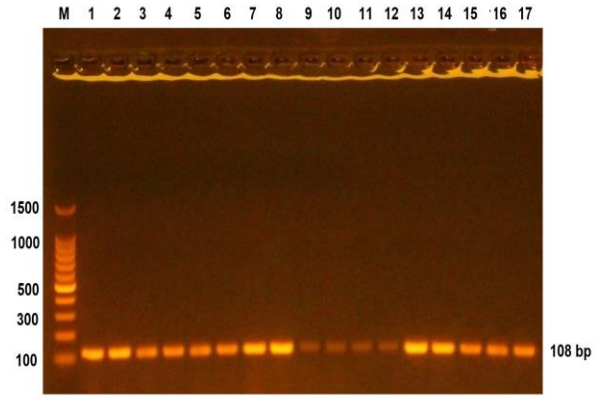


Figure2. Gel electrophoresis showing singleplex PCR product of of *aspHS* gene *A.* 108 bp

Gene expression

RNA Extraction: The experiment of quantitative PCR reaction was done by using seven isolates of *A. fumigatus*. Total RNA was successfully extracted from all samples. The concentration of RNA ranged from 7.5 to 24 ng/ µl. A good yield with a high concentration of total RNA depends on the extraction conditions where by strict aseptic techniques must be used. The utilization of TRIzol in the total RNA extraction from fungi is well recommended (24).

Heat Shock Protein gene HSPs 70 expression of *A. fumigatus* isolates by RT-PCR: The RT- PCR was adopted to detect the *HSP70* gene expression for *A. fumigatus* isolates; the samples were analyzed and standardized against the gene expression of housekeeping *aspHS* gene. The relative changes in the mRNA expression levels were determined using comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$) between the *A. fumigatus* isolates that have been grown at three different temperature include 28ºC, 37ºC and 45ºC. Amplification and detection of *HSP*70 gene of *A. fumigatus* was carried out using SYBR Green qRT-PCR method.The positive results showed amplification at CT (threshold cycle) value were in range 23.56–32.79 for housekeeping *aspHS* gene (Figure 3), 23.71-32.88 for *HSP70* gene (Figure4), respectively, the melting temperature (Tm) values obtained for isolates were in range 80.82-81.22ºC for housekeeping *aspHS* gene (Figure 5), *HSP70* were in range of 83.80 - 84.91ºC (Figure6).

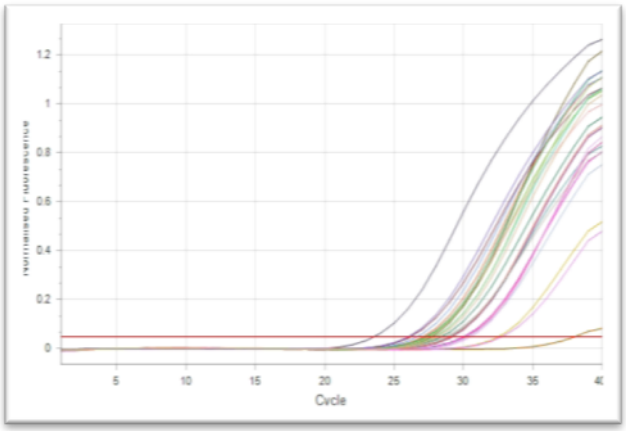


Figure3. Curve of cycling housekeeping *aspHS* gene

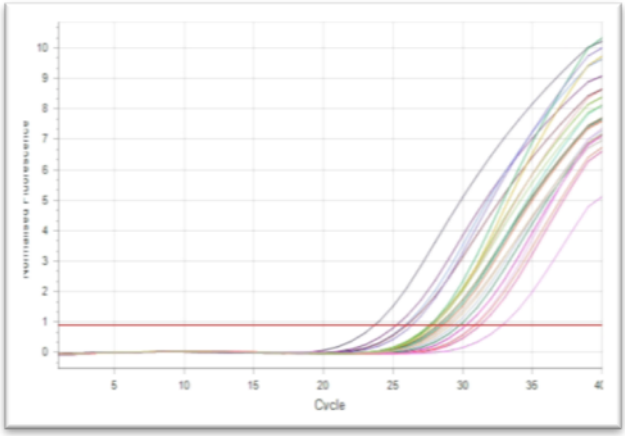


Figure4. Curve of cycling of *HSP70* gene

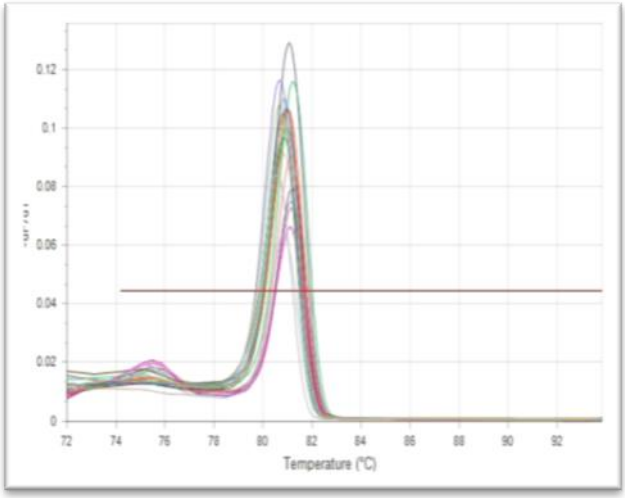


Figure5. Curve of melting housekeeping *aspHS* gene Growth at 42℃

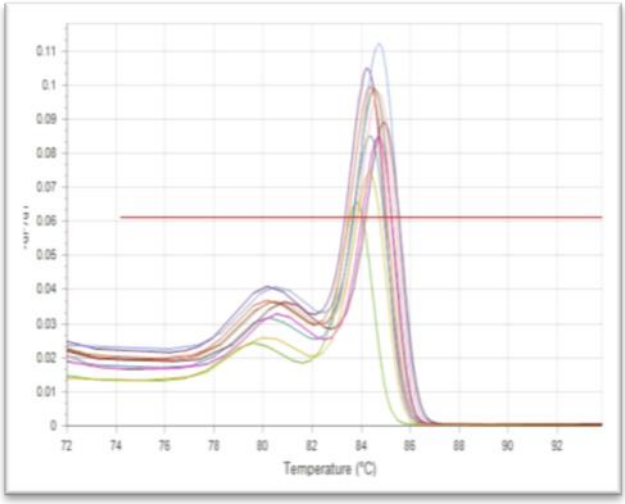


Figure 6. Curve of melting *HSP70* gene

The results of *HSP70* gene expression in Table 9 shows that there are high significant differences between the *A. fumigatus* isolates that incubated at different incubation temperatures. At 28°C of the incubation, which represented the optimum incubation temperature, the mean of folds was reached to 1.00 in all the samples. The maximum mean of folds was at 37°C of the incubation which reached to (2.44), while the maximum mean of folds was at 45°C of the incubation which reached to (1.14). This means that the level of *HSP*70 gene expression decreased when the temperature increases to 45 ˚C. These results are in accordance with Sharafi *et al*, (25), they mentioned the levels of *HSP*70 gene expression in *A. fumigatus* is highest at temperatures ranging from 37 to 42˚C

Table9. Fold of *HSP*70 gene expression of *A.fumigatus* depending on ΔΔCt method..

Temperature of Incubation	Sample	Ct of reference *aspHS*	Ct of target *HSP70* gene	ΔCt	ΔΔCt	Relative quantification (Folds)#	Mean of Relative quantification (Folds) #
	1	29.21	25.27	-3.93	0.00	1.00	
	2	29.03	28.45	-0.58	0.00	1.00	
	3	26.67	25.89	-0.78	0.00	1.00	
28	4	32.53	27.93	-4.60	0.00	1.00	1.00 ± 0.00 a
	5	28.02	28.48	0.47	0.00	1.00	
	6	27.31	27.68	0.37	0.00	1.00	
	7	26.07	26.37	0.31	0.00	1.00	
	1	27.46	27.65	0.18	4.11	0.06	
	2	27.72	28.25	0.53	1.11	0.46	
	3	30.07	30.28	0.21	0.98	0.51	
37	4	23.56	23.71	0.15	4.75	0.04	0.865 ± 0.32 ab
	5	30.27	29.45	-0.82	-1.29	2.44	
	6	32.79	32.88	0.10	-0.27	1.21	
	7	27.99	27.88	-0.11	-0.42	1.34	
	1	27.12	28.63	1.51	5.44	0.02	
	2	27.28	27.77	0.49	1.07	0.48	
	3	26.20	26.03	-0.17	0.61	0.66	
	4	30.23	31.27	1.04	5.65	0.02	0.495 ± 0.14 b
45	5	28.41	29.82	1.41	0.94	0.52	
	6	28.73	28.91	0.18	-0.19	1.14	
	7	28.17	29.15	0.98	0.67	0.63	
	LSD value	---	---	---	---	0.561 *	0.539 *

.* (P<0.05).. #Fold = $2^{-\Delta\Delta Ct}$

Conclusion

1- *Aspergillus fumigatus* was the most frequent species of *Aspergillus* isolated from aspergillosis patients (46.9%), followed by *A. niger* (28.6 %) and *A. flavus* (24.5 %).

2- The patient group (50-59) years noticed to be more vulnerable to aspergillosis diseases, and this infection was more abundant in male than female.

3- *HSP70* gene expression showed the level increased at 37 ˚C but decreased when the temperature increases to 45 ˚C.

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