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Phenotype and Genotype Identification of Fungal Isolates in Otomycosis Patients with Emphasis on their Enzymatic Activity

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Detection of the fungal causative agents in the external auditory canal could be valuable to determine the potential risk of the disease. The aim of the present study was to isolate and identify the mycoflora of the human auditory canal in sampled patients of Saudi Arabia with emphasis on their enzymatic activity. A total number of 150 patients were clinically examined for the presence of mycotic otitis in Al-Dawadmi governorate (Saudi Arabia). The clinical samples were cultured on Sabouraud dextrose agar media. Fungal isolates were then identified using phenotypic and genotypic techniques. The enzymatic activities of some fungal isolates were also detected. Eighty six fungal isolates were recovered in this study. These isolates included sixteen fungal species belonging to eight genera. *Aspergillus spp*. were recorded in sixty one cases (70.93%), *Penicillium* (19.76%), *Candida* (2.3%) and *Fusarium* (2.3%). The disease was more prevalent among 21-30 years old (46.51%). Pruritus was the most common symptom. Extracellular proteolytic and lipolytic enzymes were produced by (90.9 %) and (75.75%) of the fungal isolates respectively. Hydrolytic enzymes that are considered the most important factors influencing virulence and pathogenicity of opportunistic fungal infections were detected in most of the fungal cultures tested herein.

Key words: Hydrolytic enzymes, ear, *Aspergillus*, *Penicillium*, *Fusarium*

Otomycosis refers to a superficial fungal infection of the human external auditory canal. Sometimes is associated with bacterial infection as an opportunistic agent. The epidemiology of otomycosis is worldwide; however, the hot, humid and dusty environment of the tropics and subtropics makes otomycosis more prevalent[1]. The infection may be acute, sub-acute or chronic and usually present with itching of the ear, otalgia, aural fullness, hearing impairment and tinnitus. The accompanied inflammation is associated with superficial epithelial exfoliation and formation of masses of debris containing hyphae, which further worsen the discomfort and sometimes culminate into frank suppuration in the affected ear[2].

Most severe cases of otomycosis are associated with tympanic membrane perforation, middle ear and sometimes the whole temporal bone involvement are observed with immunosuppression[3]. 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

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present in more than 90% of the patients[4,5]. Other common symptoms are otalgia, hearing loss, tinnitus and aural discharge[4].

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 hence itching may follow pain[6]. The use of broad-spectrum antibiotics, presence of trauma, persistent otorrhoea, alterations in immunity, use of steroids, dermatological diseases, loss of cerumen, hearing aids and swimming have been documented as predisposing factors[1,7,8].

A wide variety of fungi have been implicated in the causation of mycotic infection of the ear. The most common organisms include *Aspergillus* and *Candida* species. Other less frequently involved fungi include *Penicillium*, *Mucor* and *Rhizopus* species[6,9]. Pure or mixed fungal isolates could be responsible for either a unilateral or bilateral otomycosis[5].

Fungi are known to elaborate extracellular enzymes based on the substrate they utilize for growth. Extracellularly produced enzymes have been described in certain fungi such as *Candida sp*.[10] and *Aspergillus* species[11]. Production and secretion of hydrolytic enzymes, such as proteases, lipases and phospholipases are very important factors for virulence. These enzymes play an important role in nutrition, tissue damage, fungal dissemination within the human body, iron acquisition and overcoming the host immune system which strongly affects fungal pathogenicity[12]. Moreover, secretion of enzymes into extracellular environments might be an important adaptive mechanism during the life cycle of fungi[13]. Earlier studies on fungal enzymatic activities aimed at establishing the role of enzymes in fungal pathogenicity as well as their capacity to induce inflammatory reactions in the host tissues[14]. It is logical to suppose that these enzymes could act by enabling tissue invasion easier, but they could also participate in causing infection by impairing some mechanisms of the immune system and/or assist in obtaining nutrients, thus causing injury to the host[15,16].

In Saudi Arabia, knowledge on otomycosis is not fully elucidated and still very limited. Critical observations in phenotypic and genotypic identification of the fungal causal agents are also ill-defined. Therefore, the present experimental work was aimed to identify fungal species involved in otomycosis as well as their ability to produce proteolytic and lipolytic enzymes.

MATERIALS AND METHODS

Study population and sample collection

One hundred and fifty patients attending the outpatient clinic of Otolaryngology Department, Alhusseni Hospital in Al-Dawadmi governorate were clinically examined for otomycosis presence during the period from December 2012 to April 2013. Sterile cotton swabs were used for collecting debris, fungal elements, and earwax from the external auditory canal of patients showing symptoms of otomycosis. For each case, a specific questionnaire was performed containing personal information, history of otalgia, history of residual water in the ear canal after bathing or swimming and previous mycological report. Samples were immediately transferred to the Microbiology Laboratory at the College of Applied Medical Sciences of Shaqra University for primary investigation and further isolation and identification of fungi .

Mycological analysis

Culturing

Direct smears from swabs were prepared and examined using the mounting fluid Lactophenol Cotton Blue stain (LPCB) as recommended by Ellis *et al.*[17]. Fresh swabs were streaked on plates containing Sabouraud's Dextrose Agar medium (SDA).The composition of the medium per litter: peptone, 15 g; dextrose, 40 g and agar, 20 g. Plates were then incubated at 28° C for 7-15 days until fungal colonies appear. All fungi were stored on sabouraud dextrose agar (Oxoid) slants in the refrigerator at 4 °C prior to use.

Identification of fungi

Phenotype identification

Upon the completion of the incubation period, mycological examination of the fungal isolates was conducted based on the colony morphology and microscopic characterization. These fungal isolates were further re-identified and confirmed accurately at Assuit University Mycological Center according to the following key references[18-21].

Genotype identification

Twenty one 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 100 ¼l autoclaved distilled water in 2ml sterile vials and boiled at 100°C for 15 minutes and stored at -70° C. Samples were sent to SolGent Company (Daejeon, South Korea) for rRNA gene sequencing. The DNA of fungi 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 then sequenced in the sense and antisense directions using ITS1 and ITS4 primers[22]. 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 (SolGent Company, Daejeon, South Korea).

Screening of fungal isolates for extracellular enzyme production

Proteolytic activity

Test tubes containing modified **c**asein hydrolysis medium were used. The medium composition (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. The tested fungi were inoculated and cultures were incubated at 25°C for 7 days. Degradation of milk protein was measured as depth of clear zone (mm).

Lipolytic activity

The medium of Ullman and Blasins[23] 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). 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 of fungal isolates. After incubation at 25℃ for 7 days, the lipolytic enzymatic 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 (mm) was measured.

RESULTS AND DISCUSSION

Eighty six fungal isolates were recovered in this study (Table 1). These isolates include sixteen fungal species belonging to eight genera. The genus *Aspergillus* was represented by 6 species (*A.niger*, *A.flavus*, *A.brasiliensis*, *A.parasiticus*, *A.fumigatus* and *A.terreus*) followed, by the genus *Penicillium* which was represented by 3 species (*P.chrysogenum*, *P.aurantiogriseum* and *P.brevicopactum*). *Aspergillus spp*. were recorded in sixty one cases (70.93%), *Penicillium* (17cases,19.76%),*Candida* (2cases,2.3%), *Fusarium* (2 cases,2.3%) and the remaining genera were recorded only in one case for each of them(Table 1). Twenty one fungal isolates were re-identified by rRNA gene sequencing. Phylogenetic tree (Fig.1) showed eight clades; for *Aspergillus niger, A.terreus, A.flavus, P.chrysogenum, P.aurantiogriseum, Eurotium rubrum, Candida parapsillosis* and *Galactomyces candidum* which showed close relationship with similar strains deposited in the Gene Bank. It is worthy to mention that the genotypic identification based on rRNA gene sequencing (Fig.2) was showed close

Table 1. Incidence of fungi recovered from patients with otomycotic infections

Fungal species	Number of isolates	% incidence
Aspergillus spp.	61	70.93
A.niger	33	38.37
A.flavus	9	10.46
A. parasiticus	6	6.97
A. brasiliensis	6	6.97
A.fumigatus	5	5.81
A.terreus	2	2.32
Penicillium spp.	17	19.76
P.chrysogenum	12	13.95
P.aurantiogriseum	3	3.48
P.brevicopactum	2	2.32
Fusarium proliferatum	2	2.32
Candida sp.	2	2.32
Eurotium sp.	1	1.16
Galactomyces candidum	1	1.16
Scopulariopsis candida	1	1.16
Trichosporon beigelii	1	1.16

Table 2. Gene sequencing for some fungal isolates

Fungal isolate	Gene sequence
A.flavus	AUMC9400, ITS1) CGTGGNTTCTAGCGAGCCAACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCCGCCATT CATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACCACGAACTCTGTCTGATCTA GTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCG ATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAA CGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACG GCTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTC CGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATC AATCTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA GGAA AUMC9400, ITS4 NNNAANGNCACCTACTGATCCGAGGTCACCTGGAAAAGATTGATTTGCGTTCGGCAAGCGCCGGCCGG GCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTGGGG CCCGTCCCCCCCGGAGAGGGGACGACGACCCAACACACAAGCCGTGCTTGATGGGCAGCAATGACGCT CGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACGGAA TTCTGCAATTCACACTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTG TTGAAAGTTTTAACTGATTGCGATACAATCAACTCAGACTTCACTAGATCAGACAGAGTTCGTGGTGTCT CCGGCGGGCGCGGGCCCGGGGCTGAGAGCCCCCGGCGGCCATGAATGGCGGGCCCGCCGAAGCAACTA AGGTACAGTAAACACGGGTGGGAGGTTGGGCTCGCTAGGAACCCTACACTCGGTAATGATCCTTCCGCA GGTTCACCTACGGAAG
A.niger	AUMC9510, ITS1 GNGGTTCGACTCGGTCTTTGGGCCACCTCCCATCCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCGC CGCTTGTCGGCCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAAC ACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTG GTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCAT CGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCC CTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGGACGGGCCCGAAAGGCAGCGGCG GCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCG ACGTTTTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATC AATAAGCGGAGGAA AUMC9510, ITS4 NNNACTGGCAGCTACCTGATCGAGGTCACCTGGAAGAATGGTTGGAAAACGTCGGCAGGCGCCGGCCA ATCCTACAGAGCATGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTCGG

GCCCGTCCCCCCGGAGAGGGGGACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCT
CGGACAGGCATGCCCCCCCGGAATACCAGGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAAT
TCTGCAATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGT
TGAAAGTTTTAACTGATTGCATTCAATCAACTCAGACTGCACGCTTTCAGACAGTGTTCGTGTTGGGGTC
TCCGGCGGGCACGGGCCCGGGGGGCAGAGGCGCCCCCCCGGCGGCCGACAAGCGGCGGGGCCCGCCGAA
GCAACAGGGTACAATAGACACGGGATGGGAGGTTGGGCCCAAAGGACCCGCACTCGGTAATGATCCTTC
CGCAAGTTCA
CCCTACGGAG

P.chrysogenum

AUMC9514, ITS1
AGAAGACTCTGGGTCACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTAACTGG
CCGCCGGGGGGCTTACGCCCCCGGGCCCGCCGCCGAAGACACCCTCGAACTCTGTCTGAAGATTGT
AGTCTGAGTGAAAATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAG
AACGCAGCGAAATGCGATACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACAT
TGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGTG
TTGGGCCCCGTCCTCCGATCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGA
GCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAAATTTTTATCCA
GGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

AUMC9514, ITS4
ANGNNNGGGNACCTNCTGATCCGAGGTCACCTGGATAAAAATTTGGGTTGATCGGCAAGCGCCGGCCG
GGCCTACNGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCCGCCGCTGCCTTTCGG
GCCCGTCCCCCGGGATCGGAGACGGGGGCCCAACACACAAGCCGTGCTTGAGGGCAGAAATGACGCTC
GGACAGGCATGCCCCCCGGAATACCAGGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATT
TGCAATTCACATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTG
AAAGTTTTAAATAATTTATATTTTCACTCAGACTACAATCTTCAGACAGAGTTCGAGGGTGTCTTCGGCG
GGCGCGGGCCCGGGGGGCGTAAGCCCCCCGGCGGCCAGTTAAGGCGGGCCCGCCGAAGCAACAAGGTAA
AATAAACACGGGTGGGAGGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGNTTC
ACCT ACGGAA

Scopulariopsis sp.

AUMC9540, ITS1
CGGGTCTTTGGGCCACCTCCCATCCGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGG
CCGCCGGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAA
AGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCAT
CGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTG
ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCG
GCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGGCCCGAAAGGCAGCGGCGGCACCGCGTC
CGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCCGGCGCCTGCCGACGTTTTCCAA
CCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA GGA
AUMC9540, ITS4

ANTGGCTCTACCTGATCGAGGTCACCTGGAAGAATGGTTGGAAAACGTCGGCAGGCGCCGGCCAATCCT
ACAGAGCATGTGACAAAGCCCCATACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCG
TCCCCCCGGAGAGGGGGACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGAC
AGGCATGCCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTCTGC
AATTCACATTAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTGAAA
GTTTTAACTGATTGCATTCAATCAACTCAGACTGCACGCTTTCAGACAGTGTTCGTGTTGGGGTCTCCGG
CGGGCACGGGCCCGGGGGGCAGAGGCGCCCCCCCGGCGGCCGACAAGCGGCGGGCCCGCCGAAGCAA
CAGGGTACAATAGACACGGATGGGAGGTTGGGCCCAAAGGACCCGCACTCGGTAATGATCCTTCCGCA
GGTTCACCCTACGGAAG
Galactomyces candidum AUMC9543, ITS1
ATATATATTTGTGAATTTCACAAACAACATCAATTTTATAGTCTATTATTTTTAATTAAAACTTTTAACAA
TGGATCTCTTGGTTCTCGTATCGATGAAGAACGCAGCGAAACGCGATATTTCTTGTGAATTGCAGAAGT
GAATCATCAGTTTTTGAACGCACATTGCACTTTGGGGTATCCCCCAAAGTATACTTGTTTGAGCGTTGTT
TCTCTCTTGGAATTGCTTTGCTCTTCTAAAATTTCGAATCAAATTCGTTTGAAAAACAACACTATTCAACC
TCAGATCAAGTAGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA
AUMC9543, ITS4
TGGATCTACTTGATCTGAGGTTGATAGTGTTGTTTTTCAACGAATTTGATTCGAAATTTTAGAAGAGCAA
AGCAATTCCAAGAGAGAAACACAACGCTCAAACAAGTATACTTTGGGGGATACCCCAAAGTGCAATGTGC
GTTCAAAAACTGATGATTCACTTCTGCAATTCACACAAGAAATATCGCGTTTCGCTGCGTTCTTCATCGATA
CGAGAACCAAGAGATCCATTGTTAAAAAGTTTTAATTAAAAATAATAGACTATATAAAATTGATGTTGTTTG
TTGAAATTTCACAAAATATTATTAATTCATAAATGATCCTTCCGCAGGTTCACCTACGGAAG
Candida parapsilosis AUMC9548, ITS1
TAAAGTGCTTACTGCATTTTTTCTTACACATGTGTTTTTCTTTTTTTGAAAACTTTGCTTTGGTAGGCCTTC
TATATGGGGCCTGCCAGAGATTAAACTCAACCAAATTTTATTTAATGTCAACCGATTATTTAATAGTCAA
AACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATG
AATTGCAGATATTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATG
CCTGTTTGAGCGTCATTTCTCCCTCAAACCCTCGGGTTTGGTGTTGAGCGATACGCTGGGTTTGCTTGAA
AGAAAGGCGGAGTATAAACTAATGGATAGGTTTTTTTCCACTCATTGGTACAAACTCCAAAACTTCTTCC
AAATTCGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAA
AUMC9548, ITS4
CGCTGTCTACCTGATTTGAGGTCGAATTTGGAAGAAGTTTTGGAGTTTGTACCAATGAGTGGAAAAAAAC
CTATCCATTAGTTTATACTCCGCCTTTCTTTCAAGCAAACCCAGCGTATCGCTCAACACCAAACCCGAGG
GTTTGAGGGAGAAATGACGCTCAAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAA
AGATTCGATGATTCACGAATATCTGCAATTCATATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCG
AGAACCAAGAGATCCGTTGTTGAAAGTTTTGACTATTAAATAAATCGGTTGACATTAAATAAAATTTGGTT
GAGTTTAATCTCTGGCAGGCCCCATATAGAAGGCCTACCAAAGCAAAGTTTTTCAAAAAAAGAAAAACA
CATGTGTAAGAAAAAATGCAGTTAAGCACTTTTCATTCTGTAATGATCCTTCCGCAGGTTCACCCTACGGAAG

Eurotium rubrum

AUMC9397, ITS1
CGACCTCTGGGNCCACCTCCCATCCGTGTCTATCTGTACCCTGTTGCTTCGGCGTGGCCACGGCCCCGCCG
GAGACTAACATTTGAACGCTGTCTGAAGTTTGCAGTCTGAGTTTTTAGTTAAACAATCGTTAAAACTTTC
AACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAATTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCCTGGTATT
CCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCTTCCGTCCCT
GGCAACGGGGACGGGCCCAAAAGGCAGTGGCGGCACCATGTCTGGTCCTCGAGCGTATGGGGCTTTGTC
ACCCGCTCCCGTAGGTCCAGCTGGCAGCTAGCCTCGCAACCAATCTTTTTAACCAGGTTGACCTCGGATC
AGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGG
AUMC9397, ITS4
GNACTGGGGTCTACCTGATCCGAGGTCACCTGGTTAAAAGATTGGTTGCGAGGCTAGCTGCCAGCTGGA
CCTACGGGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCAGACATGGTGCCGCCACTGCCTTTTGGG
CCCGTCCCCGTTGCCAGGGACGGAAGCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGG
ACAGGCATGCCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGAACTCGATGATTCACTGAATTCT
GCAATTCACATTAATTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGA
AAGTTTTAACGATTGTTTAACTAAAAACTCAGACTGCAAACTTCAGACAGCGTTCAAATGTTAGTCTCCG
GCGGGCCGTGGCCACGCCGAAGCAACAGGGTACAGATAGACACGGATGGGAGGTTGGACCCAGAGGGC
CCGCACTCGGTAATGATCCTTCCGCAGGTTCCCCTACGGAAGG

agreement with phenotypic identification of some fungal isolates studied in the present investigation. From the present study it was observed that *Aspergillus niger* was showed high percentage of incidence (38.37)%, followed by *Penicillium chrysogenum* (13.95%) that were the dominant fungal species involved in otomycosis. These findings are in close agreement and harmony with the previous reports whereas *Aspergillus* species are the most commonly identified fungal pathogens in otomycosis[24,25]. The majority of the fungal pathogens isolated from the ear swabs were belonged to the taxon *Aspergillus*, represented by *A. niger*, *A. terreus*, *A. flavus* and *A. fumigatus.* The pathogenic fungi involved in otomycosis in south eastern part of China, are similar to India, Turkey and other countries where the majority of the pathogenic fungi involved in otomycoses belonged to *Aspergilli*[4,26]. Among *Aspergilli*, *A. niger* has been found to be the most common etiological agent and reported as the major cause of otomycosis[4,27] but there is a slight difference from the findings of Kaur *et al*.[2] who reported *A. fumigatus* as the most common cause of otomycosis. This mould has been considered more pathogenic than *A. niger* as *A.fumigatus* produces a haemolytic exotoxin which has the ability to alter skin resistance. Jia *et al*.[28] also reported that the most common cause of otomycosis were *Aspergillus* spp. that accounted for 73.04% of total fungal isolates and *A.niger* (54.78%) was the dominant fungi of otomycosis.

Most of the reports indicated 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[29] as well as in Spain[30] and Iraq[31]. According to Márquez *et al*[32] and Araiza *et al*[33] 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.

From 86 positive cases of otomycosis, 50 were male (58.13%) and 36 were female (42.35%). Men in the present study were more often affected

Table 3. Percentage incidence of otomycosis according to age and sex

Age group	Gender		Patients with otomycosis	
Years	Male	Female	N= 86	%
≤ 10	0	0	0	0
11-20	4	2	6	6.97
21-30	25	15	40	46.51
31-40	13	12	25	29.06
41-50	8	7	15	17.44

Table 4. Percent incidence and frequency of otomycosis symptoms in some patients*

Symptoms	Number of patients	Incidence (%)
Pruritus	61	70.9
Otalgia	50	58.1
Fullness of Ear	45	52.3
Pain	30	34.8
Otorrhea	23	26.7
Headache	20	23.2

* Number of patients was 86

Table 5. Lipolytic and proteolytic enzyme activity of certain fungi

AUMC No.	Fungal isolates	Lipase activity		Protease activity	
		Depth of clear zone (mm)	Level	Depth of turbid zone (mm)	Level*
9378	*Aspergillus brasiliensis*	32	H	11	L
9396	*A. brasiliensis*	15	M	15	M
9401	*A. brasiliensis*	50	H	0	N
9377	*Aspergillus flavus*	10	L	23	H
9391	*A.flavus*	51	H	17	M
9400	*A. flavus*	8	L	19	M
9385	*A. flavus*	8	L	20	M
9382	*A.flavus* var. *columnaris*	10	L	15	M
9393	*A. flavus* var. *columnaris*	15	M	16	M
9536	*A. fumigatus* var. *ellipticus*	50	H	0	N
9548	*A. fumigatus* var. *ellipticus*	0	N	5	L
9554	*A.fumigatus* var. *ellipticus*	10	L	14	M
9379	*A. niger*	30	H	13	M
9559	*A.niger*	35	H	13	M
9510	*A. niger*	30	H	19	M
9530	*A. niger*	50	H	6	L

by otomycosis, and such figures were closer to those observed by Kaur *et al*[2], Ho *et al*[32] and Yehia *et al*[35] who found 60%,56% and 52.5%,respectively in males. However, these data are in disagreement from the findings by Fasunla *et al*[36] who studied 5784 patients with ear diseases and found that 378 (6.54%) had otomycosis which consisted of 145 (38.36%) are males and 233 (61.64%) females. They recorded that the percent of otomycosis in male lower than that in female and this may suggest that the females generally seek medical helps for their ailment more than males. Other reasons could be attributed to some cultural practices such as the traditional head scarf and hijab commonly worn by women. This practice is usually associated with the prolonged covering of the external auditory canal which increases the humidity within the ear canal and hence predisposes to otomycosis[37]. Besides, the use of dryer in the washing and setting of hairs by women also increases the humidity in the external auditory canal and this encourages otomycosis. The age of otomycotic patients was ranging from 17 to 50 years. The prevalence of otomycosis regularly decreased with the increase of age of patients. The largest number of cases in the 21-30 years old age group (46.51%) followed by 31-40 age group (29.06%) and 41-50 age group (17.44%) (Table 2). Otomycosis was seen in patients aged between 2 and 66 years. Nonetheless, 50% of the cases were diagnosed in patients between 2 and 15 years of age. Occurrences of 70% to 41.1% were seen in

patients within the age range of 16 to 30 years[2,35,38]. Baratie *et al*[39] found that otomycotic patients in their fourth decade of life made up the biggest group (30.4%) followed by 21-30 age group (22.2%).

Moreover, all of the patients had unilateral otomycosis, 56 (65.11%) right and 30 (34.88%) left ears, these results are nearly close to those reported by Fasunla *et al*[36] who found that the percentage incidence of otomycosis in right ears(46.03%) was higher than that in left ears (41.80%). Paulose *et al*[40] and Mugiliston & O'Donoghue[41] reported that otomycosis is predominantly a unilateral disease suggesting it is not highly infectious. The infection in right ears may be attributed to majority of patients being right handed.

None of positive cases in this study was suffer from any chronic disease and immunocompromised (receiving chemotherapeutic agents or systemic steroids). The common symptoms presenting solely, or in combination of each other encountered in the study group have been summarized in (Table 4). Pruritis was the most common complaint encountered followed by otalgia, fullness of ear, pain, otorrhoea and headache. These results are nearly close to that which were recorded by Pontes *et al*[42] whereas the most clinical signs were: pruritus (60%), otalgia (45%),otorrhea(30%) and hypacusis (30%) (Multiple responses). Jia *et al*[28] also found that the important symptoms was pruritus (57.41%) followed by otorrhea (53.70%), ear fullness (48.15%), hearing loss (34.26%), otalgia (12.96%) and tinnitus (11.11%).

Out of 33 isolates which were chosen randomly to test their ability to produce lipase enzyme, 25 (75.75%) were able to produce lipase but with variable capabilities (Table 5). High lipase production was exhibited by 8 isolates (32%) which were mainly belonging to genus *Aspergillus.* Three isolates (12%) which include *A.brasiliensis* (AUMC 9396), *A.flavus var.columnaris* (AUMC

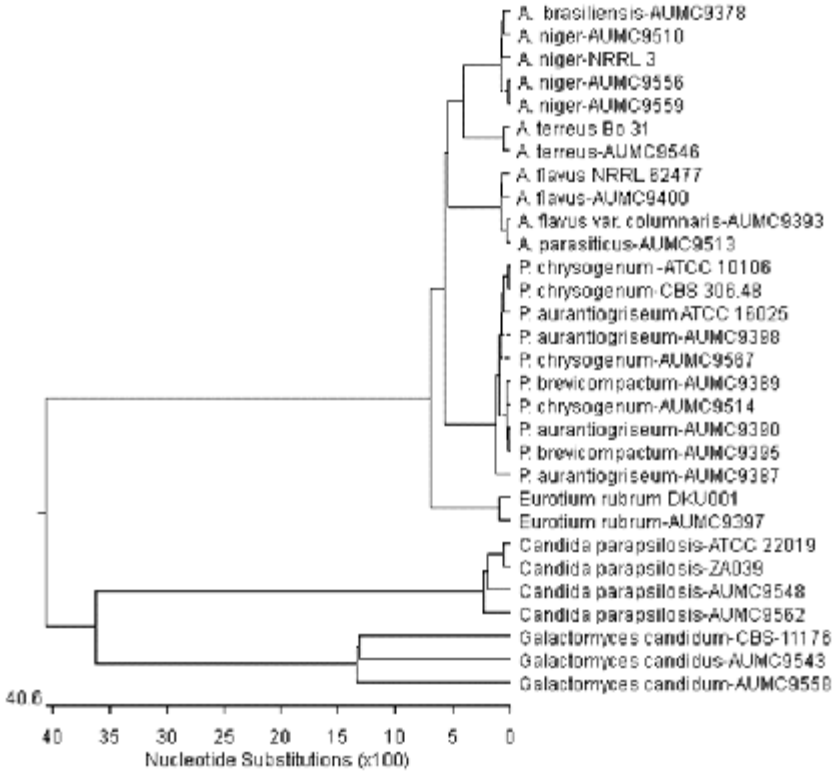

Fig. 1. Phylogenetic tree of fungal species isolated from otomycotic cases (given AUMC numbers), A= *Aspergillus*, P= *Penicillium*. Reference strains of corresponding fungi are involved in the tree (given ATCC, Bo, CBS, DKU, NRRL or ZA numbers)

9393) and *P.aurantiogriseum* (AUMC 9398) were moderately able to produce lipase. Fourteen isolates (56%) which were belonging to *Aspergillus*, *Candida*, *Penicillium* and *Scopulariopsis* genera exhibited low activity of lipase. 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[43-46] found that extracellular lipases play a role during microbial infections and suggested their role is to digest lipids for nutrient acquisition by pathogenic microbe and that these enzymes help the microbe (bacteria or fungi) to grow in environments where lipids are the sole carbon source.

Data indicated that 90.9 % of tested isolates (30 out of 33) had the ability to produce protease enzymes. High protease production was exhibited by 4 isolates (13.3%) which were belonging to the genera *Aspergillus* and *Penicillium*. These isolates include, *A.flavus* (AUMC 9377), *A.parasiticus* (AUMC 9517) and 2 isolates of *Penicillium brevicompactum* (AUMC 9389 and AUMC 9389). 22 isolates (73.3%) which include 2 isolates of *A.terreus* (AUMC 9546 and AUMC 9549), 3 isolates of *A.flavus* (AUMC 9385, AUMC 9400 and AUMC 9391),3 isolates of *P.aurantiogriseum* (AUMC 9398, AUMC 9390 and AUMC 9387), 3 isolates of *A.niger* (AUMC 9510, AUMC 9559 and AUMC 9379), 4 isolates of *P.chrysogenum* (AUMC 9514, AUMC 9567,AUMC 9547 and AUMC 9388),2 isolates of *A.flavus var.columnaris* (AUMC 9393 and AUMC 9382)and one isolate for each of the following species *A.brasiliensis* (AUMC 9396), *A.fumigatus var.ellipticus* (AUMC 9554),*Candida sp*.(AUMC 9561) and *Scopulariopsis candida* (AUMC 9540) were moderately able to produce protease. The remaining isolates exhibited low activity of protease production (Table 5). These results indicated that most of the tested isolates exhibited moderate protease capabilities. Abdoul-Nasr *et al*[47] recorded that more than 66% of tested isolates (73 out of 110) of five fungal genera; 3 *Aspergillus spp*. (55 isolates), one *Cladosporium sp*. (5 isolates), 2 *Fusarium spp*. (23 isolates), one *Stachybotrys sp*. (26 isolates) and one isolate of *Myrothecium sp*. had the ability to produce protease. Salyers and Witt[48] reported that microbial cells secrete hydrolytic enzymes that destroy the constituents of host cell membranes leading to membrane dysfunction, physical disruption as well as aid in the invasion of host tissues. Proteolytic degradation of lung tissues has been suggested as one of the key events involved in the physiopathology of *A. fumigates*[49]. Also, several species of*Aspergillus* such as *A. fumigatus, A. flavus, A. oryzae* and *A. sojae* are known to secrete protease as reported by Monod *et al*[50].

CONCLUSION

Clinical suspicion of otomycosis is important to prevent unnecessary use of antibiotics. Otolaryngologists should remain alert for otomycosis and should consider obtaining cultures when this disease is suspected. Mycological diagnosis is important since symptoms (pruritus, otalgia and Otorrhea) are not specific. Hydrolytic enzymes which are considered the most important virulence factors influencing the pathogenicity of opportunistic fungal infections were detected in most of the cultures of fungal isolates tested herein.

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