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## Fungi Associated within Human Otomycosis

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### ABSTRACT

Some diseases are so tightly correlated to allergic reactions to specific fungi. Several fungi are the causative agents of otomycosis. The knowledge of fungal otitis in Egypt is still quite poor. The main aims of this study are to isolate and identify fungal species involved in ear infection, examine their abilities to produce proteolytic and lipolytic enzymes as well as their sensitivity to some antifungal therapeutic agents. A total of 80 clinical otitis samples were recruited from the Ear, Nose and Throat Department, Minia University Hospital, Minia University, Minia during the period from January 2017- December 2018. The obtained results showed isolation of 152 fungal isolates related to 15 species representing 10 genera from the collected samples and were tested for their capabilities to synthesize protease and lipase enzymes. *Aspergillus* and *Candida* were the most prevalent identified species from different isolates. All the tested fungal isolates were very sensitive to Nystatine and Itraconazole and resistant to Muconazole. Moderate resistance to Turbinafine and Fluconazole was obtained by most of these fungi. However, Griseofulvin did not show any antifungal activity against the tested fungal strains. *Aspergillus*, *Alternaria* and *Fusarium* species showed the highest producer fungi for both enzymes.

### INTRODUCTION

Otomycosis is a superficial mycotic infection of the outer ear canal that affects 10% of otitis externa patients. Inflammation, pruritus, scaling, and extreme discomfort are among symptoms of the illness, which can be subacute or acute. Inflammation, superficial epithelial exfoliation, masses of debris containing hyphae, suppuration, and discomfort are all symptoms of mycosis (Bassiouncy *et al.*, 1986)

According to Conant *et al.* (1971), only 15 to 20% of ear infections are true otomycosis, with the majority being caused by bacteria. Otomycosis is caused by a number of different fungus. Aspergilli, of which *Aspergillus fumigatus* was one of the first identified and most frequently isolated species, are said to be responsible for 90% of cases of fungal infection of the human ear (Raper and Fennell 1965 and Thammahong *et al.*, 2015).

The most prevalent aspergilli are regularly detected in the air and from sources such as home dust, hay, and straw (Raper and Fennell 1965). Furthermore, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus* are the most commonly reported fungi (Collee *et al.*, 1996). *Aspergillus niger* is thought to be the primary cause of ear infections (Al-Doory 1980). According to Pradhan *et al.* (2003), all cases were caused by *Aspergillus* or *Candida* species. Also, *Scopulariopsis*, *Polypaecilum*, *Mucor*, *Rhizopus*, *Penicillium*, and *Dermatophytes* are aetiologic agents in rare occasions, usually as a result of face dermatophytosis (Kaur *et al.*, 2000).

The filamentous fungus may successfully release a variety of hydrolytic enzymes, the most common of which are protease and lipase. Soil is a dynamic medium for microbial and biological activities, and the number and kind of microorganisms present in soil are influenced by environmental conditions (Prescott *et al.*, 1993). Since they are recognized for their ability to secrete significant quantities of enzymes in their growing environment, microbial proteases from *Aspergillus* species have been examined in depth. Several of these secreted enzymes have been widely used in the food and beverage industry for decades, thanks to large-scale submerged fermentation (Biesebeke *et al.*, 2005).

Azole medications have become the preferred treatment for oropharyngeal and vulvovaginal candidiasis in recent years. Recent research has linked treatment failures to the production of efflux pumps that diminish drug accumulation, changes in the structure or concentration of antifungal target proteins, and changes in membrane sterol composition to some fungus and related species (Sanglard and Odds 2002, CLSI 2010 and Herasym *et al.*, 2016). Polyene medicines like Amphotericin and Nystatin were the only treatment options for invasive fungal

infections for a long time. Nystatin is still the most widely available antifungal medicine in underdeveloped countries.

The main goal of this study was to detect fungal species that are associated with ear infections, as well as to investigate their ability to produce proteolytic and lipolytic enzymes, and also their sensitivity to antifungal treatment drugs.

## MATERIALS AND METHODS

### Collection of Samples:

Samples were collected using cotton swabs from inside the ears of 80 clinical otitis patients after the clinical examination under the supervision of the doctor at Ear, Nose and Throat Department, Minia University Hospital, Minia University, Minia during the period from January 2017- December 2018. Those infected patients were categorized into three groups according to the age, group (1) from 18-30 years, group (2) from 31-50 years and group (3) from 51-80 years. A form has been prepared for each patient, in which some important information was proven, such as age and sex. The conference was done with the help of the specialist doctor. The collected samples were then transferred to the laboratory of Microbiology at the Department of Botany and Microbiology, Faculty of Science, Assiut University for the purpose of isolation and identification of fungi.

### Isolation of Fungi:

Potato Dextrose Agar (PDA) has been used for the growing and isolation of fungi by streaking the swab samples on surfaces of Petri-dishes of sterilized solidifying PDA medium. The plates were incubated at a temperature of 28° C for a period of 4 to 21 days.

### Identification of Fungal Genera and Species:

The obtained fungal isolates were identified according to macro- and microscopic features on the bases of the following keys:

1- Domsch *et al.* (2007), and Moubasher (1993), for soil fungi.

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- 2- Ellis (1971 & 1976), for Dematiaceous Hyphomycetes, Simmons (1967), for *Alternaria*, *Stemphylium* and *Ulocladium*.
- 3- Booth (1971) and Leslie and Summerell (2006), for *Fusarium* species.
- 4- Raper and Fennell (1965) and Samson and Varga (2007), for the genus *Aspergillus* systematics in the genomic era, Christensen and Raper (1978), for synoptic key to *Aspergillus nidulans* group species and related *Emericella*.
- 5- Raper and Thom (1949), Raper and Fennell (1965) and Pitt (1979) for the genus *Penicillium* and its teleomorphic states, *Eupenicillium* and *Talaromyces*.

#### Screening For Protease and Lipase Enzymes Production:

The collected fungal isolates were examined for their abilities to produce both protease and lipase enzymes. Using a casein hydrolytic media, the isolated fungi's proteolytic activity and ability to generate protease enzymes were assessed (Paterson and Bridge, 1994). The lipolytic activity of the isolated fungi was determined using a modified version of the Ullman and Blasins (1974) method. Instead of Tween 20, Tween 80 (poly oxy-ethylene sorbitan monooleate) was used.

#### Antifungal Activity of Some Drugs:

Six different antifungal agents (Nystatin, Fluconazole, Griseofulvin, Terbinafine, Muconazole and Itraconazole) were selected to carry out the antifungal susceptibility against the examined fungal isolates (Table 1).

**Table 1:** list of tested antifungal agents.

Antifungal name	Disc potency	Active agents
Nystatin	100 unit	Polyenes
Fluconazole	10 µg	Azoles
Griseofulvin	125 mg	Griseofulvin
Terbinafine	10 µl	Allylamines
Muconazole	10 µl	Azoles-Imidazole
Itraconazole	10 µg	Azoles

### RESULTS

#### Fungi Recovered from Otitis Patients:

Eighty patients suffering from external ear infections were examined in this study. Out of these patients, 48 cases (60%) were positive for fungal mycelial growth. From the positive patients, 28 cases (58.3%) were males and 20 cases (41.7%) were females. The highest incidence of age group is 18-30 yrs (25 cases, 52.1%) and

the least incidence was seen in the age group of 31-50 yrs (13 cases, 27.1%) and 51y-80 yrs (10 cases, 20.8%). The mycological analysis of the tested patients indicated the isolation of 152 fungal isolates related to 15 species representing 10 genera. *Aspergillus* and *Candida* species were the most prevalent in ear infections (Table 2), plates 1, 2 & 3.

**Table 2:** Number and percentage of the appearance of fungal isolates from otitis patients on potato dextrose agar medium at 28°C.

Genera and Species	No. of isolates	%
<i>Acremonium strictum</i>	3	2.0
<i>Alternaria alternata</i>	5	3.3
Total <i>Aspergillus</i>	104	68.4
<i>A. carbonarius</i>	2	1.3
<i>A. flavus</i>	26	17.1
<i>A. fumigatus</i>	13	8.6
<i>A. nidulans</i>	8	5.3
<i>A. niger</i>	48	31.6
<i>A. terreus</i>	7	4.6
<i>Cladosporium cladosporioides</i>	3	2.0
Total <i>Fusarium</i>	3	2.0
<i>F. oxysporum</i>	2	1.3
<i>F. solani</i>	1	0.7
<i>Mucor heimalis</i>	2	1.3
<i>Penicillium corylophyllum</i>	1	0.7
<i>Rhizopus stolonifer</i>	5	3.3
<i>Scopulariopsis brevicaulis</i>	4	2.6
Yeasts	22	14.4
Total isolates		
No. of genera = 10	152	100 %
No. of species = 15		

**Protease Production:**

A total of 129 fungal isolates was tested for their abilities for protease production. The obtained results revealed 119 (92.28%) % able to produce the enzyme. From those positive isolates, 79 isolates (61.2%) % have high proteolytic production whereas, 29 (26.3%) were moderate producers. The remaining positive isolates 11 (8.5%) were weak producers. The results revealed that isolates included *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. terreus*, *Fusarium oxysporum* and *Scopulariopsis brevicaulis* exhibited high enzyme production. Whereas, isolates belonging to *A. carbonarius*, *Cladosporium cladosporioides*, and *Mucor heimalis* achieved moderate production. The rest of the tested isolates appertaining to *Acremonium strictum*, and *Penicillium funiculosum* were weak producers (Table 3).

**Lipase Production:**

A total of 129 isolates tested for their abilities for lipase production 107 (82.9%) were able to produce the enzyme. From those positive isolates, 74 isolates (69.1 %) have a high capability for enzyme production whereas, 24 (22.4 %) were moderate producers. The remaining positive isolates 9 (8.4%) were weak producers. The results revealed that isolates included *A. flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Fusarium oxysporum*, *Cladosporium cladosporioides* and *Rhizopus stolonifera* exhibited high enzyme production. Whereas, isolates belonging to *Acremonium strictum*, *Alternaria alternata*, *Mucor heimalis*, and *Penicillium funiculosum* achieved moderate production. The rest tested isolates appertaining to *Mucor* (Table 4).

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**Table 3:** Protease production by fungal isolates recovered from otitis patients.

Genera and Species	NIT	Protease activity			
		NIP	H	M	W
<i>Acremonium strictum</i>	3	1	-	-	1
<i>Alternaria alternata</i>	5	4	2	1	1
<i>Aspergillus carbonarius</i>	2	1	-	1	-
<i>A. flavus</i>	26	26	23	2	1
<i>A. fumigatus</i>	13	13	10	2	1
<i>A. nidulans</i>	8	3	-	2	1
<i>A. niger</i>	48	48	35	10	3
<i>A. terreus</i>	7	7	2	4	1
<i>Cladosporium cladosporioides</i>	3	1	-	1	-
<i>Fusarium oxysporum</i>	2	5	3	1	1
<i>Mucor heimalis</i>	2	2	-	2	-
<i>Penicillium funiculosum</i>	1	2	-	1	1
<i>Rhizopus stolonifer</i>	5	3	2	1	-
<i>Scopulariopsis brevicaulis</i>	4	3	2	1	-
Total isolates	129	119	79	29	11

NIT= Number of isolates tested, NIP= Number of isolates positive, H= High activity (2.3-3.5 cm), M= Moderate activity (1.6-2.2 cm), W= weak activity (0.2-1.5 cm).

**Table 4:** lipase production by fungal isolates recovered from otitis patients.

Genera and Species	NIT	Lipase activity			
		NIP	H	M	W
<i>Acremonium strictum</i>	3	1	-	1	-
<i>Alternaria alternata</i>	5	3	1	2	-
<i>Aspergillus carbonarius</i>	2	1	1	-	-
<i>A. flavus</i>	26	26	23	2	1
<i>A. fumigatus</i>	13	13	10	2	1
<i>A. nidulans</i>	8	4	1	2	1
<i>A. niger</i>	48	40	30	7	3
<i>A. terreus</i>	7	7	4	2	1
<i>Cladosporium cladosporioides</i>	3	1	1	-	-
<i>Fusarium oxysporum</i>	2	4	2	2	-
<i>Mucor heimalis</i>	2	2	-	1	1
<i>Penicillium funiculosum</i>	1	1	-	1	-
<i>Rhizopus stolonifer</i>	5	2	1	1	-
<i>Scopulariopsis brevicaulis</i>	4	2	-	1	1
Total isolates	129	107	74	24	9

NIT= Number of tested isolates, NIP= Number of positive isolates, H= High activity (2.3-3.5 cm), M= Moderate activity (1.6-2.2 cm), W= weak activity (0.2-1.5 cm).

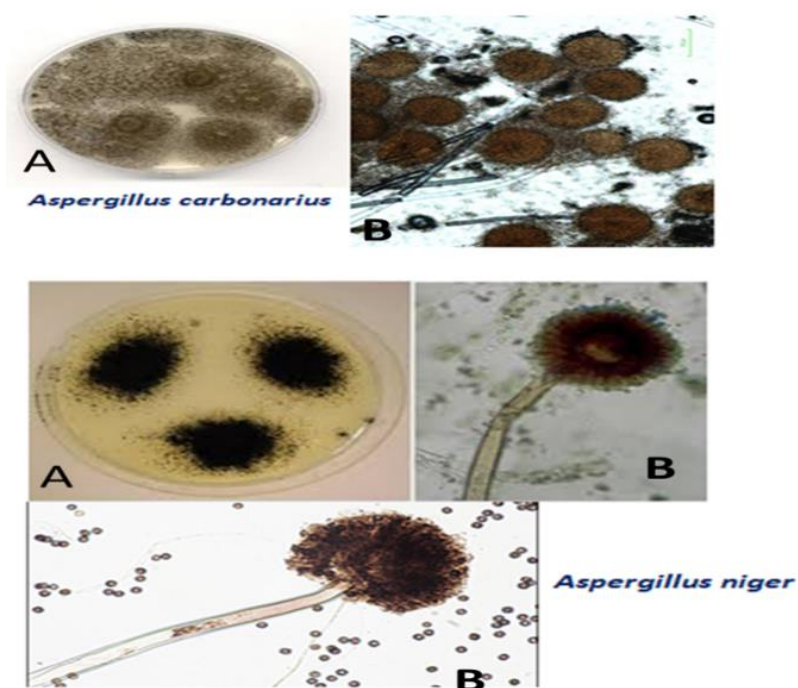
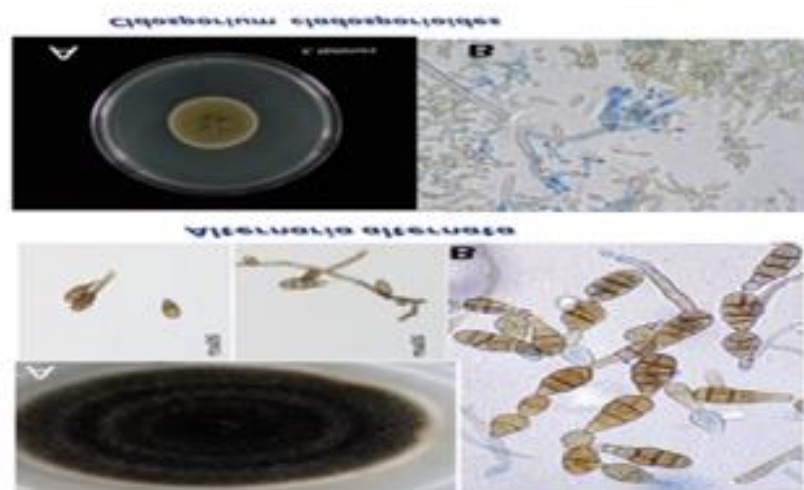
### Antifungal Agents:

Antibiotic sensitivity test by disc method showed a significant difference in inhibiting of growth of tested fungi. Griseofulvin has not shown any antifungal activity against the tested fungal strains.

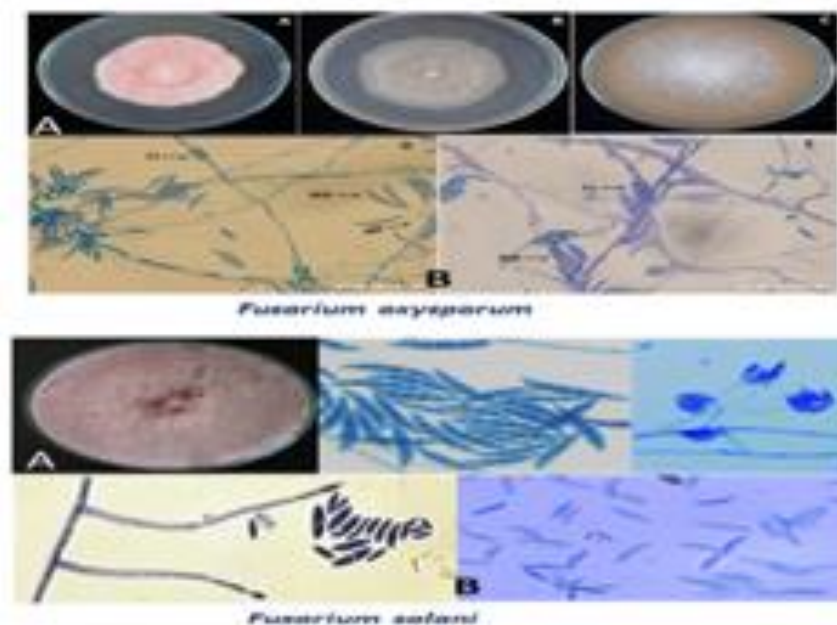
Also, all fungal isolates tested were very sensitive to nystatin and itraconazole and resistant to muconazole. Also, most of these fungi showed moderate resistance to terbinafine and fluconazole.

**Table 5:** Sensitivity of some fungi isolated from otitis patients towards antifungal agents in terms of inhibition zones measured in millimeters.

Fungal isolates	Antifungal agents					
	Terbinafine	Fluconazole	Nystatin	Griseofulvin	Itraconazole	Muconazole
<i>Alternaria alternata</i>	14	15	35	-	30	8
<i>Aspergillus flavus</i>	10	17	23	-	16	9
<i>A. fumigates</i>	15	24	32	-	21	7
<i>A. niger</i>	10	21	37	-	23	13
<i>A. terreus</i>	13	19	36	-	34	24
<i>Cladosporiumcladosporioides</i>	24	25	38	-	27	16
<i>Fusarium oxysporum</i>	19	27	29	-	32	15
<i>Penicillium funiculosum</i>	11	14	32	-	25	21
<i>Scopulariopsisbrevicaulis</i>	10	10	21	-	23	18
Species = 9	9	9	9	0	9	9

**Plate 1:** Colonies texture (A) and conidiophores and conidia (B).**Plate 2:** Colonies texture (A) and conidiophores and conidia (B).

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**Plate 3:** Colonies texture (Left) and conidiophores and conidia (Right).

#### DISCUSSION

In the current study, eighty patients suffering from an external ear infection (mycotic otitis externa) were examined for the presence of fungi. Out of these patients, 48 cases (60%) were positive for fungal mycelial growth. Of which, 28 cases (58.3%) were males and 20 cases (41.7%) were females. The highest incidence of age group is 18 - 30 yrs (52.1%) and the least incidence was seen in the age group of 31 - 50 yrs (27.1%) and 51- 80 yrs (20.8%). Young men were more likely to get the infection because they spend more time outside and are therefore more exposed to fungal spores. In this respect, fungi may occur in the external ear canal as saprobe or commensal, but in a certain condition, the fungus begins active reproduction, the ear canal can fill with dense fungal debris, causing pain that is not removed until the fungus gets a ride from the canal by antifungal treatment. About 9 to 25% of ear-related patients were caused by fungi (fungal otitis or otomycosis), while most cases were related to bacterial infection (Mugliston and O'Donoghue 1985 and Stern and Lucente 1988).

Otomycosis is a fungal infection of the external ear canal. Only a few studies are concerned with its frequency in Egypt. In this investigation, 60% of studied cases were infected by fungi. In accordance with this result, findings of each of Kaur *et al.* (2000), Nwabuisi and Ologe (2002), Kumar (2005), Aneja *et al.* (2010) and Barati *et al.* (2011), reported, respectively, 78%, 63.2%, 78%, 69% and 75.92% of the patients with the positive fungal diagnosis. Also, Kaur *et al.* (2000), Nwabuisi and Ologe (2002) and Pradhan *et al.* (2003) observed 76.8%, 54% and 81.3% of otomycosis, respectively.

#### Enzymes Production:

The ability of common fungi (129 isolates) collected in the current study for protease and lipase production was studied. The results revealed that most isolates tested were able to produce both protease (119 isolates) and lipase (107 isolates). From the positive isolates, 79 (66.4%) and 74 (69.1%) exhibited high proteolytic and lipolytic production, respectively. whereas 29 (24.4%) and 24 (22.4%) isolates showed moderate production of both enzymes, respectively. In addition, 11 isolates (9.2%) showed weak activity for the production of protease



enzyme and 9 (8.4%) were weak lipase producers. *Aspergillus*, *Alternaria* and *Fusarium* species showed the highest producer fungi for both enzymes. It is worthy to mention that lipolytic and proteolytic activities are considered the main virulence factors involved in the pathogenicity of fungi causing human and animal mycosis diseases and this has a major impact on public health (Cox *et al.*, 2000, Tomee and Kauffman 2000, Watanabe *et al.*, 2008, Karkowska-Kuleta 2009 and Farhan *et al.*, 2019).

#### **Antifungal Agents:**

Antibiotic sensitivity test by disc diffusion method showed a significant difference in inhibiting the growth of tested fungi using six antifungal agents. Data showed that all fungal isolates tested were very sensitive to nystatin and itraconazole, but resistant to muconazole. Furthermore, the majority of these fungi were resistant to terbinafine and fluconazole. Fungal isolates which exhibited high sensitivity towards most antifungal agents tested were related to *A. niger*, *A. terreus*, *C. cladosporioides*, *F. oxysporum* and *U. atrum*. On contrast, *P. funiculosum*, *S. brevicaulis* and *S. chartarum* showed higher resistance against the antifungal agents tested. It could be concluded that nystatin, itraconazole and fluconazole have a significant inhibitory effect against the growth of most tested isolates. Hence, the Sequence of antifungal activity against fungal isolates was descendingly arranged according to their antimicrobial potentiality as follows: nystatin>itraconazole>fluconazole >terbinafine>muconazole and griseofulvin. Kiakojouri *et al.* (2015) observed that after two weeks of utilising boericke alcohol in combination with Miconazole ointment, neither the intervention nor the control groups showed an improvement in their ear fungal infection. Differences in antifungal activity can be attributed to pathogenic etiological agent sensitivity, immune system state, and drug diffusion. After two weeks of utilising boericke alcohol and Miconazole ointment, neither the

intervention nor the control groups showed an improvement in their ear fungal infection. Differences in antifungal activity can be attributed to pathogenic etiological agent sensitivity, immune system state, and drug diffusion (Omran *et al.* 2018).

#### **CONCLUSION**

Up to the best of our knowledge, this is the pioneering work that provides valuable information about the incidence of pathogenic fungi in otomycosis in Minia Governorate. One of the risk factors for the transmission of fungal infections of the ear or nose is the spread of fungi in ecosystems in close proximity to human activities. *Aspergillus* and *Candida* species were proved to be of great value in otomycoses and mycotic sinusitis. As a result, it is critical to consider the various strategies for preventing or treating fungal infections. The first step is to assess the incidence and distribution of those diseases, and more knowledge of the predisposing variables and contamination sources in the manufacturing environment would be advantageous to control the human fungal disease. In our study higher incidence was found among males. The identification of a predisposing factor is also a crucial step in preventing recurrence. Cleaning the ear with wooden sticks or infected fingers, which disseminate fungal debris into the external auditory canal, should be avoided since it affects the normal lining epithelium, which is the body's natural defense against infection. The identification of a predisposing factor is also a crucial step in preventing recurrence. Cleaning the ear with wooden sticks or infected fingers, which disseminate fungal debris into the external auditory canal, should be avoided because it affects the normal lining epithelium, which acts as a natural defense against infection.

#### **RECOMMENDATIONS**

1-The job and geographical distribution in addition to gender were found to affect the infection with the ear. So, treatment involves the elimination of predisposing

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- factors and reducing the use of antibiotics.
- 2- In general, the discovery and identification of various species of fungi from the ear does not suggest that all of the fungi retrieved are disease-causing organisms. However, it is critical to distinguish these situations from those in which fungi are the cause of sickness.
  - 3- The presence of these fungi in many ecosystems that are closely linked to human activities is a risk factor for illnesses caused by these fungal species. As a result, it's critical to think about the many strategies for preventing fungal growth. It would be good to have a better understanding of the contamination sources in these environments in order to be able to manage their presence.
  - 4- Characterisation of pathogenic fungal species involved in otitis disorders requires ongoing collaboration between otolaryngologists and microbiologists.
  - 5-Future antifungal agents should have a broad spectrum of fungicidal activity without causing host harm due to the mechanism. A golden target is required to find a "golden" antifungal medication. The value of an in-vitro sensitivity test in selecting the most effective antifungal medicines cannot be overstated.
  - 6-Patients should also avoid removing ear wax with metallic or rigid objects since this might result in cuts, which can lead to microbial infections. In addition, otomycosis can be avoided or reduced by keeping the ear canal dry.
  - 7-Ear examination and immunological examination should be applied to patients as additional examinations on the suspicion of fungal infections.
  - 8- To attain the best results, early detection, correct diagnosis, and tailored treatment options are required.
  - 9- This research suggests that a microbiological abundance of protease and lipase-producing fungi has the power to digest proteolytic materials and induce infections in the ears and nose.

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