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Otomycosis in Assiut, Egypt

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Abstract: During the period from October 2011 to August 2012 a total of 124 patients were clinically examined for mycotic otitis at the outpatient clinic of Otolaryngology Department, Assiut University Hospitals (AUH). The mycological analysis of ear swabs revealed the isolation of 18 fungal species and one variety belonging to 7 genera. Among the 92 mycologically-positive cases, 56 were males (60.9%) and 36 were females (39.1%). The disease was more prevalent among persons between 21-30 years where it was diagnosed in 29 patients matching 31.5% of total positive cases. *Aspergillus* infection was confirmed in 84.8 % of total cases. Fungi belonging to section *Nigri* were associated with infection of 39 cases (42.4%). Other species related to sections *Flavi* and *Terrei* were involved in 35 and 4 cases (38 % and 4.3% of the total cases respectively). *Candida* species (*C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis*) were involved in 16.3% of otomycotic cases. The remaining fungal species comprised *Chrysosporium keratinophilum*, *Cladosporium sphaerospermum*, *Mucor circinelloides*, *Phoma epicoccina* and *Stemphylium vesicarium* were rare etiologic agents of otomycosis. Extracellular proteolytic, lipolytic and ureolytic enzymes were produced by 95%, 76.2% and 36.6% of fungal isolates respectively. Aflatoxins were detected in cultures of 72.5% of *Aspergillus* species belonging to section *Flavi*. In-*vitro* sensitivity test showed that Terbinafine, Clotrimazole and Clove oil were the most effective antifungal agents.

Key words: Otomycosis, *Aspergillus*, *Candida*, protease, lipase, urease, aflatoxins, antifungal agents.

Introduction

Otomycosis is the fungal infection of the ear. Although it affects the external ear canal, the middle ear may be involved in case of a perforated tympanic membrane. The mastoid cavity may be affected if an open cavity mastoid surgery is performed previously (Ozcan 2011). Presence of itching in external ear canal must raise a high index of suspicion for otomycosis since it is the most frequent symptom. In some studies, itching was present in more than 90% of the patients (Ozcan *et al.* 2003a and Pradhan *et al.* 2003). Other common symptoms are otalgia, hearing loss, tinnitus and aural discharge (Ozcan *et al.* 2003a). Hearing loss and tinnitus are usually due to obstruction of the external ear canal by aural discharge or fungal hyphae. In some patients, fungal infection may supervene on bacterial external otitis and in that case itching may follow pain (Ozcan 2011).

Otomycosis shows a worldwide distribution and is particularly frequent in hot and humid regions (Kaur *et al.* 2000 and Ozcan *et al.* 2003a). The prevalence of otomycosis is as high as 54% in hot and humid regions (Than *et al.* 1980) whereas the prevalence decreases to 9% in temperate climates (Mugliston and O'Donoghue 1985). Wearing turban or other clothes on head has been reported as a risk factor for otomycosis in Bahrain, Turkey and India (Paulose *et al.* 1989, Ozcan *et al,* 2003a, Kumar 2005 and Ozcan 2011)). Swimming is also reported as a risk factor for otomycosis (Paulose *et al.* 1989, Ozcan *et al.* 2003a and Wang *et al.* 2005). Perforation of the tympanic membrane and previous ear surgery have also been reported as important risk factors for otomycosis (Falser 1984, Paulose *et al.* 1989, Ozcan *et al.* 2003a and Ho *et al.* 2006). The prevalence of dermatomycoses in patients with otomycosis was 36.5% in Turkey (Ozcan *et al.* 2003b) and 51% in India (Kumar 2005). In Turkey, 50% of patients who were refractory to otomycosis treatment had dermatomycoses such as tinea pedis and the same pathogenic fungi were isolated from dermatomycoses and otomycosis in nearly half of these patients (Ozcan *et al.* 2003b). Use of topical antibiotics has been reported as a predisposing factor for otomycosis by Jackman *et al.* (2005). Both absence (Paulose *et al.* 1989) and presence (Kaur *et al.* 2000) of earwax in the external ear canal have been accused as predisposing factors for otomycosis. Some authors suggested that cleaning external ear canal with matchsticks or cotton bud swabs was a predisposing factor (Kaur *et al.* 2000). Other proposed predisposing factors are immunosuppression, radiation therapy, cancer chemotherapy and prolonged treatment with systemic antibiotics (Falser 1984).

In Egypt, knowledge on otomycosis is still very limited. The present study aimed at identifying fungal species involved in otomycosis as well as their ability to produce proteolytic, lipolytic and ureolytic enzymes and their sensitivity to some antifungal therapeutic agents.

Materials and Methods

Patients

One hundred and twenty-four patients attending the outpatient clinic of Otolaryngology Department, Assiut University Hospitals (AUH)

were clinically examined for otomycosis during the period from October 2011 to August 2012.

Collection of samples

Sterile cotton swabs were used for collecting debris, fungal elements, and earwax from the external ear canal of patients showing symptoms of otomycosis. Samples were transferred immediately to the Assiut University Mycological Centre (AUMC) for further studies.

Mycological analysis

a) Direct microscopic examination: Direct smears from swabs were prepared and examined using Lactophenol Cotton Blue stain (LPCB) as recommended by Ellis *et al.* (2007).

b) Culturing of samples: Swabs were streaked on Sabouraud's dextrose agar medium (SDA) with the composition of (g/l): peptone, 15; dextrose, 40 and agar, 20 (Ellis *et al*. 2007). Cultures were incubated at 28° C for 7-15 days until fungal colonies appear. Cultures were also preserved on SDA slant agar at 4° C for further studies.

c) Identification of fungi

a) Phenotypic identification: Fungi were identified on the basis of macro-and microscopic features. The following references were consulted: Raper and Fennell (1965), Moubasher (1993), Barnett *et al*. (2000) and Domsch *et al.* (2007).

b) Genotypic identification: Some fungal isolates were selected and individually grown on yeast malt agar (YM) and incubated at 28° C for 3 days. A small amount of fungal growth was scrapped and suspended in 100µl autoclaved distilled water in 2ml sterile vials and boiled at 100°C for 15 minutes. Samples were sent to SolGent Company (Daejeon, South Korea) for rRNA gene sequencing. Fungal DNA was extracted and isolated using SolGent purification bead. Prior to sequencing, the ribosomal rRNA gene was amplified using the polymerase chain reaction (PCR) technique in which two universal fungal primers ITS1 (forward) and ITS4 (reverse) were incorporated in the reaction mixture. Primers used for gene amplification have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). PCR products were sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White *et al.* 1990). Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

Enzymatic activities of otomycotic fungi

a) Proteolytic activity: Test tubes containing modified **c**asein hydrolysis medium (Paterson and Bridge 1994) were used. The medium comprised (g/l): KH_2PO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.2; $CaCI_2.2H_2O$, 0.1; 15% skimmed milk, 25 ml; glucose, 10; and agar, 20. After inoculation with the tested fungi, cultures were incubated at 25°C for 7 days. Degradation of milk protein was measured as depth of clear zone (in mm).

b) Lipolytic activity: The medium of Ullman and Blasins (1974) was used which has the following composition (g/l): peptone, 10; $MgSO_4$.$7H_2O$, 0.2; $CaCI_2.2H_2O$, 0.2; and agar, 20). The medium was sterilized by autoclaving at 121°C for 20 minutes. Tween 80 (10 ml) was autoclaved separately and added to the sterile and cooled basal medium. The medium was dispensed aseptically in test tubes (10 ml/tube) followed by inoculation by fungal isolates. After incubation at 25°C for 7 days, the lipolytic ability was observed as a visible precipitate due to the formation of crystals of calcium salt of the oleic acid liberated by the enzyme. The depth of precipitate (in mm) was measured.

c) Urease Activity: Christensen urea medium described by Ellis *et al* (2007) was employed with some modifications. The medium was prepared in 2 separate parts. The first part contained (g/l): peptone, 1; KH_2PO_4, 1; KCl, 0.05; yeast extract, 1; and phenol red, 0.012. These components were dissolved in 800 ml distilled water. The second part was composed of (g/l): glucose, 5; $MgSO_4.7H_2O$, 0.5; and distilled water, 200 ml. The two parts were mixed after autoclaving and cooled to 50°C. Aliquots of 5 ml of 40% solution of sterilized urea were added to each 100 ml of the medium which was then poured into sterile 5 ml test tubes (3 ml for each). After inoculation, cultures were incubated at 25°C for 3-5 days. Results were recorded as positive after appearance of a deep pink color in the broth medium.

Screening for aflatoxin production

a) Culturing of selected fungi: Thirty six fungal isolates belonging to *A. flavus* (26 isolates), *A. flavus* var. *columnaris* (9) and *A. parasiticus* (1) were cultivated in 250 ml conical flasks containing 50 ml of Potato Dextrose Broth (PDB) followed by sterilization, inoculation and incubation at 28C for 7 days (El-Kady and Moubasher 1982).

b) Extraction and detection of aflatoxins: Chloroform was the solvent for mycotoxin extraction. Pre-coated TLC silica plates were used for separation of mycotoxins followed by visualization under UV light (254 or 365 nm). Aflatoxins B and G when present fluoresce blue and greenish blue respectively. The intensity of the sample spots was compared with that of the standard aflatoxins (El-kady and Moubasher 1982 and Dorner 1998).

***In-vitro* sensitivity of otomycotic fungi to antifungal agents**

Disc diffusion method (CLSI 2010) was employed with some modifications as Mueller-Hinton II agar was replaced by Sabouraud's

dextrose agar medium. Inhibition zone around each disc was measured in mm and the fungal isolates were classified as sensitive, intermediate or resistant according to Ellis (2011) and Al-Hussaini *et al*. (2013).

Results and Discussion

Fungi recovered from cases of otomycosis

Eighteen fungal species and one variety belonging to seven genera were isolated and identified. The genus *Aspergillus* was represented by 9 species and one variety with the majority attributed to sections *Nigri* (39 isolates belonging to 6 species), *Flavi* (35 isolates of 2 species and one variety) and *Terrei* (one species). *Candida* was represented by 4 species whereas the remaining genera comprised only one species for each as shown in Table (1). Identification of some fungal isolates was confirmed by rRNA gene sequencing. Phylogenetic tree (Fig.1) showed three clades; one for *Aspergillus terreus* which showed close relationship with similar strains deposited in the GenBank. The second and third clades supported the identification of *A. flavus* and *Mucor circinelloides*. The majority of mycotic otitis cases were associated with *Aspergillus* (78 cases representing 84.8 % of total cases). Fungi belonging to section *Nigri* were associated with infection in 39 cases (42.4 %). Similarly fungal species related to sections *Flavi* and *Terrei* were involved in 35 and 4 cases (38 % and 4.3% of the total cases respectively). These findings are in great harmony with the previous reports from Brazil (Pontes *et al.* 2009), Iraq (Khammas *et al.* 2010), Iran (Barati *et al.* 2011 and Saki *et al.* 2013), Kingdom Saudi Arabia (Abou-Halawa *et al.* 2012), India (Sarvan *et al.* 2012, Gokale *et al*. 2013 and Panchal *et al.* 2013) and Spain (Garcia-Agudo *et al.* 2011).

Most reports indicate that *Aspergillus* species are involved in about 70% of fungal otitis cases, reinforcing the importance of *Aspergillus* otitis. Although *A. niger* is the globally most frequently recovered species, *A. flavus* occurs in equal frequency in Mexico (Bonifaz *et al*. 2010) as well as in Spain (Garcia-Agudo *et al*. 2011) and Iraq (Al-Abbasi *et al*. 2011). According to Marquez *et al*. (1999) and Araiza *et al* (2006) otitis caused by *A. fumigatus, A. terreus, A. clavatus, A. candidus,* and *A. nidulans* has been described. They also stated that several other hyalohyphomycetes (species of *Scopulariopsis, Penicillium* and *Fusarium*), phaeohyphomycetes (*Alternaria* spp., *Cladosporium* spp.) and *Candida albicans* have been isolated from patients with otomycosis. As mentioned by Araiza *et al.* (2006) growth of *Aspergillus* species is usually limited to the stratum corneum and produces a mild inflammatory process. In patients with excessive cerumen, the fungal filamentous masses intermingle to form a plug that may mechanically impair hearing. Actually this is a very superficial process which does not constitute a parasitic infection, but rather a saprotrophic condition to the auditory canal. Accordingly, all ornamentations and reproductive forms of *Aspergillus* spp. are seen in cases of *Aspergillus* otitis, in a similar proportion to what occurs in the environment and on culture media.

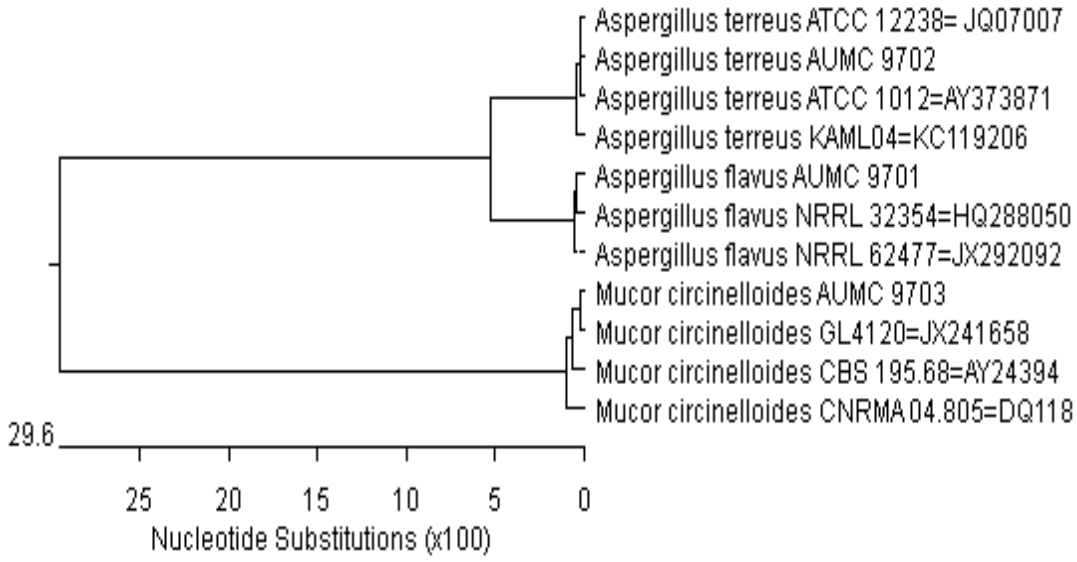

Fig.1 Phylogenetic tree for fungal species isolated from cases of otomycosis (given AUMC Numbers). Reference strains are included in the tree (given ATCC, KAML, NRRL, GL, CBS and CNRMA numbers).

Table (1): Incidence of fungi recovered from otomycotic infections.

Fungal species	Incidences out of 92 cases and (%)
***Aspergillus* species section *Flavi* (Total isolates)**	35 (38)
Aspergillus flavus Link	25 (27.1)
Aspergillus flavus var. c*olumnaris* Raper & Fennell	9 (9.7)
Aspergillus parasiticus Speare	1 (1.1)
***Aspergillus* species section *Nigri* (Total isolates)**	39 (42.3)
Aspergillus brasiliensis Raper & Fennell	28 (30.4)
Aspergillus carbonarius (Bainier) Thom	1 (1.1)
Aspergillus lacticoffeatus Frisvad & Samson	2 (2.2)
Aspergillus niger van Tieghem	4 (4.3)
Aspergillus piperis Samson & Frisvad	2 (2.2)
Aspergillus tubengensis Mosseray	2 (2.2)
Aspergillus terreus Thom	4 (4.3)
Cladosporium sphearospermum Penzig	2 (2.2)
Chrysosporium keratinophilum (Frey) Carmichael	1 (1.1)
Candida albicans (C.P Robin) Berkhout	2 (2.2)
Candida glabrata (Anderson) Meyer & Yarrow	1 (1.1)
Candida krusei (Castellani) Berkhout (Telemorph: *Pichia kudriavzevei* Boidin, Pignal & Besson)	10 (10.8)
Candida tropicalis (Castellani) Berkhout	2 (2.2)
Phoma epicoccina Punith., Tulloch & Leach (Synanamorph: *Epicoccum nigrum* Link)	1 (1.1)
Mucor circinelliodes van Tieghem	3 (3.2)
Stemphylium vesicarium (Wallr.) Simmons (Teleomorph: *Pleospora allii* (Rabenh.) Ces. & de Not.	1 (1.1)

Incidence of otomycosis in relation to sex, age and health status of patients

Among the 92 mycologically positive cases, 56 were males (60.9%) and 36 were females (39.1%). The disease was more prevalent among adults between 21-30 years where it was diagnosed in 29 patients (31.5% of total cases). The prevalence of otomycosis regularly decreased with the increase of age of patients as shown in Table (2). As mentioned by Bonifaz *et al*. (2010), *Aspergillus* otitis is a cosmopolitan condition that occurs more often in warm and humid climates. It affects both genders in the same proportion, with a slight predominance of females. The authors also added that *Aspergillus* species may be part of the habitual microbiota of the external auditory canal and *Aspergillus* otitis is almost always seen in people who practice water sports but rarely affects children. It is more frequent among adolescents and this has been related to growth spurts. However, the highest frequency is observed amongst young adults aging 20–30 years-old (Brook 1980, Muglestos and O'Donoghue 1985 and Zaror *et al*. 1991). In Nigeria, Fasunla *et al.* (2008) studied 5784 patients with ear diseases and found that 378 (6.54%) had otomycosis which consisted of 145 (38.36%) males and 233 (61.64%) females. Seventeen patients (4.50%) had recurrence within six months of treatment, 4 (1.06%) had poorly controlled plasma glucose. A significant number of the Nigerian patients, 52 (13.76%), had prior topical aural antibiotic treatment following misdiagnosis and the predominant etiological agents were *Aspergillus niger* (48.35%) and *Aspergillus fumigatus* (33.96%). In Australia, Ling and Sader (2008) reported a case of fungal malignant otitis externa caused by *A. flavus*. The case was a 77-year-old man with non-insulin dependent diabetes mellitus who presented with otalgia and otorrhea. In Iran Barati *et al.* (2011) reported that Otomycosis was confirmed after mycological diagnosis in 69% of clinically suspected patients. The highest incidence of otomycosis was in autumn and in patients aged 21-40 years old. Working in dry dusty environment was a major predisposing factor. *Aspergillus flavus* was the most common fungus in otomycosis followed by *A. niger*, *Candida albicans*, *A. fumigatus*, *A. nidulans* and *C. parapsilosis*. More recently, Saki *et al*. (2013) investigated 881 cases suspecting otomycosis in Iran. They reported that the 20–39 year age group had the highest prevalence of otomycosis. Positive cases (293 cases), comprised 162 (55.3%) women and 131 (44.7%) men. The fungal agents isolated were *A. niger* (67.2%), *A. flavus* (13%), *C. albicans* (11.6%), *A. fumigatus* (6.2%) and *Penicillium* species (2%).

In the present study (Tables 1& 2), *Candida* species were involved in 15 cases (16.3%) of

otomycotic cases. The remaining fungal species were rare etiologic agents of otomycosis. These included *Chrysosporium keratinophilum*, *Cladosporium sphaerospermum*, *Phoma epicoccina*, *Mucor circinelloides* and *Stemphylium vesicarium*. To the best of our knowledge *C. kertinophilum*, *P. epicoccina* and *S. vesicarium* are recorded for the first time in Egypt as causal agents of mycotic otitis externa. In India, Jadhav *et al*. (2003) investigated mycologically 79 patients (42 males and 37 females) clinically diagnosed as otomycotic cases. They found that *Candida albicans* was the sole pathogen in two patients (1 male and 1 female) aged 18 and 20 years respectively. Both patients had unilateral otomycosis and used antibiotic solution and removed wax with wooden sticks. Aneja *et al.* (2010) obtained *C. albicans* and *Penicillium* species from 10.2% and 2.7% of otomycotric samples respectively With reference to Table (3), only 16 out of 92 patients (17.4%) were diabetic and most of them were infected by *A. flavus.* The majority of otomycotic cases were unilateral (86 cases representing 93.4% of patients). Bilateral otomycosis was diagnosed in 6 patients, all of which were infected with *A. flavus.* Cases of disease recurrence were observed in 35 patients who were affected by *A. flavus* (48.6%) and other aspergilli attributed to Section *Nigri* (51.4%). There were 9 patients affected with a mixture of two fungi and all of them showed unilateral otomycosis. All patients infected with *Candida*, presented with unilateral non-recurrent otomycosis with the majority of them (13 out of 15, 86.7%) being non-diabetic. According to the P-value analysis, the relative frequency of infections with *Aspergillus* and *Mucor circinelloides* was significantly higher in diabetic than in non-diabitic patients. In United Arab Emirates (UAE), Hazarika *et al*. (2012) reported a case of mucormycosis of the middle ear in a 24-year-old male who was suffering from a left ear pain of 6-month-duration. Histopathology revealed non-septate, thin-walled fungal masses. Jia *et al.* (2011) reported recurrence of otomycosis in 8.89% of patients from Shanghai.

Enzymatic activities of otomycotic fungi

Lipolytic activity: Out of 101 isolates, 76 (75.2%) were able to produce lipase but with variable capabilities (Table 4). High lipase production was exhibited by 40 isolates which were mainly belonging to *Aspergillus* sections *Flavi* and *Nigri.* Although 50% of *A. terreus* isolates were moderately able to produce lipase, only 13.3% of *Candida* isolates were lipolytic showing low activity. Many investigators have emphasized the ability of several *Aspergillus* strains belonging to *A. niger*, *A. flavus*, *A. parasiticus* and *A. terreus* to produce extracellular lipases (Contesini, *et al*. 2009, Metwalli *et al.* 2013 and Srivastava and Gautamb 2013).

Proteolytic activity: Protease was produced by 96 out of 101 isolates which were almost of moderate proteolytic capabilities. All fungal isolates belonging to sections *Flavi* (35), *Nigri* (39) and *Terrei* (4) were proteolytic. On the other hand, 10 out of 15 *Candida* isolates were protease producers (Table 4). Reports from Serbia (Arsovic *et al.* 2004) showed that proteases were produced by 7 out of 8 *Candida* strains isolated from children with otomycosis and were belonging to *C. parapsilosis*, *C. famata*, *C. guilliermondii* and *C. albicans.* The authors suggested that protease enzymes enhance the ability of *Candida* spp. to colonize the skin and penetrate the host cells, which could be important in establishing the cause of the ear infection. It is known that the secreted proteases are important virulence factors in *Candida* species in cases of skin, mucosal and deep organ infections (Sohnle *et al.* 1976) and may help the yeast invasion through the keratin protective layer and facilitate the initiation of the infection of the ear canal (Negi *et al*. 1984). Fungal strains with higher proteolytic activity are considered more virulent (Hube 1996). As suggested by Kothary *et al*. (1984) variability among *A. flavus* isolates in proteinase production suggested strong correlation between isolate ability to express proteolytic activity and association with invasive aspergillosis in humans. The authors also added that *A. flavus* isolates causing invasive human lung infections are more likely to produce proteases than are environmental isolates.

Urease activity: Urease was only produced by 37.6 % fungal isolates which belong to *Aspergillus* sections *Flavi* (30 isolates) and *Terrei* (3) as well as the tested isolates of *Phoma*, *Mucor* and *Stemphylium.* Urease was not detected in fungal cultures related to *Aspergillus* sections *Nigri* and *Candida* (Table 3). The inability of *Candida* strains to produce urease was reported by Ellis *et al.* (2007). On the other hand, urease from *A. niger* was isolated and purified by Smith *et al* (1993) and Ghasemi *et al*. (2004). The enzyme was also detected in cultures of other filamentous and yeast fungi (Lubbers *et al*. 1996, Farley and Santosa 2002 and Mirbod *et al*. 2002). It is worthy to mention that fungal enzymes including lipase, protease and urease are considered as major virulence factors for pathogenecity for fungi causing human and animal diseases (Cox *et al* 2000, Karkowska-Kuleta 2009, Tomee and Kauffman 2000 and Watanabe *et al* 2008).

Table (2): Incidence of otomycotic cases and causative fungi in relation to sex and age of patients

Fungi	Age group (years)														Total cases	
	≤ 10		11-20		21-30		31 – 40		41 – 50		51 - 60		≥ 61			
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Total cases	5	2	5	2	18	11	9	8	5	10	10	3	4	0	56	36
Aspergillus section *Flavi*	3	0	2	0	8	4	4	2	1	4	5	1	1	0	24	11
Aspergillus section *Nigri*	1	2	2	0	7	7	3	6	2	4	4	1	0	0	19	20
Aspergillus section *Terrei*	0	0	0	1	0	0	1	0	0	0	0	1	1	0	2	2
Candida species	2	1	1	0	3	0	0	3	1	1	0	0	3	0	10	5
Chrysosporium keratinophylum	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Cladosporium sphaerospermum	0	0	1	0	1	0	0	0	0	0	0	0	0	0	2	0
Phoma epicoccina	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Mucor circinelloides	0	0	0	0	0	0	0	1	1	0	1	0	0	0	2	1
Stemphylium vesicarium	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0

M= Males, F= Females

Table 3**:** Incidence of otomycotic fungi in relation to health status of patients

Fungal species	No. of cases	Diabetic	P -value	Non diabetic	Unilateral infection	Bilateral	Recurrence infection
Aspergillus section *Flavi*	35	12 (34.3%)	0.001*	23 (65.7%)	29	6	17
Aspergillus section *Nigri*	39	0 (0%)	0.000*	39 (100%)	39	0	18
Aspergillus section *Terrei*	4	0 (0 %)	0.792	4 (100%)	4	0	0
Candida species	15	2 (13.3%)	0.935	13 (86.6%)	15	0	0
Chrysosporium keratinophilum	1	1(100%)	0.387	0 (00%)	1	0	0
Cladosporium sphaerospermum	2	0 (0%)	0.512	2 (100%)	2	0	0
Phoma epicoccina	1	0 (0%)	0.645	1 (100%)	1	0	0
Mucor circinelloides	3	3 (100%)	0.002*	0 (0%)	3	0	0
Stemphylium vesicarium	1	0 (0%)	0.645	1 (100%)	1	0	0
Mixed fungal infection	9	2 (22.2%)	0.687	7 (77.8%)	9	0	0
Total number of cases	92	16	----	76	86	6	35

N. B.: mixed fungal infections were diagnosed in 9 cases

Table (4): Enzymatic activities of fungi recovered from otomycosis

Fungal species	Lipase					Protease					Urease				
	H	M	L	P	N	H	M	L	P	N	H	M	L	P	N
Total species (n=101)	40	28	8	76	25	1	87	8	96	5	16	9	13	38	63
Section *Flavi* (n= 35)	10	17	4	31	4	0	35	0	35	0	9	9	12	30	5
Section *Nigri* (n= 39)	30	4	1	35	4	0	37	2	39	0	0	0	0	0	39
Section *Terrei* (n=4)	0	2	0	2	2	0	4	0	4	0	2	0	1	3	1
Candida spp. (n=15)	0	0	2	2	13	0	5	5	10	5	0	0	0	0	15
Chrysosporium keratinophilum (n=1)	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1
Cladosporium sphaerospermum (n=2)	0	2	0	2	0	0	2	0	2	0	0	0	0	0	2
Phoma epicoccina (n=1)	0	0	1	1	0	0	0	1	1	0	1	0	0	1	0
Mucor circinellioides (n=3)	0	1	0	1	2	1	2	0	3	0	3	0	0	3	0
Stemphylium vesicarium (n=1)	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0

Aflatoxins produced by otomycotic fungal isolates

Aflatoxins were produced by 26 out of 36 isolates representing 72.2% of tested fungi. Isolates of *A. flavus* 19 (73%) were able to produce aflatoxins B_1, B_2 and G_1 with variable levels. Relatively high levels of aflatoxin B1 was produced by 3 isolates of *A. flavus* as shown in Table (5). Six isolates of *A. flavus* var. *columnaris* produced low to intermediate levels of aflatoxins B1, B2 and G1. *A. parasiticus* produced low levels of aflatoxin B1. It is well established that the most potent of aflatoxins is B1 which is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC 2002). Production of these toxins by fungi isolated from human corneal ulcers in Egypt has been demonstrated by Gharamah *et al.* (2013).

Sensitivity of fungi to antifungal agents

Data in Table 5 showed that Terbinafine (TER) followed by Clotrimazole (CC) and clove oil were the most effective antifungal agents where 55.1% – 68.3 % of total tested isolates were sensitive to these compounds. Results showed also that 90.8% of isolates were resistant to Fluconazole (FLC). Also, 50 - 54 % of isolates were resistant to Cetrimide (CET), Itraconazole (IT) and Amphoteicin B (AP). *Aspergillus* species belonging to sections *Flavi*, *Nigri* and *Terrei* were sensitive to Clotrimazole, Cetrimide, Terbinaine, Tioconazole and clove oil. On the other hand, most of these fungi showed resistance to Amphotericin B, Fluconazole, Itraconazole, Ketoconazole and Nystatin. *Candida* spp. showed variable degrees of sensitivity to Amphotericin B, Clotrimazole, Fluconazole, Ketoconazole and Nystatin. Fungal isolates related to *Chrysosporium*, *Phoma* and *Mucor* responded variably to the different antifungal agents as shown in Table (6). In this respect, Bonifaz *et al*. (2010) reported that topical antifungal agents are very helpful and should be started after proper cleaning of the auditory canal is obtained. The most widely used agents are Amphotericin B (1mg/ml which is active against most fungi but causes irritation) and Nystatin (100,000 U/ml). These agents are extremely effective in the treatment of *Aspergillus* otitis, while Imidazole derivatives such as Clotrimazole, Miconazole and Ketoconazole are less useful options. Jackman *et al*. (2005) used terbinafine for this purpose. Most patients with invasive *Aspergillus* otitis require long-term therapy with systemic antifungal drugs, which is usually associated with a complete recovery. The most widely recommended agent is Itraconazole at 100-200 mg/day for 15-30 days. In-*vitro* resistance to Itraconazole was described in isolates of *A. niger* causing otomycosis while susceptibility was retained to Voriconazole (Kaya and Kiraz 2007).

Conclusion: It is advised to establish a continuous collaboration between otolaryngologists and microbiologists for characterization of the pathogenic fungal species involved in otomycosis. In-*vitro* sensitivity test is of great importance to choose the most active antifungal agents. Patients are also advised to avoid removing of ear wax by metallic or stiff materials that may cause wounds subjecting ears to microbial infections. Keeping ear canal dry can also prevent or reduce the chance of otomycosis.

Table (5): Mycotoxins produced by otomycotic *Aspergillus* isolates

Fungal species	Aflatoxins	No. of producing isolates	Level of production (µg/L)			Non-producers
			L	M	H	
A. flavus (n=25)	B1	11	9	0	2	
	B2	1	0	1	0	
	B1 + B2	4	1	2	1	
	B1 + G1	2	2	0	0	
	G1	1	0	1	0	
	Total	19	12	4	3	6
A.flavus var. *columnaris* (n=9)	B1	2	2	0	0	
	B1 +B2	2	1	1	0	
	B1 + G1	1	1	0	0	
	G1	1	0	1	0	
	Total	6	4	2	0	3
A. parasiticus (n=1)	B1	1	1	0	0	0
Total isolates tested (n=35)	B1	14	11	0	3	
	B2	1	0	1	0	
	G1	2	3	0	0	
	B1 + B2	6	2	3	1	
	B1 + G1	3	1	0	0	
	Total	26	17	6	4	9

Levels of aflatoxin production (µg/L): L= Low (≤ 100), M= Moderate (>100- <500), H= High (≥ 500**).**

Table 6: Degree of sensitivity (DS): S = Susceptible, I = Intermediate, R= Resistant of common otomycotic fungal isolates to different antifungal agents.

Fungi tested (No. of isolates)	DS	AP	CC	CET	FLC	IT	KT	TER	NS	SER	TIO	CO
Aspergillus section *Flavi* (35)	S	3	29	14	1	1	8	34	8	19	31	22
	I	12	6	15	0	21	16	1	6	11	2	11
	R	20	0	6	34	13	11	0	21	5	2	2
Aspergillus section *Nigri* (39)	S	6	15	5	0	1	2	28	6	6	15	21
	I	13	16	8	1	7	8	6	13	17	11	9
	R	20	8	26	38	31	29	5	20	16	13	9
Aspergillus section *Terrei* (4)	S	0	2	1	0	1	1	3	0	0	3	3
	I	0	2	0	0	2	2	0	1	0	0	0
	R	4	0	3	4	1	1	1	3	4	1	1
Candida spp. (15)	S	6	12	2	7	3	15	2	10	0	3	5
	I	7	3	0	0	7	0	6	5	2	0	4
	R	2	0	13	8	5	0	7	0	13	12	6
Chrysosporium kertinophilum (1)	S	0	1	1	0	1	1	1	0	1	1	1
	I	0	0	0	0	1	1	1	0	0	0	0
	R	1	0	0	1	0	0	0	1	0	0	0
Mucor circinelloides (3)	S	2	0	0	0	0	0	0	0	1	0	2
	I	0	0	0	0	0	0	0	3	0	0	0
	R	1	3	3	3	3	3	3	0	2	3	1
Phoma epicoccina (1)	S	0	0	1	0	1	1	1	0	0	1	1
	I	0	0	0	0	0	0	0	1	0	0	0
	R	1	1	0	1	0	0	0	0	1	0	0
Total isolates (98)	S	17	59	22	8	7	27	67	24	27	54	55
	I	32	27	23	1	38	27	15	29	30	13	24
	R	49	12	53	89	53	44	16	45	41	31	19

AP:Amphotericine-B, CC: Clotrimazole, CET: Cetrimide, FLU: Fluconazole, IT: Itraconazole KT: Ketoconazole, TER: Terbinafine, NS: Nystatin, SER: Sertaconazole, TIO: Tioconazole, CO: Clove oil.

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