# Isolation, Characterization, and Antifungal Sensitivity Pattern of *Candida* Species Causing Otomycosis

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**Abstract-** Otomycosis is one of the overwhelming diseases both for patients and specialists with a high recurrence rate despite adequate and proper treatment. This study aims to investigate further the various types of fungi involved in otomycosis and test their susceptibility against common antifungals. In total, among candidiasis-suspected patients, 60 samples were incorporated into the study. PCR method was used for *Candida* species detection. Broth microdilution method of Clinical and Laboratory Standards Institute document M60 was applied to assess MIC values of rampant antifungals. We used SPSS software (version 16.0) for statistical analysis. In this survey, 20, 3, and 1 type of *Candida albicans*, *Candida parapsilosis*, and *Candida glabrata* were identified, respectively. All 20 *C. albicans* isolates were sensitive to amphotericin B (range 0.03-1 µg/ml), voriconazole, (0.03-1 µg/ml), and itraconazole (0.03-0.5 µg/ml.); moreover, one isolate was resistant to fluconazole. Two isolates out of three isolates of *C. parapsilosis*, were susceptible to all agents while the other one isolate was resistant to fluconazole. *C. glabrata* isolate was susceptible to all agents. In summary, the results conveyed the importance of clinicians remaining vigilant in diagnosing otomycosis due to its non-specific manifestations. To manage effectively otomycosis and avoid complications or recurrence, it is imperative to diagnose the condition at the earliest time, confirm its virulence through various tests, and identify antifungal susceptibility patterns. Despite this, relapse is often seen and achieving complete remission can prove to be a major hurdle in individuals who have had mastoidectomy and those with weakened immune systems.



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## Introduction

Fungal otitis externa is a fungal disease that specifically targets the external auditory canal (1,2).

Otomycosis is one of the overwhelming diseases both for patients and specialists with a high recurrence rate despite long and adequate treatment (3,4). *Aspergillus* species(spp.) and Candida spp. are considered the most common causative agents of otomycosis (2,5-7). *Aspergillus niger* is the flagship species among *Aspergillus* spp., followed by *Aspergillus* section *flavui*, *Aspergillus* section *fumigati*, and *Aspergillus terreus* (7-9). *Candida albicans* is the second most familiar species, following non-albicans *Candida*. It is joined by *Penicillium* spp., *Mucor* spp., *Rhizopus* spp., *Cladosporium* spp., and *Chrysosporium* spp (2). Fungal species show diverse levels of susceptibility towards currently available antifungals. A significant number of non-albicans *Candida* spp. exhibit resistance to these medications (8). The recognition of the contributing fungi plays a vital role in the successful prescription of antifungal medication for a cure. Thorough investigations into various otomycosis-causing fungi and their susceptibility to current antifungal treatments will aid clinicians in diagnosing and improving the treatment of fungal otitis externa. The purpose of this study was to

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give a more thorough understanding of the multiple fungi varieties associated with fungal otitis externa and their response to existing antifungal treatments.

## Materials and Methods

### Collection of samples and initial examination

In order to gather debris, fungal components, and cerumen from the outer ear canal of patients with otomycosis symptoms, two sterile cotton swabs were utilized. If syringing is done, the aspirate can be used as a sample. It is crucial to process the samples without delay. Direct microscopic inspection was performed on the debris using a 10% potassium hydroxide to search for fungal elements. The samples were also cultured on Sabouraud Dextrose Agar (SDA) from Merck, Germany and incubated at 35° C for a period of one week.

### Molecular identification

In total, 25 µl of amplified polymerase chain reaction (PCR) was used, including 25 pmol, 1 µl of each reverse and forward primer, 2 µl of DNA, 12.5 µl of amplicon master mix (Amplicon, Denmark), and water added to reach the final volume. The gene region was successfully amplified using the internal transcribed spacers (ITS) 1 and 4 primers, following the given protocol: 10 min of primary denaturation at 95º C, 40 cycles of denaturation at 95º C for 20 sec, annealing at 62º C for 20 sec, an expansion at 72º C for 20 sec, and an ultimate extension of 72º C for a period of 5 minutes. Eventually, the products were run on a 2% agarose gel. Purification and sequencing of the PCR products were carried out using the Sanger dideoxynucleotide method. The flanking primers (ITS 1-4) and the internal primers (ITS 1-4) were used to obtain overlapping sequences in consecutive runs. The Mega Sequence analysis software was utilized to assemble and analyze the sequence data. Additionally, individual nucleotide-nucleotide searches were carried out on the National Center for Biotechnology Information website using the BLASTn algorithm. (http://www.ncbi.nlm.nih.gov/BLAST/). In accordance with the previous method, the PCR technique was employed using the WHP1 gene and complementary primers HWP1-F (5′GCTACCACTTCAGAATCATCATC-3′) and HWP1-R (5′-GCACCTTCAGTCGTAGAGACG-3′) for *C. albicans* complex.

### Antifungal susceptibility assay

The minimum inhibitory concentrations (MIC) of miconazole, fluconazole, itraconazole, voriconazole, posaconazole, amphotericin B, caspofungin, and tolnaftate (all antifungal were obtained from Sigma Aldrich, USA) were appraised based on the Clinical and Laboratory Standards Institute document M60 (CLSI) method. We used the following medium: RPMI-1640 2×with l-glutamine and without sodium bicarbonate (Sigma-Aldrich, USA) supplemented with 2% w/v glucose (Sigma-Aldrich, USA) and buffered to pH 7.0 with MOPS (Sigma-Aldrich, USA).

Each well of flat bottom 96-well microtiter plates was filled with $5\times10^5$ cells/mL of *Candida*. Following a 24-hour incubation at 35° C, the microtiter plate reader was used to measure the absorbance at a wavelength of 590 nm. Quality control was performed on *Candida parapsilosis* ATCC 22019.

### Statistical analysis

Statistical analysis was conducted by SPSS software (version 16.0). Testing for association involved using the chi-squared test and calculating the corresponding *P*. A *P* of 0.05 or less was considered statistically significant. The MIC range and MIC 90 were also calculated.

## Results

An analysis of 65 cases of otomycosis suspected clinically revealed that 40 of them exhibited fungal growth. The age group with isolated fungus ranged from 30 to 60 years. The age group of 30-41 years had the highest number of cases reported. Out of the total positive samples, 24 were from females and 16 were from males. According to the findings, the leading contributing factor in 65% of the cases was the repeated use of unsterile items, including earbuds, safety pins, and match sticks, for ear cleaning. This was deemed statistically significant with a *P* below 0.001. The results indicate that 35 out of 40 positive cultures were KOH positive. In addition, 16 of the isolated fungi were filamentous fungi and 24 were identified as *Candida* spp. Out of all the identified fungal species, *A. niger* was the predominant isolate (n=13, 32.5%), followed by *A. fumigatus* (n=3, 7.5%). The colonies' color on CHROMagar revealed the presence of 20 *C. albicans* isolates, 2 *C. parapsilosis* isolates, 1 *Candida glabrata* isolate, and 1 *Candida krusei* isolate. Sequencing analysis confirmed the CHROMagar assessment. Through the application of HWP1 gene primers, it was determined that all *C. albicans* complex strains were of the *C. albicans* spp.

In the testing of 20 isolates of *C. albicans*, it was found that all were susceptible to amphotericin B (range 0.03-1 µg/ml), voriconazole (0.03-1 µg/ml), and

itraconazole (0.03-0.5 µg/ml.), except for one isolate which displayed resistance to fluconazole.

Of the three *C. parapsilosis* isolates, two were sensitive to all agents, but one was resistant to fluconazole. Furthermore, the *C. glabrata* isolate was also susceptible to all agents.

## Discussion

Out of the 65 suspected cases of otomycosis, 40 were found to have fungal growth on culture analysis. In this study, there was a female predominance as 24 positive samples had been isolated from females and 16 from males.

Our findings corroborate the studies conducted by Barati *et al*., (9) and Aneja *et al*., (10) studies, with 60% of the 40 fungi isolated being identified as *Candida* spp. and the remaining 40% as filamentous fungi. This indicated that the leading isolates were filamentous fungi. Despite this, it contradicts the results of da Silva Pontes *et al*., (11) and Kumar H *et al*., (12) researches, which showed a higher prevalence of *Candida* isolates compared to filamentous isolates. 20, 3, and 1 out of the 24 isolated *Candida* spp. were *C. albicans*, *C. parapsilosis*, and *C. glabrata*, respectively.

Unlike Aspergillus infections, *Candida* infections do not have a distinct appearance, making it more difficult to diagnose clinically. This can manifest as otorrhea that does not improve with aural antimicrobial therapy. Despite the fact that several in vitro studies have been conducted, there is still no agreement on which antifungal agent is the most effective. In the testing of 20 isolates of *C. albicans*, it was found that all were susceptible to amphotericin B (range 0.03-1 µg/ml), voriconazole (0.03-1 µg/ml), and itraconazole (0.03-0.5 µg/ml.), except for one isolate which displayed resistance to fluconazole. All three isolates of *C. parapsilosis* were tested, with two exhibiting susceptibility to all agents while one isolate was resistant to fluconazole. It should be mentioned that *C. glabrata* isolate was susceptible to all agents. Shokoohi *et al*., conducted a comparison between luliconazole and efinaconazole (two new azoles) and nine commonly used antifungal drugs against clinical samples of *Aspergillus* and *Candida* spp. obtained from individuals diagnosed with otomycosis, luliconazole, and efinaconazole were found to have the lowest GM, MIC values against the species studied, as reported (13).

This study was limited by the growing prevalence of COVID-19 within our geographic region, resulting in a smaller sample size.

In summary, the results conveyed the importance of clinicians remaining vigilant in diagnosing otomycosis due to its non-specific manifestations. To manage more effectively otomycosis and avoid complications or recurrence, it is imperative to diagnose the condition at the earliest time, confirm its virulence through various tests, and identify antifungal susceptibility patterns. Despite this, relapse is often seen and achieving complete remission can prove to be a major hurdle in individuals who have had mastoidectomy and those with weakened immune systems.

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