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MOLECULAR IDENTIFICATION OF *ASPERGILLUS FUMIGATUS* BY DETECTION *ASPERGILLUS* HEMOLYSIN *ASPHS* GENE

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Abstract

One hundred forty sputum specimen were collected from patients suspected have infection with aspergillosis attended from National Center for Thoracic and Respiratory Diseases (NCTRD), Baghdad Teaching Hospital, Oncology Teaching hospital and Imamein Kadhimein madical. from the beginning of September 2018 to the end of February 2019. Out of 140 (100%) samples, 49(35%) samples have been detected for the aspergillosis. The results of fungal culturing was found that *Candida* spp. was the most common fungi isolated from sputum specimen (40%) followed by *Aspergillus* spp. (35 %) and *Penicillum* spp. (10%). The frequent species of *Aspergillus* identified included *A. fumigatus* 23(16.43%), *A. niger* 14 (10%), followed by *A. flavus*12(8.57%), depending on the morphologic characteristics of these species on the culture media including Sabouraud's Dextrose Agar with chloramphenicol (SDAC), *Potato Dextrose* Agar (PDA) and, Corn Meal Agar (CMA) and microscopic characteristics after staining by Lactophenol cotton blue. The age group (50-59) years appeared to be more susceptible to infected by aspergillosis with percentage at (24.5)% while the age group less than 20 years was recorded 5 with percentage(10.2 %). The results also revealed that no significant differences between male and female with aspergillosis infection. The genomic DNA of *A.fumigatus* isolates were extracted using wizard genomic DNA purification kit, the extracted genomic DNA was analyzed using 1% agarose gel electrophoresis, and then the concentration and purity of the extracted genomic DNA were determined using Nanodrop spectrophotometer device. To detect *A.fumigatus* isolates by molecular methods, the extracted genomic DNA of these isolates was submitted for amplification to detect the *aspH*S gene by the singleplex PCR method using species-specific primers for these *A.fumigatus*, to sum up 17 (23%) out of 49 samples were detected for *A. fumigatus*, by observing the singleplex PCR product of *aspH*S gene with ~108bp, in the agarose gel electrophoresis. *A.fumigatus* have the ability to live and tolerance at different degrees of temperatures (28-45)°C. The Pathogenicity of *A. fumigatus* depends on immune status of patients. This species has numerous virulence factors such as gliotoxin, hemolysin and phospholipase enzymes, that enables this species to invade host tissues causing opportunistic infections. The aim of this study was to detect *A. fumigatus* from sputum specimen by traditional method and molecular method. 140 sputum specimen were isolated from patients with Pulmonary diseases (excluded from patients suffering from tuberculosis) as well as patients with leukemia, Lymphoma, lung cancer and other types of cancers. A total of 131 culture of fungal isolate *A.fumigatus* represented 23 (16.43). The identification of *A.fumigatus* by traditional method is a routine work based on the phenotype and color of colony on SDACC but they do not give accurate results, so result of molecular method (PCR) represent 17 (12.14%). The molecular method (PCR) is a rapid, sensitive and specific method for detection of *A.fumigatus*.

Key words: *Aspergillus fumigatus*, hemolysin, aspHS gene

Introduction

Aspergillus spp. are worldwide filamentous environmental molds and can cause abroad spectrum of infections in humans, including hypersensitivity reactions, chronic pulmonary infections, and acute life-threatening infections, the latter occurring primarily in immune-compromised individuals (Cornely *et al.*, 2017). Among 250 species of Aspergillus spp., less than 40 species are oblivious to cause diseases in humans (Geiser, 2009). Including *A. fumigatus, A. niger, A. terrus, A. nidulans* and *A. flavus*. From these pathogenic species, *A. fumigatus* is the most pathogenic and responsible for approximately 90% of all invasive aspergillosis infection in immunocompromised individual or allergic for individual with atopic immune system (Marr *et al.*, 2002; Howard and Arendrup, 2011). Pulmonary disease caused by Aspergillus, mainly A. fumigatus, presents with a spectrum of clinical syndromes in the lung (Soubani and Chandrasekar, 2002). This species leading to complications

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including a spectrum of diseases from allergic responses to the organism (allergic bronchopulmonary aspergillosis), to colonization with *Aspergillus* species, aspergilloma or fungus ball, other superficial conditions (external ear colonization), invasive infection (invasive pulmonary aspergillosis) and other clinical syndromes of tissue invasion) (Patterson, 2011). Patients at highest risk for invasive aspergillosis include those with prolonged neutropenia; recipients of hematopoietic stem cell transplants or solid organ transplants, especially lung transplants; patients with advanced AIDS; and those with chronic granulomatous disease (Patterson *et al.*, 2000; Segal and Romani, 2009).

Aspergillus fumigatus 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 (Jenks and Hoenig, 2018).

The hypothesis suggested that most important genes involved in high temperature tolerance is heat shock protein (HSP) gene, controlling the production of HSPs (Burnie *et al.*, 2006). HSPs are generally present in eukaryotic and prokaryotic cells (Zhang *et al*., 1998) and their expression levels increase under stress conditions.. It has been shown that the expression of HSP70 prepares a condition for fungi to adapt to new environmental situations (Allendoerfer *et al*., 1996).

The hemolysin produced by *A. fumigatus* as a hemolytic toxin is another secondary metabolites that promotes aspergillosis and it may also promote the opportunistic infections (Nayak *et al.,* 2013). Hemolysins are capable of lysing red blood cells as well as nucleated cells. One cluster of these proteins, including hemolytically active representatives has been identified as the Aegerolysin family. Aegerolysins form the largest group of fungal hemolysins that have been characterized. They are generally small in size (15 – 20 kDa) with a β-sheet structure, conserved cysteine residues, and characterized by a large number of aromatic and acidic residues. These proteins are stable over a wide range of pH but are heat labile above temperatures of 60–65°C (Nayak *et al.*, 2013). A. fumigatus possesses two aegerolysin hemolysins that belong to the aegerolysin family of proteins, and a third hemolysin that has been identified as an asp-hemolysin-like protein (Wartenberg *et al.,* 2011). Asp-HS is secreted by A. fumigatus into the environment and can kill cells that are in the vicinity of the spore and it is enables to disrupt blood cells and release of iron, an important growth factor for microbes especially during infection (Malicev *et al.,* 2007).

Meterials and Methods

Samples collection

During the period of study from September 2018 to February 2019, 100 aspergillosis sputum were collected from patients clinically diagnosed with aspergillosis, Pulmonary diseases (excluded from patients suffering from tuberculosis) as well as patients with leukemia, Lymphoma ,lung cancer and other types of cancers ,this specimens were collected from National Center for Thoracic and Respiratory Diseases (NCTRD), Baghdad Teaching Hospital, Oncology Teaching hospital and Imamein Kadhimein madical. The patient's ages ranged from 6 months to 3 years. This study was carried out after obtaining the approval from the Institute of Genetic Engineering and Biotechnology for Post Graduate Studies/ Baghdad University and Ministry of Health/ Iraq. Sputum were taken from the patients under sterile conditions and immediately transferred to the laboratory to inoculate into brain heart infusion broth for 4-6 hours, then inoculated on Sabaroud dextrose agar ,Potato dextrose agar and Corn meal agar (Hi media , India) at 28°C for 7 days then direct exam by Lactophenol cotton blue stain under light microscope (40x).

DNA extraction

Genomic DNA was extracted from the *A. fumigatus* isolates using a commercial wizard genomic DNA purification kit according to manufacturer's instructions (Promega, USA) with some modification. Briefly, 1 ml of an overnight *A. fumigatus* culture grown at 28°C in SDA broth (Sigma, USA) was transferred to a 1.5 ml micro centrifuge tube. The microcentrifuge tube was centrifuged at 14,000 rpm for 3 minutes to pellet the cells and the supernatant was removed. 600 µl of nuclei lysis solution (wizard genomic DNA purification kit) was added and gently pipet until the cells is resuspended. The microcentrifuge tube was incubated in water bath at 80°C for 5 minutes to lyse the cells; then cool to room temperature. 3 µl of RNase solution (wizard genomic DNA purification kit) was added to the cell lysate and the microcentrifuge tube was inverted for 5 times to mix. The microcentrifuge tube was incubated at 37°C for 60 minutes and cool to room temperature. 200 µl of protein precipitation solution (wizard genomic DNA purification kit) was added to the RNase-treated cell lysate and vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate. The microcentrifuge tube was incubated on ice for 5 minutes and centrifuged at 14,000 rpm for 5 minutes. The

supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600 µl of room temperature isopropanol. The microcentrifuge tube was gently mixed by inversion until the thread-like strands of DNA form a visible mass and centrifuged at 14,000 rpm for 5 minutes. The supernatant was carefully pour off and the microcentrifuge tube was drained on clean absorbent paper. 600 µl of room temperature 70% ethanol was added and then the microcentrifuge tube was gently inverted several times to wash the DNA pellet. The microcentrifuge tube was centrifuged at 14,000 rpm for 2 minutes and the ethanol was carefully aspirated. The microcentrifuge tube was drained on clean absorbent paper and the pellet was allowed to air-dry for 15 minutes. 100 µl of DNA rehydration solution (wizard genomic DNA purification kit) was added to the microcentrifuge tube and the DNA was rehydrated by incubating at 65°C for 1 hour. The solution was periodically mixed by gently tapping the microcentrifuge tube and the DNA sample was stored at - 20°C until use.

DNA quantification

The extracted DNA from the 23 *A. fumigatus* isolates was quantified spectrophotometrically at O.D. 260/ 280 nm with ratios 1.4-1.5. The sensitivity of the *A. fumigatus* -F and *A. fumigatus* -R primers was evaluated by PCR amplification for serial diluted concentrations (10-100 ng) of purified genomic DNA isolated from *A. fumigatus*.

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*., 2008), and then the general properties of these primers were checked by using Oligocalc Oligonucleotide Porperties Calculator program, This set of primers was designed based on the conserved region in *A. fumigatus*, primers were synthesized by Alpha DNA, Kanda. The primers sequence of *aspHS* gene and their size of product are shown in (Table 1).

Singleplex PCR master mix

The singleplex PCR reaction of *aspHS* gene detection of *A. fumigatus* was performed in 25 µl volumes containing 5.5 µl of nuclease free water, 12.5 µl of Go Taq Green Master Mix 2X containing (GoTaq DNA polymerase supplied in 2X Green GoTaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM MgCl2, yellow and blue dyes which function as loading dyes when reaction products are analyzed by agarose gel electrophoresis), 2.5 µl of 20 pmol *A.fumigatus* -F primer and 2.5 µl of 20 pmol *A.fumigatus* -R primer and 2 µl of the genomic DNA sample. The mixes were overlaid with 2 drops of mineral oil Table 2.

Table 1: The primers sequences of *aspHS* gene of *A.fumigatus* and their product size(Al- Bayati *et al.*, 2016).

Primer Name	Seq.	Tm	Size of product
aspHS –F	AGTCCACTGGGACTGTCCAT	58	~108bp
aspHS –R	GCACCACCATACTTGTTCCA		

Singleplex PCR program

Singleplex PCR was carried out in a thermal cycler (Applied Biosystem, 9902, Singapore) according to the PCR program described by (14), with some modification. Briefly, the amplification of 16S rRNA gene of *A.fumigatus* was carried out with initial denaturation at 95°C for 6 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for *A. fumigatus* -F and *A. fumigatus* -R primers for 90 seconds and extension at 72°C for 2 minutes. The thermal cycles were terminated by a final extension for 10 minutes at 72°C.

Singleplex PCR products analysis

The analysis of singleplex PCR products of *aspHS* gene of *A.fumigatus* were performed on 1% agarose gel. The 100 bp DNA ladder (Promega, USA) was used and the gel was run at 100 volt for 45 minutes at room temperature. The singleplex PCR products were stained with ethidium bromide and visualized by an image analyzer (ChemiImager 5500, Alpha Innotech, USA).

Results

Conventional methods

One hundred and forty sputum specimen were collected from patients with Pulmonary diseases (excluded from patients suffering from tuberculosis) as well as patients with leukemia, Lymphoma ,lung cancer In the present study the most common isolated was *Candida spp*.56(40%) then *A.fumigatus* 23(16.43%). Followed by *A. niger* 14(10%). then *A. flavus* 12(8.57%). The last fungal isolated *Penicillium spp*. 14(10%). Then culture on SDAC total of 140 specimen collected 49 identify as *Aspergillus spp*. as table 4. The conventional methods include culture of *A.fumigatus* on SDAC after 7 days incubation at 28ºC .Colonies appear powdery colony, which was at first white, but then turned dark green to gray, with a whitish boundary. The reverse side was white to tan . Ranged diameter of colony 60 to 70 mm (figure1A, B), then direct examination by using Lactophenol cotton blue stains and examined Under light microscope (40x)

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	10µM/µl	2
Nuclease free water	-	4.5
DNA sample	-	4
Total volume	-	25

Table 3: 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.	30
3	Annealing	58°C	30 sec.	
4	Extension	72°C	60 sec.	
5	Final extension	72°C	7 min.	1
6	Storage	4°C	"	-

A.fumigates is characterized by the formation of a conidiophore with columnar conidial heads consisting of flask-shaped vesicles (19-31 µm diameter), uniseriate phialides, and long chains of conidia. Conidia are bluish-green to pale green, size 2-3 µm and globose finely rough generally hydrophobic. The conidiophores are usually less than 300 µm in length and smooth Fig. 2.

Analysis of extracted DNA of *A.fumigatus* isolates

After performing of the DNA extraction from *A.fumigatus* isolates, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA using 1% agaros gel at 7 volt/ cm for 45 minutes (Fig. 4).

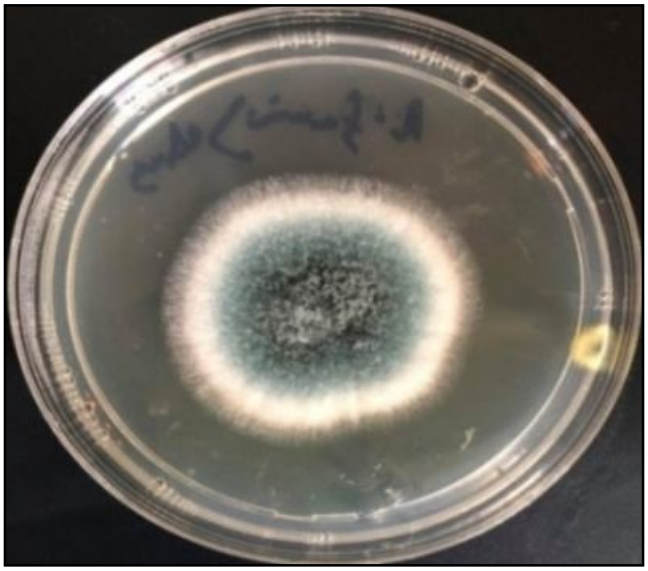

Fig. 1: *A.fumigatus* on SDAC medium after 7 days incubation at 28 ºC, Plate (A) top view.

Table 4: Fungal isolated in this study.

Samples	NO.	%
Aspergillus fumigatus	23	16.43
Aspergillus niger	14	10
Aspergillus flavus	12	8.57
Candida albican	31	22.14
Candida tropicalis	13	9.29
Candida glabrata	8	5.71
Candida parapsilosis	4	2.86
Penicillium spp.	14	10
Mix*	12	8.57
Negative	9	6.43
Total	140	100
Chi-Square (χ^2)	—	8.936 **
** (P<0.01).		

*.*Afumigatus, A.niger,A.flavus,Candida albican, Candida tropicalis, Candida glabrata, Candida parapsilosis* and *Penicillium* spp.

Analysis of singleplex PCR products of *aspHS* gene for *A.fumigatus*

On the basis of the *aspHS* gene sequence, a product of ~108 bp was amplified by singleplex PCR with *A.fumigatus* -F and *A.fumigatus* -R primers. The singleplex PCR products and 100 bp DNA ladder were resolved by electrophoresis. 5µl of the singleplex PCR product were loaded on 1.5% agarose gel and run at 100 volt/ cm for 60 minutes. The gel was stained with ethidium bromide solution (0.5 µg/ ml) for 15-30 minutes; finally, bands were visualized on UV transiluminator at 350 wave length and then photographed by using photo documentation system. The singleplex PCR result was considered positive for *A.fumigatus* when there was presence of ~108bp singleplex PCR product band of *aspHS* gene for the *A.fumigatus* on the agarose gel electrophoresis, no amplification was observed with negative control (Fig. 5). In 49 *Aspergillus spp*. clinically

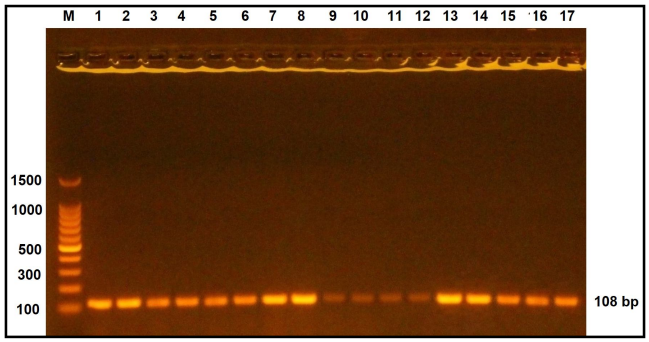

Fig. 5: Agarose gel electrophoresis of singlplex PCR amplified products for *aspHS* gene of *A. fumigatus* were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. Lane M :100bp DNA marker. Lane (1-17): PCR products of *aspHS* gene showed positive result with positive bands of 108 bp of *A. fumigatus*.

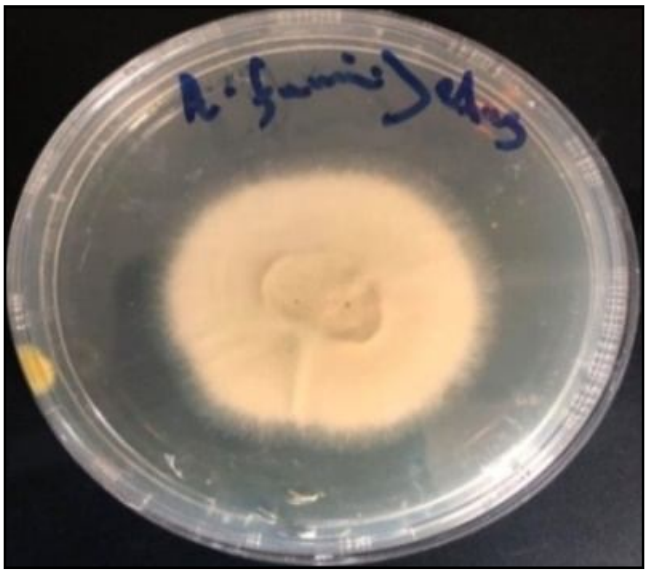

Fig. 2: *A.fumigatus* on SDAC medium after 7 days incubation at 28 ºC, Plate (B) reverse view.

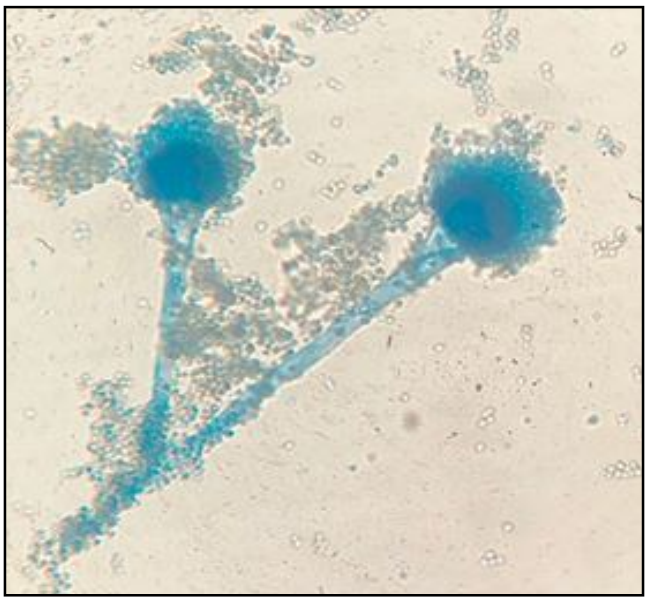

Fig. 3: *A.fumigatus* under light microscope (40x).

diagnosed with aspergillosis , the singleplex PCR method showed that *aspHS* gene (108bp) exists in 17(83.3%) of isolations out of 23(100%) that were positive identified by morphological and microscopic methods.

Discussion

Identification of this fungal isoklate by using the conventional methods include culture, Gram staining and biochemical tests which were go together with study conducted by (Raper and Fennell, 1965). Showed that the fungal cultures were positive in 131(93.6%) patients versus 9(6.4%) patients revealed negative bacterial culture, the most common isolated was *Candida spp.* 56(40%) then *A.fumigatus* 23(16.43%). Followed by *A. niger* 14(10%). then *A. flavus* 12(8.57%). The last fungal

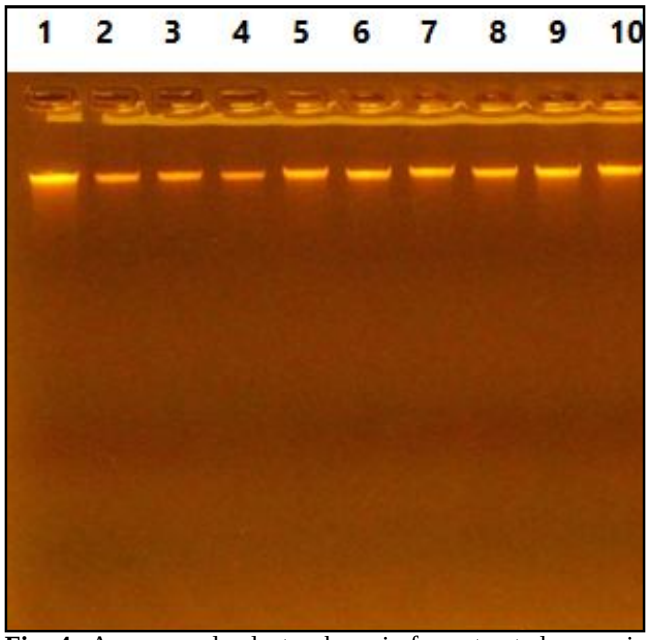

Fig. 4: Agarose gel electrophoresis for extracted genomic DNA of *A. fumigatus* isolates using 1% agarose gel at 7volt/cm for 1 hour. Lane 1-10: Extracted genomic DNA.

isolated *Penicillium spp*. 14(10%). Direct Examination of sputum by KOH 10% is a bad and old method to detected about fungi because its reaction with sputum and KOH can damage the microscope stage if the slide overflows. In addition, crystals can form on standing so that reading of smear becomes difficult. *A.fumigatus* colonies on SDAC after 7 days incubation at 28ºC, powdery colony, which was at first white, but then turned dark green to gray, with a whitish boundary. The reverse side was white to tan . Ranged diameter of colony 60 to 70 mm (Fig. 1A,B). Under light microscope (40x) *A.fumigatus* is characterized by the formation of a conidiophore with columnar conidial heads consisting of flask-shaped vesicles (19-31 μm diameter), uniseriate phialides, and long chains of conidia. Conidia are bluish-green to pale green, size 2-3 µm and globose finely rough generally hydrophobic. The conidiophores are usually less than 300 µm in length and smooth (Fig. 2). The diagnosis of A.fumigatus by traditional method is a routine work based on the phenotype and color of colony on SDAC but they do not give accurate results The identification of Aspergillus spp was conducted according to (Raper and Fennell, 1965; Ellis *et al.*, 2007).

The detection of fungal pathogens in clinical samples by PCR requires the use of extraction methods that efficiently lyse fungal cells and recover DNA suitable for amplification. The most difficult steps in the isolation of *Aspergillus* DNA is to disrupt the cell wall without causing damage to genomic DNA (Bir *et al.*, 1995) and

the isolation methods should provide a sufficient amount of pure DNA which can be further amplified by PCR while not containing inhibitors at the same time inhibitors which would block PCR (Olexová *et al.*, 2004).

The result of current study showed the important use of commercial kits such as genomic DNA extraction kit as a rapid extraction method, good quality high molecular weight DNA and very useful in avoiding the laboratory contamination during DNA extraction. The results indicated that molecular method (PCR) is a more sensitive and specific than cultural method since it is more stable system in targeting the genes and less affected by environmental circumstances, while cultural method is less sensitive and less specific than molecular method. The molecular method (PCR) to diagnosis of *A.fumigatus* by detected of *aspHS* gene is a more sensitive and specific than cultural method for this detection (Al- Bayati *et al*., 2016).

At a comparison between the conventional and molecular methods, we think that the incubation period is uncertain and inappropriate growth media, in addition to the contamination of culture in identification methods could be the reason for false positive results, this may explain the false positive results in 32(65.3%) out of 49(100%) samples clinically diagnosed with aspergillosis infection by using these conventional methodes, by using pair primers targeted the aspHS gene (~108 bp) showed a positive result in 17(34.6%) out of 49(100%) samples that were positive by the conventional methods. patients who were also gave positive results by these conventional methods. The benefits of molecular methods are more sensitive, more qualitative for results, materials available, but the drawback of molecular methods is costly. These explanations made molecular methods relatively more accurate than conventional methods (Gehlot *et al.,* 2011). 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 (Abad-Diaz-De-Cerio *et al*., 2013). Yokota *et al*., (1977) and Kumagai *et al*., (1999) they found gene encodes for a haemolysin, with activity against rabbit and sheep erythrocytes and cytotoxic effects on macrophages and endothelial cells in vitro. The *aspHS* gene is more highly expressed in vivo than in vitro (Gravelat *et al*., 2008) and it has also recently been reported as a major *in vitro*-secreted protein (Wartenberg *et al.,* 2011).

Conclusion

Aspergillus fumigatus is the mainly cause of aspergillosis and it can be molecularly diagnosed by investigating *aspHS* gene was commanly found in *A. fumigatus* as a virulence factor.

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