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Phenotypic Identification And Molecular Characterization Of Gliotoxin producing *Aspergillus fumigatus* Isolated From Hunters With Special Emphasis To Clinical Manifestations and Risk Factors In Diyala Province –Iraq

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Abstract

Aim: Isolation of *A. fumigatus* from hunters, identification of *A. fumigatus* by phenotypic characterization and PCR technique , detection of *A. fumigatus* gliotoxin by real time PCR, evaluation of relationship between *A. fumigatus* infection and possible risk factors among hunters In Diyala province –Iraq .
Methods: Ninety nine swabs from mouth, nose and ear of hunters with respiratory signs in Diyala province were included. Samples were cultured on Sabouraud Dextrose Agar. *A. fumigatus* was identified according to morphological features . Genomic DNA was isolated from fungal growth and universal primers ITS1 and ITS4 were utilized to confirm the isolates .
Results : *A.fumigatus* was isolate from hunters mouth ,nose and ear respectively . All *A.fumigatus* isolates were producers for gliotoxin .Age, education level ,economic status and source of water play no role in minimizing infection with *A.fumigatus* among hunters .Unilateral facial swelling associated with *A.fumigatus* isolation from mouth .Unilateral facial swelling, sinus congestion and pain, necrotic black lesions on the hard palate, necrotic black lesions on the nasal turbinate and drainage of black pus from eyes associated with *A.fumigatus* isolation from nose .Unilateral facial swelling, sinus congestion and pain, necrotic black lesions on the hard palate associated with *A.fumigatus* isolation from ear.
Conclusions : *A. fumigatus* infection represent serious problem for hunters . Mouth, nose and ear respectively exposed to gliotoxin producing *A. fumigatus* with certain clinical manifestation . Age, education level ,economic status and source of water play no role in minimizing infection with A.fumigatus among hunters.
Keywords : *A. fumigatus* ,Molecular diagnosis; clinical manifestations Risk factors; Hunters, Iraq

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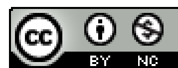

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Introduction

In recent years, aspergillosis opportunistic infections have been recognized as an important cause of morbidity and mortality in developing as well as developed nations [1, 2]. Aspergillosis is reported with increasing frequency in humans and animals from many regions of the world[3, 4]. There are about 600 species of Aspergillus, of which about 27 species of Aspergillus are found to be associated in various clinical disorders of humans and animals [5].

Disease is primarily caused by *A.fumigatus*, opportunistic filament forming moulds [3, 6]. These fungi are widely prevalent in environment and are recovered from soil, air, water, plant substrates [7].

Human constantly inhale high amounts of conidia from *A.fumigatus*, which may affected their respiratory tract after long exposure [8].*A. fumigatus* consider the leading cause of severe problems in immune-compromised populations (AIDS patients, cancer patients receiving chemotherapy, solid organ transplant/skin graft patients and victims of chronic granulomatous disease) [9].

A. fumigatus is now the second most common fungal infection found in hospitalized patients, after Candida albicans [10, 11]. *A. fumigatus* is ubiquitous, with a worldwide distribution due to the production of small spores called conidia that have an average size of 23.5 mm, resulting in the conidia dispersing in the air and remaining in the atmosphere for prolonged periods [12].Following inhalation these conidia are usually efficiently eliminated by host immune factors. However, in immuno-compromised patients A. fumigatus can cause a range of systemic diseases with mortality rates ranging from 30-90% [13]. Pulmonary infection occur in patients with cystic fibrosis causing allergic bronchopulmonary aspergillosis (ABPA), invasive disease (IA) or a fungus ball (aspergilloma) [14].

A.fumigatus is capable of producing secondary metabolites ,which can be harmful such as gliotoxin, which is also produced by several other Aspergillus species, Trichoderma species, and Penicillium species [15].Gliotoxin is a member of the epidithiodioxopiperazine (ETP) class of toxins, which are characterized by a disulfide bridge across a piperazine ring with low molecular weight (326 Da)[16] .The oxidized form of gliotoxin travels into host immune cells where it is able to affect cellular functions essential to the immune response[17].

The current study designed for isolation and identification of *A.fumigatus* by phenotypic characterization and PCR based molecular technique from hunters in Diyala province – Iraq ;Real-time based detection of gliotoxin of *A.fumigatus* from hunters; Evaluation of relationship between *A.fumigatus* infection and Clinical signs and possible risk factors for hunters infection .

Materials and Methods

Study Area And Study Population

This study was performed in Baqubah city - Diyala province 33°45'34.71"N; 44°36'23.97"E ,Northeast[18, 19] . The study included 33 hunters, age ranged (15-55 years) ,from 14 of October 2018 to 13 of January 2019. This study was conducted according to the principles of Helsinki declaration. A full explanation about the purpose of this study to all patients was done. Dully filled consent form obtained from all patients that agree to participate in the study. Approval of ethical review committee of college of veterinary medicine, University of Diyala ,Iraq was taken before initiation of the work.

Sample Collections:

Samples used were mouth ,nose and ear swabs . Sterile, clean swabs were used for sample collection, swabs was covered, to prevent contamination of air pollution and retention of the sample without contamination until cultured within a few hours if necessary. Cotton swabs were wetted with 0.85% normal saline then used to wipe the mouth, nose and ear several

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times, and then the swabs conducted for direct smear and culture purposes.

Culture:

Swabs were inoculated into Sabouraud's dextrose agar(SDA), containing 0.05gm\L chloramphenicol, Penicillin at a concentration of 0.4 ml\L and Streptomycin at a concentration of 2 ml\L . The media were incubated at 37°C for 1-2 weeks[20].

Staining

Lacto phenol cotton blue solution is added on a slide. By sterilized needle, a mycellial mat was transferred on fluid and pressed gently, then mixed with the stain. A clean cover slip had been taken and with the help of a forceps places the cover slip on mycellial mat. Observed under low to high power objectives of microscope [21, 22]

Molecular Identification of Aspergillus spp. by PCR

DNA Extraction:

DNA was extracted from *Aspergillus spp.* By using Wizard® genomic DNA Purification kit (Promega, USA) according to the protocol stated by the kit manufacturer [23]

Concentration and purity of DNA:

DNA was extracted from hundred isolated of *Aspergillus* spp. and they were concentrated in one tube. The concentration and the purity of the DNA samples were determined by Quantus Fluorometer at (9.9 ng/µl and 57 ng/µl) was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected, according to the protocol stated by the kit manufacturer [23]

Primer selection and preparation

Universal primers ITS1 (5`- TCCG-TAGGTGAACC TGCGG-3`) and ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) were used for detection of Aspergillus (Promega, USA) .

PCR working solution:

Optimization of PCR was accomplished after several trials. Thus the following mixture was adopted amplification reactions were produced in the 25µl final volume containing 12.5 µl Go Taq® master mix (Promega, USA), 2µl of the primers and 2µl DNA template and complete the volume by 8.2 ul nuclease-free water

Programmable thermal controller

Program for amplifying the 5.8S rDNA and the ITS 1region, amplified from type of ITS1 and ITS3 for *Aspergillus* spp. For identification of *Aspergillus* spp. ,an initial denaturation step at 95°C for five minutes was followed by thirty cycles of denaturation at 95°C for thirty seconds, annealing at 55°C for thirty seconds , and extension at 72°C for thirty seconds, with a final extension step of 72°C for seven minutes [24].

Agarose Gel Electrophoresis:

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria, according to the protocol stated by the kit manufacturer (Promega, U.S.A)[25]. All processes including fungi gDNA extraction, PCR amplification, and assembly, for fungi, analysis on ITS region (18S prior to update); length greater than 500 bp guaranteed .

Detection of GLIP gene expression by RT-PCR :

Extracted DNA was used for detection of GLIP gene by RT-PCR using applied Biosystems.
Hold at 95°C for 5:00.then cycling for 45 cycle according to the following
1) 95°C for 15s
2) 58°C for 15s acquiring on Green
3) 72°C for 15s

The melting step: Melt from 72°C to 95°C at 0.3°C/s

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Statistical Data Analysis : Patients demography and cross tabulation were calculated by Statistical Package for the Social Sciences for Windows version 17 (SPSS, Armonk, NY: IBM Corp.). Pearson's chi-square and Pearson's correlation coefficient were used for correlation between the variables of the two tests. P value of ≤ 0.05 and ≤ 0.01 (two tailed) was set to be statistically significant[26, 27].

Results

Table (1) shows the identification of *A.fumigatus* isolated from hunters according to morphological features on SDA .The total number of *A.fumigatus* isolated from hunters was 34/99, (34.34%) .*A.fumigatus was* isolated from 18/33 (54.5%) mouth swabs ; 24/33,(72.7%) nasal swabs and 7/33, (21.2%) ear swabs. All 34 *A.fumigatus* positive samples ,(34.34%) confirmed by conventional PCR and sequencing. Analysis of sequences and confirmation of *A.fumigatus* homogenic data using rRNA database (NCBI) after amplification of fungi's ribosomal RNA. All processes including fungi gDNA extraction, PCR amplification, sequencing, and assembly. For fungi, analysis on ITS region (18S); length greater than 500 bp guaranteed to be *A.fumigatus* as shown in figure (1).

Detection Of *A.fumigatus* Gliotoxin Isolated From Hunters by Real time PCR

As shown in figure (2), All *A. fumigatus* isolated from hunters have the ability to produce gliotoxin that detected by real time PCR. The minimum cycle threshold was 0.077 staring at cycle 1.

As shown in table (2), *A.fumigatus* was isolated more frequently from mouth of hunters at the age group (12 -20)years; (48-56) years , (15.15%). *A.fumigatus* was isolated more frequently from nose of hunters at the age group (21-29)years , (9.09%). *A.fumigatus* was isolated more frequently from ear of hunters at the age group (12 -20)years; (21-29) years , (48-56) years; (6.06%).No significant difference was reported between age groups infected with A.fumigatus .No significant correlation was reported between age group and *A.fumigatus* infection.

A.fumigatus was isolated more frequently from mouth of hunters with Primary education 9/33, (27.27%), followed by secondary and higher education, 4/33 ,(12. 12%). *A.fumigatus* was isolated equally frequently from nose of hunters with primary and secondary education, 4(12. 12%). *A.fumigatus* was isolated more frequently from ear of hunters with primary education, 3/33 (9. 09%). No significant difference nor correlation were reported between education level and A.fumigatus infection.

A.fumigatus was isolated more frequently from mouth of hunters with good economic status 11/33, (33.33%) followed by middle economic status , 5/33 ,(15. 15%). *A.fumigatus* was isolated frequently from nose of hunters with good economic status , 7/33 (21. 21%). *A.fumigatus* was isolated more frequently from ear of hunters with good economic status , 4/33 (12. 12%)%). No significant difference nor correlation were reported between economic status and A.fumigatus infection.

A.fumigatus was isolated more frequently from mouth(30.30%) ,nose,(21.21%) and ear (12. 12%) of hunters drinks filtrated water . No significant difference nor correlation were reported between source of water and A.fumigatus infection.

Possible Correlation Between Clinical Signs And Isolation Of *A.fumigatus* From Mouth ,Nose and Ear of Hunters

Positive correlation was reported between isolation of *A.fumigatus* from hunters mouth and unilateral facial swelling (p value =0.007) .

No significant correlation was reported between isolation of *A.fumigatus* from hunters

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mouth and each of the following clinical signs : headache (p value= 0.100),nasal congestion and pain (p value= 0.881), sinus congestion and pain (p value= **0.617**) , serosanguinous (bloody) nasal discharge (p value= **0.798**) ,drooping eyelid (p value= **0.451**) ,necrotic black lesions on the hard palate (p value= **0.703**) , necrotic black lesions on the nasal turbinate (nasal cavities) (p value= 0.805), drainage of black pus from eyes (p value= **0.240),** cough , chest pain and dyspnea (p value= 0.690).

Positive correlation was reported between isolation of *A.fumigatus* from hunters nose and unilateral facial swelling (p value =0.02) ., nasal congestion and pain (p value= **0.009**) , necrotic black lesions on the hard palate (p value= **0.002**), necrotic black lesions on the nasal turbinate (nasal cavities) (p value= **0.002**), drainage of black pus from eyes (p value= **0.04).**

No significant correlation was reported between isolation of *A.fumigatus* from hunters nose and headache (p value= 0.366);nasal congestion and pain (p value= 0.312) , serosanguinous (bloody) nasal discharge (p value= **0.503**), drooping eyelid (p value= **0.258**), cough , chest pain and dyspnea (p value= 0.294). Positive correlation was reported between isolation of *A.fumigatus* from ear of hunters and unilateral facial swelling and (p value =0.05), nasal congestion and pain (p value= **.022**) , necrotic black lesions on the hard palate (p value= **0.03**) .

No significant correlation was reported between isolation of *A.fumigatus* from ear of hunters and headache (p value= 0.391), nasal congestion and pain (p value= 0. 122), serosanguinous (bloody) nasal discharge (p value= **0.279**) , drooping eyelid (p value= **0.294**) , necrotic black lesions on the nasal turbinate (p value= **0.095**) , drainage of black pus from eyes (p value= **0.619),** cough , chest pain and dyspnea (p value= 0.914).

Table (1): Morphological Identification Of *A.fumigatus* Isolated From Hunters

Source of sample for hunters	Isolation status on SDA		Total No. of swabs
	No growth	*A.fumigatus*	
Mouth	15(45.5%)	18(54.5%)	33(100%)
Nose	24(72.7%)	9(27.3%)	33(100%)
Ear	26(78.8%)	7(21.2%)	33(100%)
Total	65 (65.66%)	34(34.34%)	99(100%)

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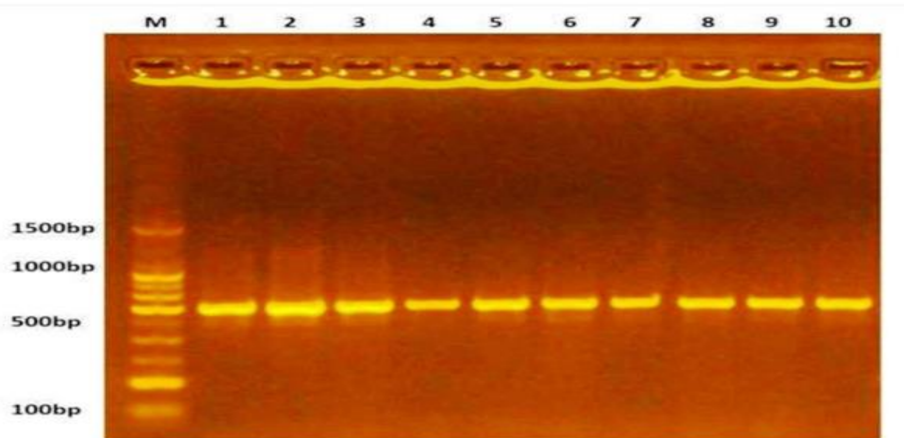
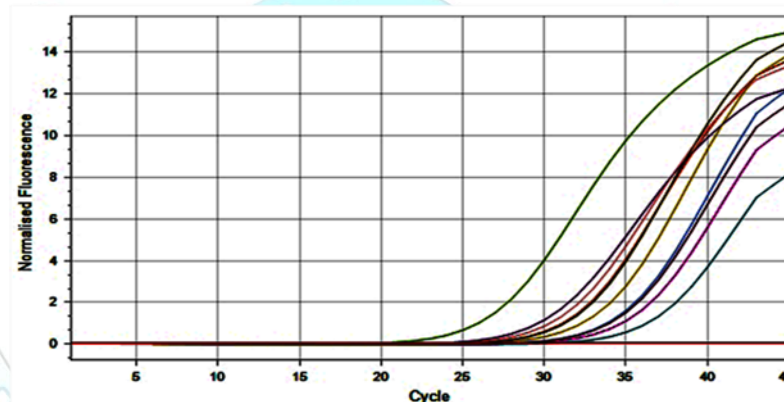


Figure (1):DNA products of *A. fumigatus* generated through ITS1 (TCCGTAGGTGAACCTGCGG), and ITS2 (TCCTCCGCTTATTGA-TATGC) primers, stained with Ethidium bromide. M : Molecular marker (100bp); lanes 1-10 (517bp) , *A. fumigatus*



Figure(2): Hunters *A.fumigatus* GLIP gene expression in real time PCR .Threshold 0.077(Automatic) starting at cycle 1

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Table (2): Possible Risk Factor Associated With *A.fumigatus* Infection among Hunters

Risk factors		*A.fumigatus* Isolated From Hunters Mouth	*A.fumigatus* Isolated From Hunters Nose	*A.fumigatus* Isolated From Hunters Ear
		A.fumigatus	*A.fumigatus*	*A.fumigatus*
Age group	12 -20	5(15.15%)	2(6.06%)	2(6.06%)
	21-29	3(9.09%)	3(9.09%)	2(6.06%)
	30-38	2(6.06%)	1(3.03 %)	1(3.03%)
	39-47	3(9. 09%)	1(3.03 %)	0(0%)
	48-56	5(15.15%)	2(6.06%)	2(6.06%)
	Total	18 (54.5%)	9(27.3%)	7(21.21%)
R		-0.030	-0.081	-0.067
P value		0.870	0.654	0.709
Edu-cation level	Illiterate	1(3.03 %)	0(0%)	0(0%)
	primary	9(27.27%)	4(12. 12%)	3(9. 09%)
	secondary	4(12. 12%)	4(12. 12%)	2(6.06%)
	higher education	4(12. 12%)	1(3.03 %)	2(6.06%)
	Total	18 (54.5%)	9(27.3%)	7(21.21%)
R		0.045	0.065	0.171
P value		0.802	0.718	0.341
Economic status	Middle	5(15.15%)	2(6.06%)	2(6.06%)
	Good	11(33.33%)	7(21.21%)	4(12. 12%)
	Very good	2(6.06%)	0(0%)	1(3.03 %)
	Total	18 (54.5%)	9(27.3%)	7(21.21%)
	R	-.027	-.071	.007
P value		.880	.694	.968
Source of water	Tap water	8(24.24%)	2(6.06%)	3(9. 09%)
	filtrated	10(30.30%)	7(21.21%)	4(12. 12%)
	Total	18 (54.5%)	9(27.3%)	7(21.21%)
	R	-.045	.250	-.005
	P value	.805	.160	.980

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Table(3): Possible Correlation Between Clinical Signs And Isolation Of *A.fumigatus* From Mouth ,Nose and Ear of Hunters

Signs	*A.fumigatus* infection No.(%) of hunters mouth	R	P Value	*A.fumigatus* infection No.(%) of hunters nose	R	P Value	*A.fumigatus* infection No.(%) of hunters ear	R	P Value
Unilateral facial swelling	16(48.48%)	0.457	0.007	9(27.27%)	0.404	0.020	7(21.21%)	.342	0.05
Headaches	10(33.33%)	0.291	0.100	5(15.15%)	0.163	0.366	4(12.12%)	.155	.391
Nasal congestion and pain	4(12.12%)	0.027	0.881	3(9.09%)	0.182	0.312	3(9.09%)	.275	.122
Sinus congestion and pain	5(15.15%)	0.090	0.617	5(15.15%)	0.447	0.009	4(12.12%)	.398	.022
Serosanguinous (bloody) nasal discharge	3(9.09%)	0.046	0.798	2(6.06%)	0.121	0.503	2 (6.06%)	.194	0.279
Drooping eyelid	6(18.18%)	-0.136	0.451	5(15.15%)	0.203	0.258	4(12.12%)	.188	0.294
Necrotic black lesions on the hard palate	6(18.18%)	-0.069	0.703	7(21.21%)	0.527	0.002	5(15.15%)	.378	0.03
Necrotic black lesions on the nasal turbinate	10(33.33%)	-0.045	0.805	9(27.27%)	0.526	0.002	6(18.18%)	.295	.095
Drainage of black pus from eyes	7 (21.21%)	-0.210	0.240	7(21.21%)	0.359	0.04	4(12.12%)	.090	0.619
Cough	6(18.18%)	0.072	0.690	4(12.12%)	0.188	0.294	2 (6.06%)	-.020	.914
Chest pain	6(18.18%)	0.072	0.690	4(12.12%)	0.188	0.294	2 (6.06%)	-.020	.914
Dyspnea	6(18.18%)	0.072	0.690	4(12.12%)	0.188	0.294	2 (6.06%)	-.020	.914

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Discussion

Current study revealed that *A.fumigatus* was isolated from hunters according to morphological features on SDA . *A.fumigatus was* isolated from (34.34%) of hunters. *A.fumigatus was* isolated from (54.5%) mouth swabs ; (72.7%) nasal swabs and (21.2%) from ear swabs .Current results considered higher than that reported by [28],who stated that *A.fumigatus* was isolated from 11.1% of oral swabs in university students . On the other hand [29] stated that *A.fumigatus was* isolated from (6.2%) ear swabs with clinical presentation of otomycosis in urmia, Iran.

Current results come in line with [30],reported that *A. fumigatus* was isolated from (27%) of otomycosis .On the other hand [31] reported (11.53%) isolation rate for *A.fumigatus* from ear. while [32] ,reported 1.5% isolation rate for *A.fumigatus* from otomycosis patients in Turkey. while [33],stated that *A.fumigatus represent 6.5%* of pathogens isolated from ear infections.

Current study revealed that *A.fumigatus was* isolated from (21.2%) of hunters ear swabs. systemic as well as local factors have been proposed to be predisposing for otomycosis such as high humidity in external auditory canal, epithelial debris accumulation , prolonged usage of broad spectrum antibiotics prolonged usage of steroid preparations, immuno suppression status, diabetes and dermatological diseases as well as instrumentation [34].On the other hand, unsterile pointed objects like match sticks and hair pins (in females). putting mustard oil and use of antibiotic ear drops [33] .

Current study come in line with others [35, 36]that using of ITS regions for amplification with The PCR technique *with common* section 18S, 5.8S, and 26S genes, coding for rRNA , was sensitive for the identification of Aspergillus. They stated the amplification product of the universal fungal primers(ITS1 and ITS4) was detected in all Aspergillus isolates, therefore the larger amplicons served to confirm the presence of a fungal target[25].

On the other hand current study agree with that reported by [37] that The ITS 1-5.8S- ITS 4 region was chosen for the design of genus and species-specific primers for identification of Aspergillus, and as a result of high nucleotide variability among genera and species, The ITS region is a good molecular target for species level identification (Landlinger et al. 2009) and is extensively used as a universal DNA barcode in fungal taxonomy studies (Suleiman et al. 2014).
Current culture and conventional PCR based diagnosis for *A.fumigatus* were followed by detection of gliotoxin production which was positive in all selected isolates via real time PCR which come in line with that reported by [16],stated that 79% of *A. fumigatus* isolated from patients with pulmonary aspergillosis produce gliotoxin that detected by PCR.

Current study revealed that *A.fumigatus* was isolated more frequently from mouth of hunters at the age group (12 -20)years as well as from those with (48-56) years , (15.15%). On the other hand *A.fumigatus* was isolated frequently from nose of hunters at the age group (21-29)years , (9.09%).While *A.fumigatus* was isolated frequently from ear of young hunters at the age group (12 -20) years; (21-29) years , as well as from (48-56) years; (6.06%).These results come in accordance with that reported by [30, 38, 39] ,stated that otomycosis due to

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A.fumigatus and other fungi was more common among young patients while [31], stated that *A.fumigatus* was isolated frequently from ear of (41-50), (51-60) ,(61-70) , (71-80) years ;representing (13.33%), (17.78%), (20%) and (31.11%) respectively.

Current study come in agreement with that reported by [30],stated that patients with otomycosis mainly came from an agricultural background where they exposed to infection frequently . The possible reason behind high rate of infection among the two distinct group of hunters between second to fifth decade of life may attributed to the fact that hunters spend more time in the environment where the aspergillus represent the common airborne fungi that were exposed to. Even though current study reported neither significant difference nor correlation between age group and *A.fumigatus* infection which might be due to the limited number of hunters under investigation beside the possibility that exposure to aspergillus conidia was independent from age .The frequent exposure of hunters of (48-56) years ,may attributed to the anatomical changes takes place in the lung parenchyma as well as for elastic fibers around the alveolar duct this process starts at the age of 50 and leads to expansion of airspaces and reduction in supporting tissue, so, over time this leads to increase the risk of acquiring a infectious that tend to be chronic[31, 40].

Current study proved that *A.fumigatus* was isolated frequently from (27.27%) of hunters mouth swabs whose education level was primary; followed by secondary and higher education ,(12. 12%). *A.fumigatus* was isolated equally from nose of hunters with primary and secondary education, (12. 12%). *A.fumigatus* was isolated more frequently from ear of hunters with primary education, (9. 09%).

A.fumigatus was isolated frequently from mouth of hunters with good economic status (33.33%) followed by middle economic status ,(15. 15%). *A.fumigatus* was isolated frequently from nose of hunters with good economic status (21. 21%). *A.fumigatus* was isolated more frequently from ear of hunters with good economic status (12. 12%)%). No significant difference nor correlation were reported between economic status and *A.fumigatus* infection. No significant difference nor correlation were reported between education or economic level and *A.fumigatus* infection.

This fact may reflect that the exposure to *A.fumigatus* continuously, whether in indoor environment or in the fields and orchards, which is characterized by being wet during the practice of hunting and constitute a good environment for the growth of fungi ;On the other hand, the status of immune cells of the body play a greater role in the initiation of infection to the hunter whether he is educated or not and regardless of economic level. The level of education and economy , whatever high, does not prevent exposure to fungi, especially if it is widespread and the size of conidial spores is small and huge as in case of aspergillus. The avoidance of infection may takes place only in the case that the fisherman has a specialized knowledge About the fungus and how it is transmitted to him by which does in reported in the studied group.

The present study proved that *A.fumigatus* was isolated frequently from mouth(30.30%) ,nose,(21.21%) and ear (12. 12%) of hunters drinks filtrated water . No significant difference nor correlation

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were reported between source of water and *A.fumigatus* infection. This result may attributed to concealment of most hunters the truth of the quality of water they drink and claim that they are drinking filtered water. On the other hand, hunting is usually takes place within the areas of orchards or farms that are far from the sources of filtered water and perhaps the process of fishing in the open land, which is characterized by rare water and dry climate. On the other hand the diversity or the wide range of growth conditions for Aspergillus such as temperature (range: 12–65°C) and pH of growth between 2.1 and 8.8 [12].Thermotolerance facilitates the growth of the fungus in decaying organic matter . The ability to thrive in this habitat needs a substantial level of thermotolerance, which is assumed to contribute to virulence of the fungus [41]. These properties might be evolved in response to competitors within the ecological niche of the organism and are unlikely to reflect specific adaptations to counter vertebrate host defense mechanisms [42].

Current study revealed positive correlation between several clinical signs and isolation of A.fumigatus from hunters .One of most common was the unilateral facial swelling was noticed in (48.48%) of hunters with positive *A.fumigatus* isolated from their mouth (p value =0.007) .Also unilateral facial swelling was noticed in (27.27%) of hunters with positive *A.fumigatus* isolated from their nose (p value =0.02);while unilateral facial swelling was noticed in (21.21%) of hunters with positive *A.fumigatus* that isolated from their ear (p value =0.05).This clinical signs was in agreement with that reported by [43, 44] that facial swelling one of the cardinal signs for nasal and paranasal sinus aspergillosis .

The present study proved positive correlation was reported between sinus congestion and pain, necrotic black lesions on the hard palate and the isolation of A.fumigatus from nose and ear of hunters which come in line with that reported by [45] ,stated that sinus pain and congestion as well as involvement of at least one paranasal sinuses were usually reported during nasal involvement via A.fumigatus. A.fumigatus infection to the nasal sinuses enhanced via presence of polyps and stagnant secretions besides other factors like neutropenia, inappropriate use of antibiotics, immunosuppressive drugs, corticosteroids, uncontrolled diabetes mellitus, human immunodeficiency virus infection, trauma, burns, and radiation therapy

On the other hand the presence of necrotic black lesions on the nasal turbinate and drainage of black pus from eyes were positively correlated with isolation of A.fumigatus .The presence of these lesion in the oral cavity reflect the portal of entry to the oral tissue which is marginal gingiva and the gingival sulcus from which the germination of conidial spore started and invasion as well as tissue destruction of gingival tissue associated with pain and ulcer formation and tissue swelling with formation of gray or violaceous hue. later extensive necrosis which present clinically as a yellow or black ulcer with facial swelling developed[46-48] .Current results come in agreement with that reported by [28, 46, 49] stated that "oral aspergillosis lesions are yellow or black in color, with a necrotic ulcerated base, classically located on the palate or posterior tongue".

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These clinical features appear to be logical according to the way of entrance of *A.fumigatus* conidia in to the body which is mainly by inhalation and as hunters usually practice their work in damping or grass land with high amount of decayed organic materials which represent a suitable environment for growth of *A.fumigatus* ,for this the primary involvement for oral cavity, nasal and paranasal sinuses, larynx ,eyes and ear were quite common [28, 49] . Several factors appear to be play a role in *A.fumigatus* colonization and subsequently development of clinical presentations which include the immunological status of hunters' mucociliary surface for upper respiratory tract which play a vital role in trapping and removing of inhaled fungal elements ,there for preventing further access. On the other hand , the continuous exposure to high dose of fungal elements ,hyphae and conidia, high levels of bioarerosols, and elevated concentrations of mycotoxins including gliotoxin, fumagillin , helvolic acid , verruculogen and trypacidin which have direct effect on ciliary movement and this effect may extended to reduce the activity of complement system and phagocytic cells (neutrophils, macrophages and dendritic cells), natural killer and γδ T-cells finally promote fungal colonization on epithelial surface [42, 50-54].On the other hand globose to subglobose morphology of conidia as well as the small size(2–3.5 µm) , enable *A. fumigatus* to bypass mucociliary clearance and facilitate reaching and adherence to the airways epithelium and distal parts of the respiratory tract [12] . Moreover, the presence of melanin in the conidial wall and highly negatively charged sialic acid residues contribute to protection of *A. fumigatus* against host cell responses [42].Thus, *A. fumigatus* conidia able to interfere with mucociliary clearance, bind effectively to respiratory epithelia and basement membrane proteins, and invade or damage epithelial cells to establish infection and potentially evade other host defenses[12, 55] .

In conclusion , *A. fumigatus* infection represent serious problem for hunters . Mouth, nose and ear respectively exposed to gliotoxin producing *A. fumigatus* with certain clinical manifestation . Age, education level ,economic status and source of water play no role in minimizing infection with A.fumigatus among hunters

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