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DETECTION OF *ASPERGILLUS* SPECIES FROM EAR AND NOSE SWABS IN A GROUP OF IRAQI DIABETES MELLITUS PATIENTS

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ABSTRACT
Fungal infections are not common in normal healthy persons; however, in the immunocompromised patients, opportunistic fungi can cause fatal infections. The aim of this study was to detect *Aspergillus spp.* in ear and nose swabs of diabetes mellitus (DM) patients and to study some of the virulence factors of *A. fumigatus* by using PCR technique. This is a prospective study conducted on 67 diabetic patients and 30 healthy persons as a control group at Ear, Nose and Throat (ENT) department of Al- Kindy teaching hospital for four month's screening for fungal isolates and the diagnosis was confirmed by relevant investigations using cultural method for identification and isolation of *Aspergillus spp*. and PCR for detecting *Aspergillus spp* virulence gene *aspHS*. The overall percentage of *Aspergillus spp.* infection in DM patients was 22.68% the infection was more in males compared with females and was more in adults compared with young patients. The *aspHS* gene was common in *A. fumigatus* isolates with a percentage of 66.67%. *Aspergillus* infection occurred commonly in adult males in diabetic patients and the *aspHS* gene was commonly found in *A. fumigatus* as a virulence factor.

KEYWORDS: *Aspergillus spp*., immunocompromised patients (ICP), *aspHS* gene.

INTRODUCTION

Fungal infections are not common in normal healthy persons; however, in the immunocompromised patients (ICP), opportunistic fungi can cause fatal infections (Badiee and Hashemizadeh, 2014). They can cause invasive infections in ICP when they invade the mucosal tissues. The ICP patients such as hematologic malignancies, a plastic anemia, Diabetes mellitus, AIDS, patients on chemotherapy, steroids and organ transplantation (Kaplan *et al*, 2009). The most common fungi which invade mucosal tissues including species like Aspergillus, *Rhizopus*, *Mucor*, *Absidia* and *Rhizomucor*. However, *Bipolaris* and *Fusarium* are less common. Fungi can reach to the sinonasal cavities by spores inhalation (Soler and Schlosser, 2012). Aspergillosis is a disease that caused by *Aspergillus* species. The common species of Aspergillosis are *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*. *A. fumigatus* is mostly seen in ICP (Mosquera and Denning, 2002; Mayr and Lass- flörl, 2011). This species has numerous virulence factors such as gliotoxin, hemolysin and phospholipase enzymes, that enables this species to invade host tissues causing opportunistic infections (Dabo and Yusha, 2007; Abad *et al*, 2010).

MATERIALS & METHODS

Samples were collected from 67 DM patients (29 female and 38 male, aged 22-82 years). Swab samples were taken from nose and ear (17 from the ear and 50 from the nose) using sterile transport swabs, control group included 30 healthy people. This study was conducted in ENT department of Al-Kindy Teaching Hospital- Unit of infectious clinical diseases- college of medicine/ Baghdad University between November 2014 and June 2015. The collected swabs were examined for the presence of fungal species. Each swab was inoculated onto two plates of Sabouraud's dextrose agar with chloramphenicol; one was incubated at (28˚C) and the other one was incubated at (37˚C) for 2 to7 days. *All Aspergillus spp. isolated from nose and ear swabs of DM patients and healthy people* were identified by the colony characteristics and morphological appearance. The identification was confirmed by the conidial morphology by preparing slides with one drop of lactophenol cotton blue stain and observed microscopically using a light microscope (magnification X40).

The data were represented by counting the number of fungal colonies growing from nasal and ear swabs. The identification of *Aspergillus spp* was conducted according to Raper & Fennell, 1965; Ellis *et al.*, 2007 and Afzal *et al.*, 2013. As colonies were detected; they were subcultured on Czapeck's dox agar (CZA) media for specific species identification according to colony characteristics and microscopical characteristics

Detection of hemolysin produced by *A. fumigatus*

All *A. fumigates* isolates were inoculated on Sheep Blood Agar (SBA) and the plates were incubated at 37˚C for 7 days. The evidence of hemolysin in the medium can be detected by the presence of a clear hemolysis in the medium as a positive result. This test can be used to confirm *A. fumigatus* that can produce hemolysin toxin (Donohue *et al.*, 2006).

The detection of *Aspergillus* hemolysin (*aspHS*) gene by polymerase chain reaction (P.C.R) technique

DNA extraction

DNA extraction was conducted depending on the attached leaflet Kit equipped with the company bio-WORLD (Fungi/ Yeast genomic DNA isolation kit). Then, samples were subjected to agarose gel electrophoresis.

Polymerase chain reaction (PCR)

PCR technique was used to amplify DNA coding for the *aspHS* gene. This gene was designed by the institute of genetic engineering and biotechnology/ University of Al-Nahrain and a pair of primers was used as shown in the Table (1):

TABLE 1: primers used in the polymerase chain reaction PCR

Primer	Nucleotide sequence	Length	Anneling
aspHS (F)	5-AGTCCACTGGGACTGTCCAT-3	20	62˚c
aspHS (R)	5-GCACCACCATACTTGTTCCA-3	20	60˚c

The interaction size was made upon 20µl depending on the leaflet attached with the Kit by Alpha DNA Company. The components of the mixture and their sizes are listed below in Table (2).

TABLE 2 Components of the mixture and their sizes

Interaction components	Size (µl)
Green Master mix	10
Primer Forward	1
Primer Reverse	1
Deionized D.W	6
DNA	2
Total	20

To amplify the DNA samples using PCR technique, the program was set out as listed below in Table 3.

TABLE 3: DNA amplification program

	Steps	Temperature (˚C)	Time	Number of Cycles
1	Initial Denaturation	95	5 min	1
2	Denaturation	95	30 sec	
3	Annealing	62	35 sec	35
4	Extension	72	35 sec	
5	Final Extension	72	7 min	1

TABLE 4. Distribution of sample study according to study group and gender

Study group	Sex	Positive carriers	Negative carriers	Total	Chi-square
DM	Male	10 (26.32%)	28 (73.68%)	38 (39.18%)	11.496 **
	Female	9 (31.03%)	20 (68.97%)	29 (29.89%)	9.071 **
Control	Male	1 (11.11%)	8 (88.89%)	9 (9.28%)	14.188 **
	Female	2 (9.52%)	19 (90.48%)	21 (21.65%)	14.372 **
Total	---	22 (22.68%)	75 (77.32%)	97 (100%)	12.944 **
** (P<0.01)					

RESULTS & DISCUSSION

The number of positive DM carriers was 19 (28.36%) [16 (84.21%) with single colony, and 3 (15.79%) with mixed colonies of *Aspergillus spp.*]. Table (4) demonstrates the distribution of fungal isolates among different study groups. It appeared that 19 out of 67 patients [10 (26.32%) males and 9 (31.03%) females] gave positive results and 48 (71.64%) gave negative results. About control group, 3 out of 30 (10%) gave positive results and 27 (90%) gave negative results.

Statistical analysis using Chi-square test revealed highly significant differences between groups ($P \leq 0.01$), which indicated that there was a relationship between the groups and the percentage of positive or negative carriers. The results also showed that there was a highly significant difference between the positive and negative carriers for each group as shown in Table (4). The fungi are usually secondary invaders of the tissue formerly rendered susceptible by bacterial infection, physical injury or excessive accumulation of cerumen in the external auditory canal (Jagadish, 2013). On the whole, moist environments are the most appropriate conditions for the yeast and spores life; however, warm climate and high humidity are the appropriate environmental conditions for otomycosis infections. These conditions are most common in the tropical region, where the warm climate and humidity are at the highest levels. On the other hand, diabetes, swimming and bathing, trauma and irrational usage of local antibiotic are considered predisposing factors for otomycosis (Kondity *et al.*, 2015).

The recent results indicated that the genus *Aspergillus* was isolated from the nose of control samples but in low levels, which could be related to the diffusion of *Aspergillus* spores in the air, dust, and soil which resulted in air pollution. The adherence of these fungi to the dust particles and the entry of these particles by inhalation in

the nose lead to cause fungal colonization. The conditions of the upper respiratory tract are being warm and moist in favor for the proliferation of the molds (Razmpa *et al.*, 2007). The imbalance in local immunity of nasal mucosa is more important than invasive fungi (Razmpa *et al.*, 2007). Patients had one or more predisposing factors to disseminate fungal infection such as diabetes mellitus and hematologic malignancy (Jahromi and Khaksar, 2005). The distribution of fungi according to the age is shown in Figure (1). It was found that the fungal infection increased with the age of patients, as the results of higher infection levels in ages over forty (36.84%) compared with the level (26.32%) in ages less than forty years old.

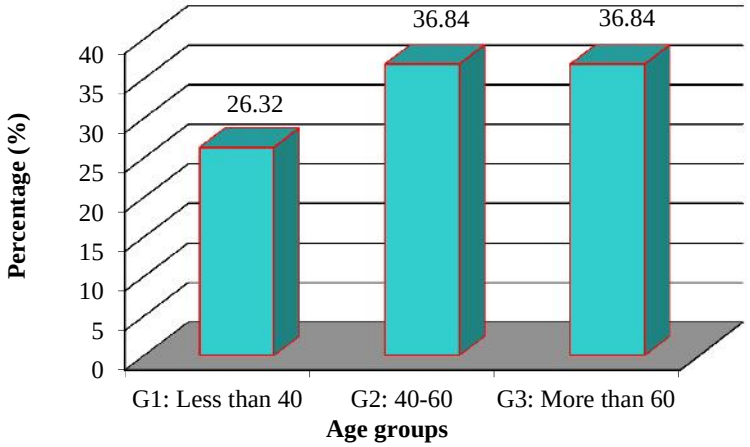


FIGURE 1. Distribution of fungi according to age groups

FIGURE 1. Distribution of fungi according to the age

The results of fungal infections are more common in the third and the fifth decade of age could be related to the high activity of these age groups as they spending more time out of their house in tracking livelihood and exposed to fungal spores more than other age groups (Venkateswarlu, 2015). It was suggested that fungal infections of the paranasal sinus are uncommon and occur in patients with diabetes or in individuals who are immunocompromised. Early detection and treatment are vital for such infections because of the high mortality rate (Vartanian and Meyers, 2012) especially in mucormycosis.

Distribution of *Aspergillus* species

Table (5) shows the distribution of *Aspergillus* species isolated from nose and ear of DM patients. The isolation rates were as follow: *A. niger* 11 (42.31%) which was the most common species, followed by *A. flavus* 8 (30.77%), *A. fumigatus* 6 (23.08%) and *A. terreus* with lowest levels 1 (3.85%). Figures 2-5 show *Aspegillus spp* colonies and their morphology.

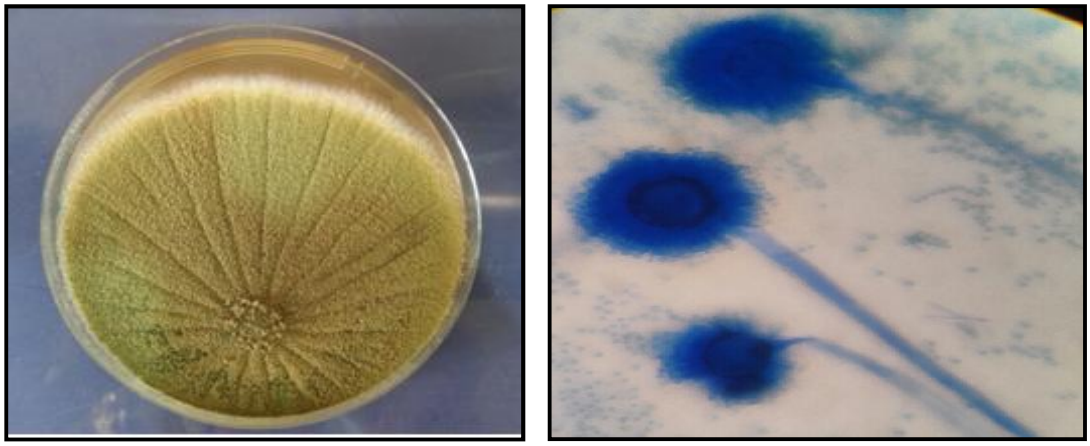
Statistical analyses revealed highly significant differences between groups DM and control groups (P<0.01), and also between the rates of different species of *Aspergillus* for each group.

TABLE 5: Distribution of *Aspergillus spp.* isolated from DM patients and control

Groups		*A.fumigatus*	*A. flavus*	*A. niger*	*A. terreus*	Total	Chi-square
DM	nose	4 (23.53%)	6 (35.29%)	6 (35.29%)	1 (5.88%)	17 (65.38%)	9.224 **
	ear	2 (33.33%)	1 (16.67%)	3 (50.00%)	0 (0.00%)	6 (23.08%)	12.084 **
Control	nose	0 (0.00%)	1 (33.33%)	2 (66.67%)	0 (0.00%)	3 (11.54%)	12.912 **
	ear	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0.00 NS
Total		6 (23.08%)	8 (30.77%)	11(42.31%)	1 (3.85%)	26 (100%)	10.436 **

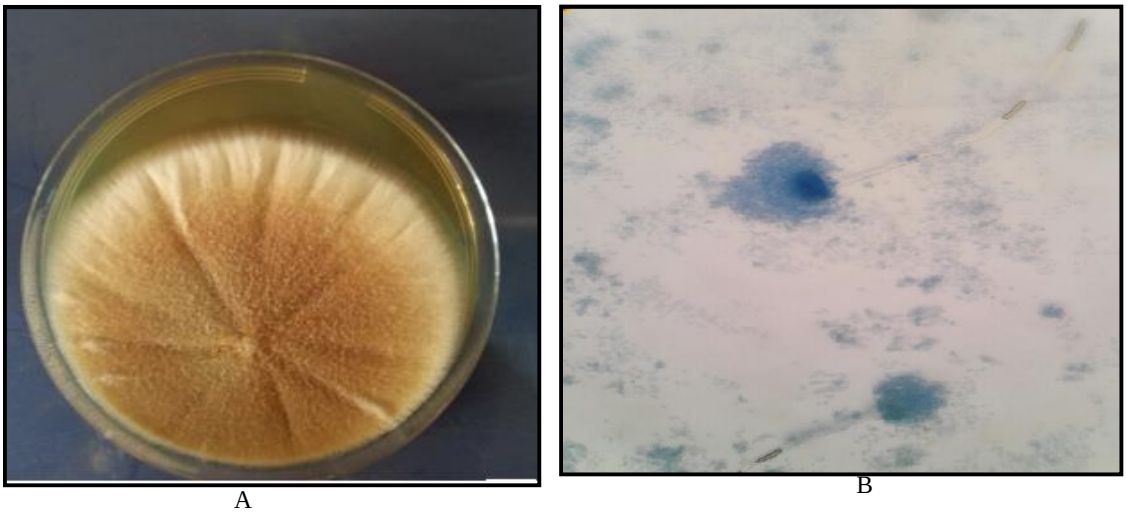
** (P<0.01)

These results agree with Majeed, (2011) who found that *A. niger* is the most common species in a chronic sinusitis infection. The present study also agrees with Razmpa *et al.* (2007) results who found that the isolation rate of *A. flavus* was more than *A. fumigatus* among nasal polyposis infections. Moreover, the results are in agreement with Venkateswarlu (2015) who found that the distribution of fungal species in otomycosis was *A. niger* followed by *A. fumigatus* and *A. flavus*. Kondity *et al.*, 2015, reported same results; they indicated that *Aspergillus spp.* cause otomycosis and the most common species was *A. niger* followed by *A. fumigatus*. However, this study disagree with Thammahong *et al.*, 2015; they found that *A. fumigatus* was the most common species isolated from invasive aspergillosis, followed by *A. flavus*; whereas, *A. niger* was the most species isolated from ear followed by *A. fumigatus* and *A. flavus*. The present results also disagree with Barati *et al.* (2011) who revealed that *A. flavus* was the most common fungus in otomycosis followed by *A. niger*, *A. fumigatus* and *A. nidulans*. Overall, the present study considered that *A. niger* is the main causative agent of otomycosis which agree with Kondity *et al.* (2015).



A B

FIGURE 2A: *Aspergillus flavus* colony on SDA after 7 days
B) *Aspergillus flavus* under light microscope fixed with LPCB 40X



A B

FIGURE 3A: *Aspergillus terreus* colony on SDA after 7 days incubation at 28˚C
B) *Aspergillus terreus* under light microscope (40X)

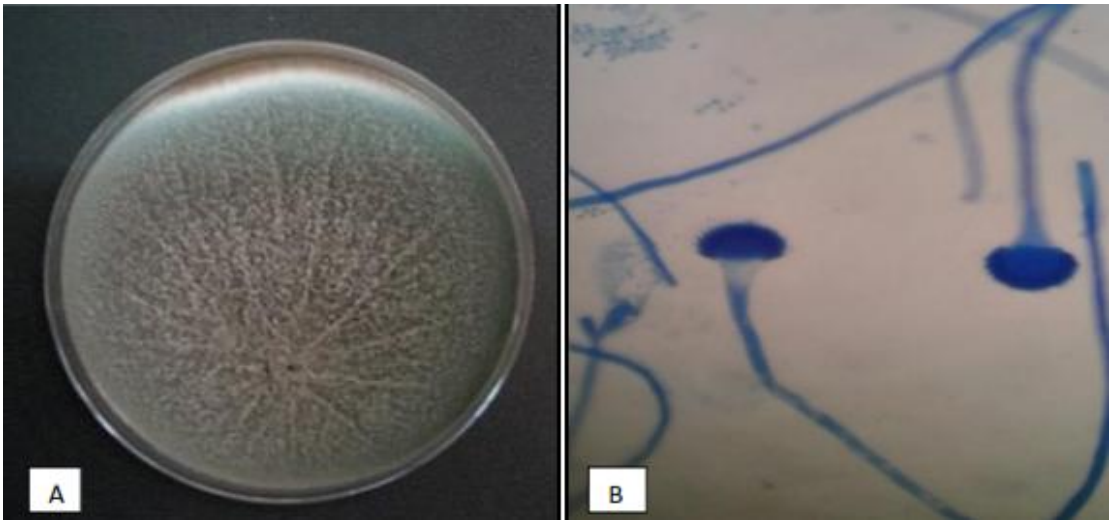


FIGURE 4A: *Aspergillus fumigatus* colony on SDA after 7 days incubation at 28˚ C
B) *Aspergillus fumigatus* under light microscope (40X)

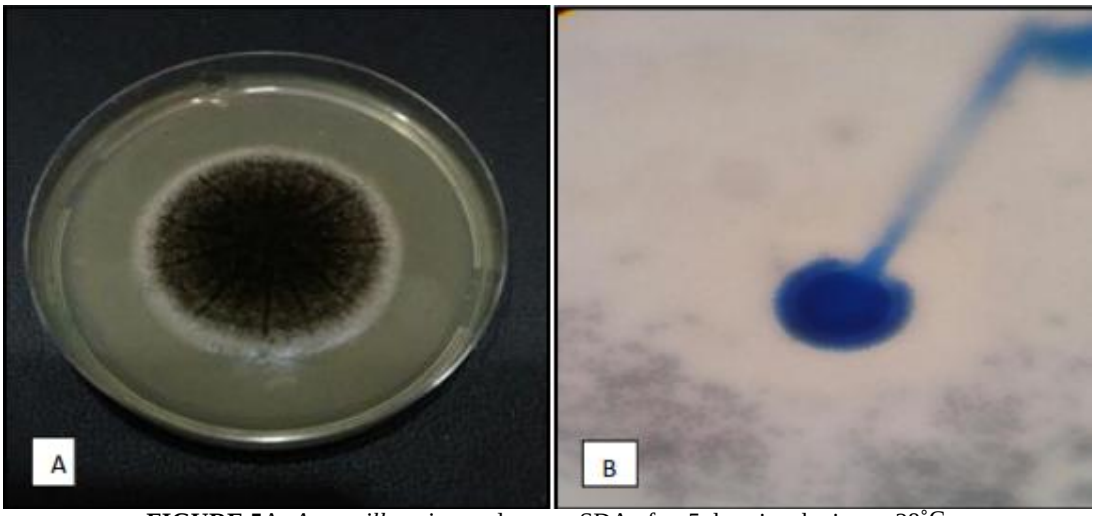


FIGURE 5A: *Aspergillus niger* colony on SDA after 5 days incubation at 28˚C
B) *Aspergillus niger* under light microscope (40X)

Detection of hemolysin produced by *A. fumigatus*

In this study, the identification of *Aspergillus spp.* was conducted by morphological and microscopical characteristics in addition to polymers chain reaction (PCR) technique as suggested by Gehlot *et al.* (2011). A comparison between the cultural and molecular (PCR) methods has been conducted for the detection of hemolysin virulence factor of *A. fumigatus*. The results revealed a significant variation between molecular and cultural methods (Table 6). It has been found that molecular method using PCR technique was able to detect hemolysin in 4 out of 6 (66.67%) isolates of *A. fumigates*, while cultural method detected 3 out of 6 (50%). The results are the same for both molecular and cultural methods with the exception of one isolate which showed positive result by using PCR method nevertheless, it gave a negative result with the cultural method as shown in Table (6) and Figs (6 and 7).

TABLE 6: A comparison between the cultural and molecular methods for the detection of hemolysin virulence factor in *A. fumigatus* isolates

Cultural method	PCR
+	+
-	-
-	-
+	+
-	+
+	+
3/6 = 50%	4/6 = 66.67%
Chi-Square = 5.027 *	

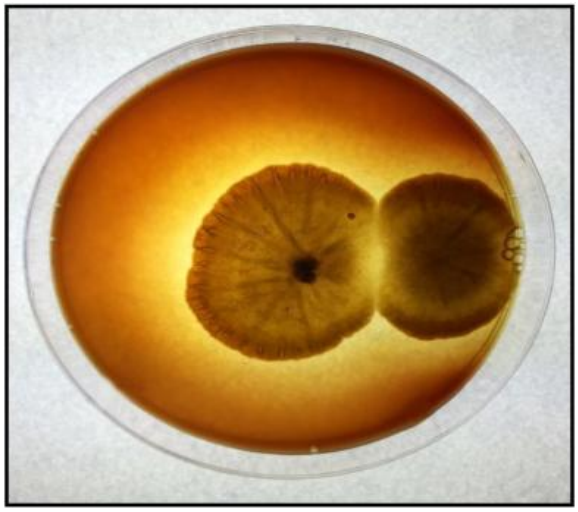


FIGURE 6: *Aspergillus fumigatus* hemolysis on sheep blood agar after 7 days incubation at 37˚C

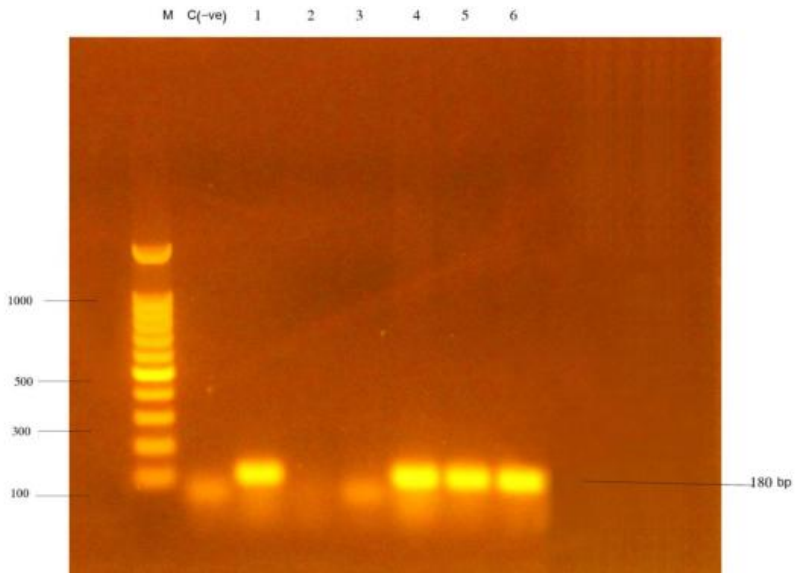


FIGURE 7: Hemolysin gene PCR product on 2% agarose gel showing 4 positive isolates out of 6 of *Aspergillus fumigatus*

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 since it is targeting the genes products. This makes cultural method more affected by environmental factors such as temperature, PH, the availability of nutrients, gene mutations and microbial contamination (Mothershed and whitny, 2006). The results also indicated that *A. fumigates* isolates have virulence factors which enable the fungus to invade the host tissues by cytotoxic and hemolytic activities and they may initiate aspergillosis in patients later (Rementeria *et al.*, 2005; Theeb *et al.*, 2015; Danohue *et al.*, 2006).

CONCLUSION

Aspergillus infection was occurred commonly in adult males in diabetic patients; *A. niger* was more common than other *Aspergillus* species and the *aspHS* gene was commonly found in *A. fumigatus* as a virulence factor.

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