ISOLATION, CHARACTERISATION AND ANTIFUNGAL SENSITIVITY PATTERN OF FUNGI CAUSING OTOMYCOSIS IN PATIENTS REPORTING IN A TERTIARY CARE HOSPITAL

Dissertation Submitted To

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI

In partial fulfillment of the regulations

For the award of the degree of

M.D. (MICROBIOLOGY)

BRANCH IV

GOVT. KILPAUK MEDICAL COLLEGE

CHENNAI

May 2018

CERTIFICATE

This is to certify that this dissertation entitled **"ISOLATION, CHARACTERISATION AND ANTIFUNGAL SENSITIVITY PATTERN OF FUNGI CAUSING OTOMYCOSIS IN PATIENTS REPORTING IN A TERTIARY CARE HOSPITAL"** is the bonafide original work done by **Dr.JAYACHITRA. J,** Post graduate in Microbiology, under my overall supervision and guidance in the Department of Microbiology, Govt. Kilpauk Medical College, Chennai, in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

Dr. K.V. LEELA, M.D.,DGO.,
Professor & H.O.D
Department of Microbiology
Govt. Kilpauk Medical College
Chennai-600010

Dr.P.VASANTHAMANI,MD.,DGO.,

The Dean
Govt. Kilpauk Medical College
Chennai-600010.

CERTIFICATE

This is to certify that this dissertation entitled **"ISOLATION, CHARACTERISATION AND ANTIFUNGAL SENSITIVITY PATTERN OF FUNGI CAUSING OTOMYCOSIS IN PATIENTS REPORTING IN A TERTIARY CARE HOSPITAL"** is a bonafide research work done by **Dr.JAYACHITRA. J,** Post graduate in Microbiology, under my guidance in the Department of Microbiology, Govt. Kilpauk Medical College, Chennai, in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

Dr. K.V. LEELA, M.D., DGO.,
Professor & H.O.D
Department of Microbiology
Govt. Kilpauk Medical College
Chennai-600010

CERTIFICATE

This is to certify that this dissertation work entitled "**ISOLATION, CHARACTERISATION AND ANTIFUNGAL SENSITIVITY PATTERN OF FUNGI CAUSING OTOMYCOSIS IN PATIENTS REPORTING IN A TERTIARY CARE HOSPITAL"** of the candidate **Dr.JAYACHITRA. J,** with registration number **201514152** for the award of **M.D Degree in Microbiology (Branch IV).** I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **1** percentage of plagiarism in the dissertation.

Dr. K.V. LEELA, M.D.,DGO.,
Professor & H.O.D
Department of Microbiology
Govt.Kilpauk Medical College
Chennai-600010

DECLARATION

I solemnly declare that this dissertation **"ISOLATION, CHARACTERISATION AND ANTIFUNGAL SENSITIVITY PATTERN OF FUNGI CAUSING OTOMYCOSIS IN PATIENTS REPORTING IN A TERTIARY CARE HOSPITAL"** is the bonafide work done by me at the Department of Microbiology, Government Kilpauk Medical College and Hospital, Chennai, under the guidance and supervision of **Dr. K.V. LEELA, M.D.,DGO.,** Professor & H.O.D of Microbiology, **Dr.THYAGARAJAN RAVINDER, M.D.,** Professor of Microbiology **Dr.M. KAVITHA, MD.,** Associate Professor, Department of Microbiology, Govt. Kilpauk Medical College, Chennai - 600 010. This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of Degree of M.D. Branch IV Microbiology examinations to be held in May 2018.

Place : Chennai.

Date : **Dr.JAYACHITRA. J**

ACKNOWLEDGEMENT

I wish to extend my profound gratitude to **Dr.P.VASANTHAMANI, M.D.,DGO.,** Dean**,** Government Kilpauk Medical College and Hospital for giving me permission to carry out my dissertation work and also to avail all the facilities available in the department.

I sincerely express my gratitude to **Dr. K.V. LEELA, M.D.,DGO.,** Professor and H.O.D., Department of Microbiology for her unreserved support, valuable advice, excellent guidance and encouragement given to me throughout this study.

I am immensely grateful to **Dr.THYAGARAJAN RAVINDER,M.D.,** Professor, Department of Microbiology for his constant motivation and guidance extended to me during my study.

My sincere thanks to **Dr. M. KAVITHA, MD.,** Associate Professor, Department of Microbiology for her help and valuable advice throughout this study.

My sincere thanks to **Dr. K.RAVI, MS.,DLO.,** Professor and H.O.D., Department of ENT for his support and valuable guidance.

I extend my sincere thanks to **Dr.M.SUGANTHI, MD., Dr.K.LAVANYA,M.D., Dr.S.HEMALATHA, M.D., Dr.C.AMUTHA, M.D., Dr.B.RAVICHANDRAN, M.D.,** Assistant Professors, Department of Microbiology for their help, support and valuable suggestions.

I would like to thank all my department colleagues for their timely help, cooperation and moral support. I express many thanks to all the technical staffs and other staff members of the Department of Microbiology for their kind co-operation during this study.

I extend my thanks to all the patients who participated in my study. I also thank my family members for their selfless love and moral support.

INTRODUCTION

Fungi constitute a vast and diverse group of eukaryotic, heterotrophic, spore producing, single celled or multi-celled organisms that live by decomposing and absorbing the organic material in which they grow[1]. Although there are over 1.5 million species of fungi on Earth, only about 300 of those are known to cause infections in humans.

Pathogenic fungi are those which are capable of causing disease in humans. They do so because of their specific metabolism and enzymes systems, which allows them to survive at elevated body temperatures and helps to overcome the host defense mechanisms[2,5].

Otomycosis

Otomycosis is a superficial, fungal infection of the external auditory canal[2,4]. Otomycosis is challenging for both patients and otolaryngologist as it frequently requires long term treatment and follow up. Inspite of proper treatment and follow up, the recurrence rate remains high[3,6].

Causative Organisms

Aspergillus and *Candida* are the most common organisms causing otomycosis[2,5,7]. Among *Aspergillus* species, *Aspergillus niger* is usually the predominant species, followed by *Aspergillus flavus, Aspergillus fumigatus* and *Aspergillus terreus*[7,9]. Among *Candida* species, *Candida albicans* is the most common isolate followed by *Candida parapsilosis, Candida glabrata, Candida*

guilliermondii and *Candida krusei*. Other fungi like *Penicillium, Mucor, Rhizopus, Cladosporium, Chrysosporium* and *Absidia* are also involved[2].

The different species of fungi have varying susceptibilities to the commonly used antifungal drugs. *Aspergillus fumigatus, Aspergillus terreus* and non-albicans *Candida* show high degree of resistance to antifungals [8]. Hence it is very important to identify and speciate the causative fungi, as it will help in choosing appropriate antifungal drugs for effective therapy.

In recent years there has been an increased awareness about fungal infections and a high degree of clinical suspicion among doctors in diagnosing otomycosis. More in-depth studies on the various otomycosis causing fungi and their degree of sensitivity to currently available antifungal drugs will help the clinician in diagnosing and refining treatment of otomycosis. This study will give us insights into the various fungi causing otomycosis in this region, their virulence factors and susceptibility to commonly used antifungal drugs.

AIM

- To isolate, identify, speciate and characterise the fungi isolated from patients with clinically suspected otomycosis.

OBJECTIVES

- To determine the prevalence of fungi causing otomycosis.
- To analyse the distribution of fungal species causing otomycosis.
- To identify the predisposing factors for otomycosis.
- To characterise the isolated fungal species.
- To determine the antifungal susceptibility pattern of the isolates.

REVIEW OF LITERATURE

Historical perspectives:

In 1835, Agostino Bassi discovered that a disease of silkworms was caused by a fungus, *Beauveria bassiana.* In 1841, David Gruby demonstrated that Favus, an infection of the scalp in humans, was caused by a fungus. By 1890, Raymond Jacques Adrien Sabouraud, the "Father of Medical Mycology" published numerous articles on fungal disorders of the skin, making huge contributions to the field of Medical Mycology.

Andrall and Gaverret, in 1843, were the first to describe fungal infection of the ear canal. The first article on "Otitis Externa" was written by Meyer in 1844. In 1851, Pacini described various preparations for the treatment of otomycosis. The term "Otomycosis" was suggested by Virchow in 1856, to describe superficial fungal infections of the ear canal[2]. Wolf in the early part of the 20th century published a list of 53 fungal species causing otomycosis.

Otomycosis:

The term Otomycosis typically describes a superficial fungal infection of external auditory canal. The infection may either be subacute or chronic[3,17]. It is generally unilateral. Otomycosis is also known as Swimmer's ear or Singapore ear.

Occurrence of otomycosis in immunocompetent individuals is usually associated with presence of a predisposing condition[4]. Immunocompromised

individuals like patients with Diabetes mellitus, HIV or lymphoma and patients with carcinoma on chemotherapy or radiotherapy are at an increased risk for developing complications associated with otomycosis[10,21].

Epidemiology

Based on various studies, an estimated 5 – 30 % of the cases of otitis externa are due to otomycosis[2,11]. The prevalence of otomycosis is related to the geographic distribution, with tropical and sub-tropical areas showing higher rates of incidence[12,13]. It has a high prevalence of over 54% in tropical regions and a low prevalence of around 9% in temperate zones[14,15] .

Higher incidence of otomycosis is seen during the rainy months followed by summer, as warmth and moisture provide a highly favourable environment for growth of fungi[16,17]. Otomycosis usually has a chronic course with acute episodes and intermittent remissions[18].

Habitat

The fungi causing otomycosis are commonly saprophytes present in soil, compost, mouldy cereal grains, etc. which serve as a source of infection[19]. These fungi present in the environment, can remain as commensals in the exposed parts of the body and cause opportunistic infections when the conditions in the ear canal are altered or when the immune status of the host is compromised.

Pathogenesis:

Mayer was the first to draw attention to infections of the external ear by fungi as early as the middle of the nineteenth century. The pathogenesis behind otomycosis is related to the anatomy, histology and physiology of external auditory canal. Being open on one side, the auditory canal is constantly exposed to fungal spores present in the atmosphere. Inspite of constant exposure to fungi, the auditory canal is protected by certain protective factors.

The inherent defence mechanisms of the ear canal include:

1. The conchal cartilage, narrow isthumus covered by hair and tragus partially covering the opening of the ear canal, prevent entry of contaminants into the ear canal [4,6,12].
2. The skin over the ear canal has a migratory property, which plays an important role as a self-cleaning mechanism. In 1882, Blake published several studies on the migratory nature of desquamated canal skin. Alberti in 1964, studied this property further and measured the rate of migration at 0.07 mm/day. As the stratum corneum reaches the cartilaginous portion of the canal, it is lifted up by the action of the hairs and the secretions of the sebaceous and ceruminous glands. This mass of desquamated epithelial cell debris and secretions coats the outer portions of the ear canal and is commonly known as the cerumen or earwax[20].

3. Cerumen or Ear wax consists of desquamated keratin, peptide and lipid secretions from the ceruminous and sebaceous glands. It also contains lysozyme, hyaluronic acid and immunoglobulin G, which helps in inhibiting bacterial and fungal growth. Cerumen has a protective role in the External auditory canal. Cerumen creates an acidic environment with a pH of about 5.7 in the external ear canal preventing growth of micro organisms. It is also hydrophobic, repelling water making the surface of the canal impermeable and thus avoiding maceration and epithelial damage. In addition to this, the sticky nature of the cerumen helps to trap fine debris[6,20].

Breakdown of the skin-cerumen barrier is the first step in the pathogenesis of otitis externa. Inflammation and edema of the skin leads to obstruction and pruritis. Pruritis encourages scratching which creates further injury. This sequence of events alters the quality and amount of cerumen produced, impairs epithelial migration, and increases the pH of the ear canal. The resulting warm, alkaline and moist ear canal becomes an ideal breeding ground for numerous organisms.

Fungal infection occurs only when the conditions are favourable, such as, presence of moisture and existing inflammation of the external ear which may favour the growth and inflammation due to fungi[21].

Direct fungal damage on tissue may occur due to tremendous force of hyphal extension into cells. Wolf and Castellani assert that inflammation can be caused by toxins secreted by the fungus. The fungi secrete various metabolic

products such as mycotoxins and enzymes like phospholipases, elastase and hemolysins[9,22,23]. These factors facilitate adherence and cause hydrolysis of cells of the host contributing to its virulence[24,25].

Mycotoxins, are secondary metabolites produced by many species of fungi, that are toxic to humans and animals.The most important mycotoxin produced by the Aspergillus group of fungi are the aflatoxins[26,27]. There are over 16 types of aflatoxins. The important ones are aflatoxins B1, B2, G1, and G2 of which aflatoxin-B1 is the most toxic. Aflatoxin has carcinogenic, mutagenic and immunosuppressive properties[28]. Aflatoxin B1 is a category 1 human carcinogen which usually enters the body through ingestion or occasionally by inhalation. Recent studies have demonstrated property of aflatoxin B1 to enter the body by permeating through the skin[29,30].

In immunocompromised patients, complications like invasive otitis externa can occur [31,32,33]. Complications like tympanic membrane perforation, mastoditis with bone erosion are also known to occur[10,37]. The pathophysiology of the tympanic membrane perforation is attributed to avascular necrosis of the tympanic membrane as a result of mycotic thrombosis in the adjacent blood vessels[10,34,37].

Malignant otitis externa is frequently caused by *Aspergillus fumigatus*. Other rare causes of fungal malignant otitis externa are *Aspergillus niger, Scedosporium apiospermum, Absidia corymbifera, Pseudallescheria boydii, Candida ciferri and Malassezia sympodialis*[2,35,36,42] . In Australia, Ling and Sader

(2008) reported a case of fungal malignant otitis externa caused by *Aspergillus flavus*.

Predisposing Factors

The fungi producing otomycosis are mostly saprophytic in nature[4].Various factors favour transformation of saprophytic fungi into pathogenic fungi[2,4,5,6]. These include:

1. Environmental factors like heat, humidity and moisture[36]. Excessive sweating during summer dilutes wax and reduces its protectiveness. Fungal spores found abundantly in the environment, are blown in the wind along with soil particles and carried by water vapours, increasing prevalence of otomycosis during rainy season when the relative humidity is as high as 80%[3,34] .
2. Increase in pH level in External Auditory Canal as occurs during swimming[38]. Water in the ears cause alteration in the pH and composition of the cerumen and predisposes to epithelial damage and proliferation of microorganisms.
3. Systemic factors Prolonged use of broad spectrum antibiotics, steroid usage, immunocompromised host, diseases like diabetes, malnutrition in children and malignancies. Co-morbid conditions like Diabetes mellitus due to alteration in the immune status and elevated glucose level in tissues increases the likelihood of fungal otitis. Patients with diabetes are more

susceptible for development of complications like invasion and malignant fungal otitis externa[39,40].

4. History of chronic suppurative otitis media (CSOM), bacterial otitis and postsurgical mastoid cavities[41,44,69]: In CSOM, the pus serves as a source of food for the fungus promoting its growth[31]. Chronic ear discharge also creates a humid condition in the ear, altering the pH to alkaline which along with the epithelial debris favours growth of fungus[41].
5. Epithelial damage due to trauma: Habitual cleaning with unsterile material and instrumentation of ear, is one of the predisposing factors to otomycosis[2,11,44]. Habit of cleaning ear with such contaminated objects leads to inoculation of fungal debris in external auditory canal [9,22,44].
6. Instillation of oil in ears: Coconut oil is reported to have sporostatic action and therefore may help preserve the viability of fungal conidia deposited in the external ear and indirectly contribute to occurrence of otomycosis[3,45].
7. Use of topical Antibiotics: Use of topical fluoroquinolone antibiotics especially ofloxacin otic drops has been reported as a predisposing factor for otomycosis[46]. Prolonged use of topical antibiotics or steroids ear drops causes suppression of the bacterial flora with subsequent emergence of fungal flora, causing fungal superinfection [16,46,47].
8. Changes in epithelial layer like dermatological diseases and fungal infections elsewhere in the body like dermatomycosis [11]: Otomycosis is known to occur in patients with untreated dermatomycosis or untreated

fungal infections like onychomycosis, vaginal candidiasis, due to autoinoculation during scratching of ears [38,48].

9. Social habits like wearing traditional head covers[49]. It causes an increase in humidity and moisture in the ear canal[50].

Symptoms[119]

The most common symptoms include:

1. Aural Pruritus[4].
2. Otalgia: Less compared to bacterial otitis.
3. Aural fullness or Ear block.
4. Tinnitus.
5. Hearing impairment and
6. Scanty ear discharge.

In case of invasion of the middle ear and mastoid, there can be deep pain and facial palsy due to invasion of the facial nerve[2].

Signs[2]:

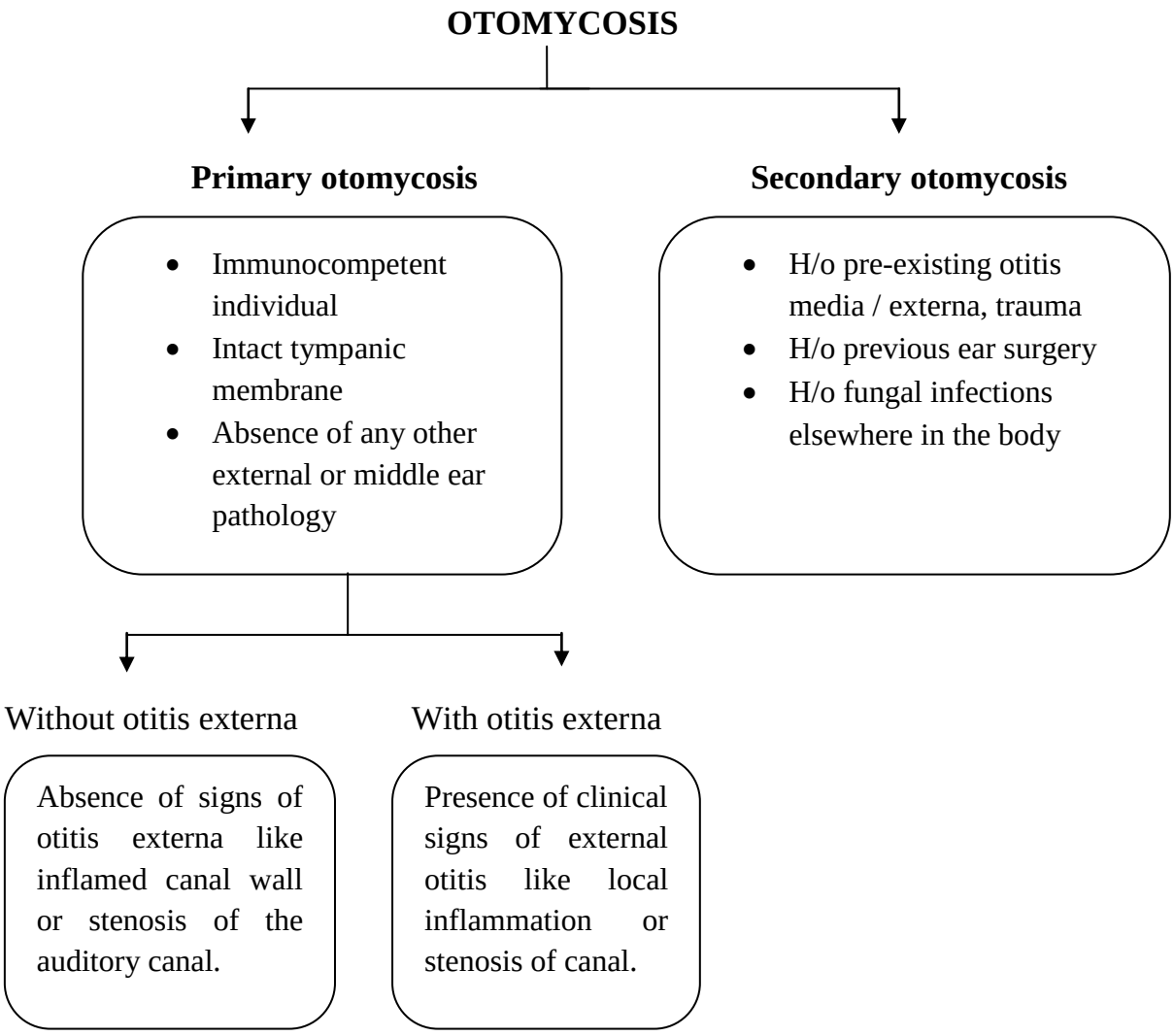
Otoscopic findings in otomycosis are as follows:

- The ear canal usually appears edematous and erythematous[7].
- *Aspergillus niger* spores appear as fine, black coal dust sprinkled in the ear canal. Characteristic findings in *Aspergillus* otomycosis is presence of mycotic elements mixed with epithelial debris covering

the auditory canal and sometimes the tympanic membrane, resembling "blotting paper" or "wet newspaper"[2,51].

- Candidal infections may present as soft, white, creamy material filling the ear canal[52]. In severe cases, the ear canal is inflamed and edematous.

Clinical Classification of Otomycosis[12]



Causative Organisms

Gregson and La Touche (1961) stated that fungal organisms were predominantly species of *Aspergillus* or *Candida*.

Medically Important Fungi Causing Otomycosis[2]

1. Filamentous fungi with septate hyphae:
    - *Aspergillus niger*
    - *Aspergillus flavus*
    - *Aspergillus fumigatus*
    - *Aspergillus terreus*
    - *Aspergillus versicolor*
    - *Aspergillus nidulans*
    - *Penicillium species*
    - *Cladosporium species*
    - *Scedosporium apiospermum*
    - *Fusarium solani*
2. Filamentous fungi with aseptate hyphae:
    - *Mucor species*
    - *Rhizopus*
3. Yeasts and Yeast-like fungi:
    - *Cryptococcus neoformans*
    - *Candida albicans*
    - *Candida parapsilosis*

- *Candida glabrata*
- *Candida guilliermondii*
- *Candida auris*
- *Candida krusei*

4. Dermatophytes:
    - *Trichophyton mentagrophytes*
    - *Trichophyton rubrum*
    - *Microsporum canis*
    - *Epidermophyton floccosum*

Species of *Aspergillus* account for 90 - 95% of the mould isolates. *Aspergillus niger* is the most common isolate followed by *Aspergillus fumigatus* and *Aspergillus* flavus [43, 53,54 .]

Wolf (1947) found that *Aspergilli* are responsible for about 90% cases of otomycosis. Other fungi isolated were *Penicillium*, *Candida* and *Mucoraceae*.

Fungi: Opportunist / Contaminant or Commensal ?[55]

Opportunistic fungi are widely distributed in the environment. Hence a reliable diagnosis requires more than just a simple identification of the organism on culture. To prove that the fungi is indeed an opportunist and not just a contaminant / a commensal the following criteria has to be met [55,56].

- The symptoms should be consistent with a fungal infection.
- Fungal structures should be seen in direct examination of the specimen which is compatible with the morphology of the fungus isolated in culture.

- The fungus should be isolated in cultures from the clinical sample, preferably from two samples from the infected site.
- Morphology of the isolated fungus should be consistent with the symptoms.
- Validity of the diagnosis is maximum when fungal elements are seen in direct examination of the specimen and when the organism is isolated from multiple cultures of the infected material.

LABORATORY DIAGNOSIS

Collection of samples:

Two sterile cotton swabs are used for collecting debris, fungal elements and earwax from the external ear canal of patients showing symptoms of otomycosis. If syringing was done, the aspirate can be used as specimen. Samples should be processed immediately.

The diagnosis can be made by direct microscopy and culture. Histopathology may be required in some cases[18].

Processing of samples.[53]

Filamentous fungi can be identified by:

1.) **Direct Examination of Samples:** It can lead to an early presumptive diagnosis. If sufficient material is not provided, culture is preferable as it is more sensitive.

- Direct Microscopic examination of the debris in 10% potassium hydroxide: It can be done to look for fungal hyphal elements. KOH acts

as a clearing agent, dissolving the cellular debris without affecting the fibrillar glucan and chitin structure of the fungal cell wall.

- Microscopic Examination using Calcofluor : Can be used with or without KOH to increase the sensitivity and specificity of detecting fungi in clinical specimens but requires the use of a fluorescence microscope[42]. Calcofluor fluoresces when exited with ultraviolet light at a emission wavelength of 450 nm and excitation wavelength of 347 nm. It binds to chitin, cellulose and other ß-1,4 linked polysaccharides and produce bright fluorescence. If Evans blue counterstain is used fungal elements appear blue against a red background.
- Direct Gram stain is done to look for yeast cells.

2.) **Fungal Culture:** The specimens are cultured on two sets of Sabouraud's Dextrose Agar (SDA) with a pH of 5.6 containing antibiotics Gentamicin 50 µg/ml or Chloramphenicol 50 µg/ml. Emmon's Sabouraud Dextrose Agar with a reduced dextrose content of 20 gms/litre and a pH of 6.9± 0.2 can also be used. The neutral pH of Emmon's SDA increases retrieval of fungi. These are incubated at 25°C for a period of three weeks. All cultures are checked every day during first week and twice a week during the next two weeks. The mycelial isolates are identified by their colony characteristics and microscopic appearance by making a teased Lactophenol Cotton Blue (LPCB) stained mount. Fungi can also be cultured on Potato Dextrose Agar (PDA) which promotes sporulation.

3.) **Riddle's Slide culture:** is also done to study the morphological details for identification. Slide culture provides greater clarity and details of the fungal elements without distortion[53].

4.) **Banana Peel Culture[57]:** Fungi belonging to ascomycetes, basidiomycetes and coelomycetes can be cultured on ripe banana peel. Autoclaved pieces of banana peel can be used as a cheaper and readily available substrate which promotes growth of slow growing fungi as well as production of sexual spores.

5.) **Scotch Tape Mount/ Adhesive Cellophane Tape Method[53]:** A drop of Lactophenol Cotton Blue solution is placed on a slide. Wearing gloves a 3 cm length of the cellophane tape is held between 2 fingers. The sticky side of the tape is then placed over the top of the fungal colony. A part of the colony adheres onto the tape. The tape is then placed on the slide covering the LPCB solution. Both ends of the tape are then pressed onto the slide. This method provides a better picture of the fungal morphology compared to the Tease mount.

6.) Automated Systems[53] :

- Mycosis IC/F Medium and the BACTEC System
- BacT/ALERT-D

Yeast isolates can be identified by the standard tests like[111]:

- Gram stain: Shape and Size of cells, Type of Budding, Presence of Arthroconidia
- Germ tube test
- Culture characteristics : Colony colour, shape and texture,
- Morphological characteristics on Corn Meal Agar,
- Colour of colony on CHROM agar[58],
- Sugar fermentation tests,
- Sugar Assimilation tests,
- Nitrate Assimilation tests,
- Urea Hydrolysis test,
- Rapid Tests for Yeast Identification

  - BactiCard Candida: Measures N-acetyl galactosaminidase and L-proline aminopeptidase activity for identification of *Candida albicans*
  - Rapid Trehalose Test for *Candida glabrata*

- Commercial Yeast identification Panel Kits:

  - API 20C
  - VITEK 2 YST

- Rapid Yeast Plus
- Peptide Nucleic Acid - Fluorescent insitu Hybridization (PNA-FISH)
- MicroScan Rapid yeast Identification Panel

Tests for Virulence[59,60]

a) Proteolytic activity[22]: Can be tested by inoculating fungi in modified casein hydrolysis medium. After inoculation cultures are incubated at 25°C for 7 days. Degradation of milk protein can be measured as depth of clear zone (in mm).

b) Lipolytic activity[22]: Test fungi are inoculated onto Ullman and Blasins media. After incubation at 25°C for 7 days, the lipolytic ability is 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) can be measured.

c) Urease Activity[22]: Can be demonstrated by inoculation onto Christensen urea medium. After inoculation, cultures are incubated at 25°C for 3-5 days. Appearance of a deep pink color indicates a positive test.

d) Hemolytic Activity[23]: Can be demonstrated by growing the fungal colonies on Sabouraud Dextrose agar with 7% sheep blood and Gentamicin. Appearance of a clear zone of hemolysis surrounding the fungal growth following incubation indicates production of hemolysin which is an important virulence factor responsible for spread of fungal infections.

d) Screening for aflatoxin production:[22,23] Aflatoxin can be extracted from the cultured fungi using solvents like chloroform or methanol. Aflatoxins can be detected by ELISA or high performance liquid chromatographic method (HPLC). Extracted mycotoxins can be visualised under UV light (254 or 365 nm). Aflatoxins B and G when present fluoresce blue and greenish blue respectively. The intensity of the fluorescence is compared with that of the standard aflatoxins[61,62].

e) Biofilm Formation:[64,120] Formation of Biofilm is one of the most important virulence factors of *Candida* species. Yeast cells can detach from the adherent biofilms causing dissemination of fungi, with development of serious systemic infections. Biofilm forming *Candida* are resistant to antifungal treatment.

Biofilms play a major role in persistence of fungal infections not responding to therapy [64].

Antifungal susceptibility testing:

With the increase in incidence of fungal infections and the rising number of antifungal drugs, laboratory methods to guide the selection of antifungal therapy are the need of the hour.

A disk diffusion method for testing *Candida* species has been developed and published as CLSI document M44A[66].

A Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi was developed and published as CLSI document M38A [67].

Antifungal Susceptibility Testing Methods:

Antifungal susceptibility testing methods are available to detect antifungal resistance and to determine the best treatment for a specific fungus. It can be done by various methods:

1.) **Broth Microdilution Method[66,67,68]:** CLSI document M27-A3 "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard" is used for antifungal susceptibility testing of yeasts[66].

CLSI document M38-A2 "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard" is the currently acceptable standard for testing filamentous fungi[67].

Microdilution methods are the gold standard for antifungal susceptibility testing. Two organizations, the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI) have standardized methods to perform antifungal susceptibility testing. But these methods are restricted to reference laboratories as they are laborious and expensive.

2.) **Broth macrodilution methods:** These methods are also available for susceptibility testing of both yeasts and moulds. It is very cumbersome and not used much nowadays.

3.) **Disc Diffusion Method[66]:** Disk diffusion tests on agar are inexpensive and easy to perform and serve as an ideal screening test. The disk diffusion method to test antifungals for yeasts (CLSI Standard: CLSI M44 A) has been developed and validated for species of *Candida*. It recommends the use of Mueller-Hinton agar supplemented with 2% glucose and 0.5 mg/L methylene blue dye medium which enhances the zone edge definition, thereby minimizing the trailing effect[63]. The pH of the medium should be between 7.2 and 7.4 with an agar depth of upto 4cm. The yeast inoculum is standardized to 0.5 McFarland and plates should be incubated at 35 ºC for 24 hours.

The standard disk diffusion test for non-dermatophyte filamentous fungi isolates (M51-A and supplement M51-S1) gives qualitative results in 8-24 hours when amphotericin B, triazoles and caspofungin are used, faster than the Broth Microdilution Method. Among *Aspergillus* species, a lower

agreement by disk diffusion susceptibility tests was observed with *Aspergillus flavus* and amphotericin B or voriconazole. Amphotericin B to test *Aspergillus fumigatus* susceptibility also showed a lower agreement when compared to the reference microdilution method[70].

Though qualitative results provided by the disk diffusion method are useful in the routine clinical laboratory, quantitative MIC data provides a more accurate method for identifying susceptible and resistant strains.

4.) **E Test:** It is a commercially prepared plastic strip which contains predefined gradient of antifungal drug concentrations. This method can be used for routine antifungal susceptibility testing of isolates but not recommended for categorisation of resistant strains [53,71,72,73].

5.) **Automated Methods[53]:** Several automated or semiautomated commercial methods based on agar diffusion or colorimetric indicators as in Etest, Sensititre Yeast One, Fungitest or Vitek are available. Automated systems significantly reduce the biologist hands-on time, turnaround time, and variability due to the standardized format. However these methods have to be evaluated with reference methods to determine breakpoint agreement cutoffs.

6.) Newer methods: [53]

- Flow Cytometry: By Flow Cytometry, the effects of a given antifungal drug can be detected by observing alterations in the fungal cell viability (rather than the growth inhibition as in conventional methods) causing changes in the measured cell fluorescence.

- Matrix Assisted Laser Desorption Ionisation – Time of Flight (MALDI-TOF): Using MALDI-TOF MS, a simple and rapid AntiFungal Susceptibility Test (named MS-AFST) was established to identify susceptible and resistant isolates.
- Isothermal Micro Calorimetry (IMC) : IMC was establshed for susceptibility testing of *Aspergillus* species in Real Time , by measuring thermal variations induced by the action of antifungals and expressed as minimal heat inhibitory concentration (MHIC).

Molecular Diagnosis:

1) **Polymerase Chain Reaction (PCR)**[74,75,76,99]**:** It is a primer initiated, enzymatic amplification of specific DNA sequence. It has 3 steps:

- **Extraction of DNA:**
- **Amplification:** There are 4 phases in amplification.
  a) Initial Denaturation.
  b) Denaturation.
  c) Annealing.
  d) Extension.
- **Electrophoresis:**

2) **Gene Sequencing**[76,77]**:**

Sequencing of the ribosomal DNA is a useful diagnostic tool for the rapid detection and identification of fungi. In addition to speciation it also helps in detecting genes responsible for virulence and drug resistance[78].

One of the most common ribosomal targets for sequencing is the Internal Transcribed Spacer (ITS) region of the rDNA[79]. This region has two regions, ITS1 and ITS2, located between the 18S and 28S ribosomal subunits and separated by the 5.8S ribosomal subunit. The amplicon is usually 400 to 700 bp in length. D1/D2region is a second variable region within the ribosomal DNA (rDNA) cluster, which can also be amplified. The D1/D2 region is located toward the 5' end of the ribosomal subunit (26S or 28S). The conserved sequences at the ends of the D1/D2 and ITS regions provide universal PCR priming sites, while the variable internal regions provide species-specific sequences for identification.

Sequence analysis: The sequence data are assembled and analyzed by the use of Sequence analysis software. Individual nucleotide-nucleotide searches are done with the BLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). The outputs from the BLAST searches are sorted on the basis of the maximum identity and recorded as they appear. Sequence-based identities with a cutoff of 97% or greater are considered significant, and the best hit is defined as the sequence with the highest maximum identity to the query sequence.

Treatment Of Otomycosis[2,6,7]:

1. The first step in treatment is identification and elimination of existing predisposing factors.

2. Cleaning and thorough debridement of external auditory canal is done preferably by suction evacuation .

3. Syringing of external auditory canal should be avoided as it may sometimes lead to flare up of the infection to deeper anatomical sites. Syringing if done, should be done under strict aseptic conditions using normal saline mixed with antifungal powder.

4. **Topical Therapy:** Topical antifungals like Clotrimazole cream, powder or solution (1%), Amphotericin B (3%), Econazole cream (1%) and Flucytosine (10%) can be used. Nystatin cream, ointment or powder, Ketoconazole, Fluconazole, Miconazole 2% cream, thiomersal (Merthiolate) or Cresyl acetate solution can also be used. Clotrimazole has an antibacterial effect, and this is an added advantage when treating mixed bacterial-fungal infections[52]. In case of perforated tympanic membrane, Tolnaftate 1% solution (Tinactin) is preferred.

5. **Systemic antifungal therapy[2]:** Systemic antifungal therapy is required only in cases of failure with topical therapy, invasive external otitis or in immunocompromised patients. Oral Itraconazole can be used for superficial otitis externa not responding to topical clotrimazole therapy[81]. Systemic Amphotericin B has also been used in invasive otitis externa. Voriconazole is used for treatment of invasive otitis externa including perforation of the tympanic membrane.

6. Tympanoplasty might be needed if the perforations do not heal spontaneously[37].

Efficacy of Clotrimazole and miconazole topical applications have been proven in the treatment of otomycosis [52,80,82,83,84].

Antifungal Resistance:

Despite increase in the spectrum of antifungal agents now available, the choice of suitable antifungal agents may be limited due to various factors like emergence of resistant fungal species[85]. Antifungal resistance can be primary (intrinsic) or secondary (acquired).

Acquired Itraconazole resistance in *Aspergillus fumigatus* and *Aspergillus niger* have been described in isolates causing otomycosis[86,87]. Such isolates may also be resistant to econazole. Some isolates may be resistant to voriconazole and posaconazole[88,89,90] .

Innately Resistant Fungi[18,53]:

Among filamentous fungi, members of the order Mucorales are usually not susceptible to Echinocandins. Fusarium is resistant to Itraconazole and Echinocandins, it requires treatment with high dose Amphotericin B or Voriconazole. *Pseudallescheria* and *Scedosporium* are resistant to Amphotericin B even at high concentrations. *Aspergillus terreus* is resistant to Amphotericin B both in vitro and in vivo.

Among yeasts, *Candida krusei* is multidrug resistant, with decreased susceptibility to Fluconazole, Amphotericin B and Flucytosine. *Candida glabrata* is less susceptible to Amphotericin B and Fluconazole[91].

According to studies by, resistance rates among *Candida albicans* isolates for amphotericin B, fluconazole , flucytosine , itraconazole , voriconazole were 2.9%, 5.9%, 0.0%, 4.2% and 2.5%, respectively.

MATERIALS AND METHODOLOGY

- This study was conducted at the Government Kipauk Medical College for a period of one year from January 2016 to December 2016.
- Patients attending the ENT department with symptoms of Otomycosis were selected as the study population.

Study Design: Cross Sectional Descriptive study

Duration of Study: 1 year (Jan 2016 to Dec 2016)

Statistical Analysis:

- Statistical analysis was done using SPSS software 16.0 version. Chi square test was used to test association and p value was calculated. A p value of 0.05 or less was considered significant.

Inclusion criteria:

- Both male and female patients of all age groups with clinically suspected otomycosis attending the ENT OPD
- All clinically diagnosed and untreated cases of otomycosis were included in the study.

Exclusion criteria:

- Bacterial cause of otitis externa.
- Patients with otomycosis on antifungal treatment.

Ethical committee approval: Approval was obtained from the Instituitional Ethical Committee before starting the study on 22.12.2015.

- Patients with clinically suspected otomycosis were identified. Consent for the study was obtained.
- History of otalgia, aural pruritis, ear discharge, diminished hearing, ear block, and tinnitus was elicited. History of any predisposing factors like habitual cleaning with unsterile materials, swimming, instillation of oil in ear, previous ear infections, ear surgery or trauma and diabetes mellitus was also elicited.
- Under strict aseptic precautions the debris in the external auditory canal was collected using 3 sterile swabs, after cleaning the pinna and adjacent areas with antiseptic solution. A repeat swab was taken on the same day after 2 hours to rule out contamination.
- The collected specimen was sent to the lab promptly along with a proper requisition slip and processed within 30 minutes.

Direct Microscopy With 10% Pottasium Hydroxide Mount[2,53]

- The specimen was placed in 1- 2 drops of 10% KOH on a clean glass slide.
- A clean and thin cover slip was placed over the preparation without any air bubbles.
- The slide may be gently heated to clear the debris.

- The preparation was examined using 10x and 40x magnification of a bright field microscope.
- Presence of fungal elements (hyphal elements, Conidiophore, spores) was looked for.

Fungal Culture[2,53]:

- The specimen was inoculated onto Sabouraud's dextrose agar slopes in duplicate. Cycloheximide / Actidione was not added to the Sabouraud's dextrose medium, as they inhibit growth of saprophytes. Gentamicin 50mg was added to one litre of SDA to minimize bacterial contamination.
- All inoculated media were incubated at 25°C and were observed daily for upto 4 weeks to look for fungal growth.
- Macroscopic features of the fungus like surface, texture, pigmentation on obverse and reverse, rate of growth were noted.
- The fungal growth was then subjected to microscopic examination using LPCB.
- An alcohol – sand flask was used to remove excess fungal material from the needle. The sand flask was made by placing 200 ml of coarse, washed sand into a 250 ml flask and filling it nearly to the top with 95% ethanol. It was kept closed with a lid to prevent evaporation. Excess fungal material in the needle can be removed by vigorously agitating it in the sand bath before flaming it.

Lactophenol cotton blue mount (Wet mount)[53,55]:

- A drop of lacto phenol cotton blue was placed in the centre of a clean glass slide. A small portion of the fungal growth was kept on it and teased using teasing needle.
- A thin, clean cover slip was applied over it, without any air bubbles.
- It was then viewed using light microscope first under low power and then under high power objective for identifying microscopic morphological features,
- Slide culture was done wherever necessary.

Riddle's Slide Culture[53]

- A sterile Petri dish labelled with the patient identification and date of inoculation was taken.
- A sterile V or U shaped glass rod was placed inside the petridish over which a sterile grease free glass slide was kept.
- Sabouraud dextrose agar or Potato dextrose agar was poured to a depth of 4mm in a sterile petridish.
- 1×1 cm block of the agar was cut and transferred aseptically onto the sterile glass slide.
- From the fungal growth the four sides of the agar block were inoculated. A sterile glass cover slip was placed over the agar block.

- A small amount of distilled water was placed inside the Petri dish to keep the atmosphere humid.
- The plate was then incubated at 27°C till adequate growth of the fungus was obtained.
- When spores were well developed, the coverslip was removed with a forceps and placed onto a drop of lactophenol cotton blue on a second slide.
- A second preparation was made by removing the agar block from the slide culture and doing a lacto phenol cotton blue preparation on the slide.
- The microscopic morphological features like hyphae, Conidiophores and Conidia were noted using low power and then high power magnification of the microscope.

Banana Peel Cuture[57]:

- Ripe banana peel was taken and cut into small squares of approximately 2cms.
- The peel was sterilized by autoclaving twice at 121°C, 15 lbs for 15 min on two consecutive days.
- The sterile peels were placed flat in a sterile glass petridish.
- The fungal growth was inoculated onto the peel. A sterile, glass coverslip was placed over the peel.
- Few drops of sterile water was left inside the petridish to provide moisture.

- The plates were incubated at 25°C, for 3-5 days till sufficient growth of fungus was observed.
- Once the spores were well developed, the coverslip with the adherent fungal growth was gently lifted with a forceps and placed onto a sterile glass slide with a drop of lactophenol cotton blue.
- The undistorted microscopic morphology was observed under low and high power objectives and the fungus was identified.

IDENTIFICATION OF YEASTS:[53,55,92,93]

- If the growth was opaque, creamy white, Grams staining was done.
- If gram positive budding cells were seen *Candida* was identified.
- It was further speciated by Germ tube test, Urease test, Sugar Fermentation test, Sugar Assimilation test, Chlamydospore formation on Corn Meal Agar and colour of colonies on CHROM agar.

Germ Tube Test (Reynolds Braude phenomenon):[55]

Growth from the yeast colonies was suspended in 0.5 – 1 ml of human serum and incubated at 37°C for 2- 3 hours.

A drop of the suspension was kept on a slide and examined microscopically for the presence or absence of germ tubes. The germ tube can be seen as a long tube like projections arising directly from the yeast cell having parallel walls, without any constriction at their point of origin.

Germ tube positive colonies were tested for formation of germ tubes after incubation at 42°C to differentiate *Candida albicans* from *Candida dublinensis*.

Colour of colonies on CHROM agar:[58,94,95,96,97]

- Using sterile loop the yeast colony was inoculated onto CHROM agar plate.
- The plates were incubated at 25°C for 3 – 5 days.
- The colour of the colonies were noted.

Chlamydospore Formation on Corn Meal Agar (Dalmau Plate Technique)[92]

- The Corn Meal Agar plate was divided into 4 quadrants and labelled. Using a sterile straight needle, the yeast colony was touched lightly and 2 -3 streaks of approximately 4 cm long and 1 – 2 cms apart were made. A flame sterilized and cooled 22mm square cover slip was placed over the streaks.
- The plates were incubated at 25°C for 3 – 5 days.
- The lid of the petri plate was removed and the plate was placed on the microscopic stage. The edge of the cover slip was observed using low power and high power objective.
- Morphological features like pseudohyphae and chlamydospores were noted.

Sugar Fermentation Test:[92]

- Sugar fermentation medium containing different sugars like dextrose, lactose, maltose, sucrose in a concentration of 2% were taken, in test tubes. Inverted Durham's tube was placed to note the collection of gas.
- Inoculum preparation was done by suspending a heavy inoculum of the yeast grown on sugar free medium. The test tubes were inoculated by adding 0.1ml of the *Candida* suspension.
- The sugar tubes were incubated for upto a week at 25°C.
- If the sugar was fermented the colour of medium was changed to yellow.

Sugar Assimilation Test:[92]

- A heavy inoculum of a 24 – 48 hrs old yeast culture was suspended in 2 ml of Yeast Nitrogen Base. The suspension was then added to 18 ml of molten agar (cooled to 45° C) and mixed well. The entire volume was poured into 90 mm petri plate and allowed to solidify.

- The carbohydrate impregnated discs were placed onto the agar surface and the plates were incubated at 37 °C for 3 – 4 days. Presence of growth around the disc was considered as positive for that particular carbohydrate. Growth around the glucose disc serves as positive control for viability of the yeast.

TESTS FOR VIRULENCE:

Urease Activity[22]

- Using a sterile loop, yeast colony or mould colony was inoculated onto Christensen urea medium and incubated at 25°C for 3-5 days.
- Appearance of a deep pink color indicates a positive test.

Hemolysin production[23]

- Colonies of *Aspergillus* species were inoculated onto Blood agar plate containing 7% sheep blood mixed in Sabouraud Dextrose Agar.
- The plates were incubated at 25°C for 3-5 days.
- Appearance of hemolysis surrounding the fungal growth indicates a positive test.

Biofilm formation[64,120]:

- Using sterile loop, *Candida* colonies from a 18 -24 hours culture on SDA was taken.
- It was inoculated into 5ml of Sabouraud's Dextrose broth and the turbidity was adjusted to o.5 Mac Farland standard.
- The tubes were incubated at 37°C for 18 - 24 hours.
- The broth was poured out and washed with Phosphate Buffer Saline.
- 0.5% crystal violet solution was added and stained for 10 minutes.
- A visible and uniform violet coloured film in the tube indicates presence of biofilm.

Toxicity Testing for Aflatoxin by High Performance Liquid Chromatography (HPLC)[26,27]:

- Fungus to be tested was cultured on Sabouraud's Dextrose Agar slope
- 50 gms of the test portion was transferred into a 500 ml Erlenmeyer flask and 5gms of NaCl was added.
- The test portion was extracted with 300 ml methanol extraction solvent and 100 ml hexane in a blender at high speed for 3 minutes.
- The extract was filtered through a fluted filter paper.
- 10 ml of clear filtrate was added to 60 ml of Phosphate buffered saline (PBS) and applied to the affinity column containing antibodies to aflatoxins B1, B2, G1 and G2.
- 10 ml of the PBS solution with the filtrate was applied to the top of the column and allowed to pass at a speed of 2-3ml/min through the column by gravity.
- Aflatoxins were eluted from the column with 0.5ml methanol.
- Post column derivatization was done using pyridinium hydrobromide perbromide (PBPB).
- 200 mL of aflatoxin standards were eluted through the liquid chromatograph. Aflatoxins elute in the order G2, G1, B2, and B1 with retention times of 6, 8, 9, and 11 min respectively.
- 200 mL of the test extract was injected into the injector and each aflatoxin peak and retention times in the chromatogram was identified and compared

with the corresponding reference standard by fluorescence detection. Quantity of each aflatoxin was also derived from the standard curve.

ANTIFUNGAL SUSCEPTIBILITY TESTING:

MICROBROTH DILUTION METHOD FOR FILAMENTOUS FUNGI[67]:

Inoculum Preparation[67]

- **Moulds:** The fungi was subcultured on Potato Dextrose Agar tubes and incubated at 25°C for 7 days. Using a sterile Pasteur pipette the surface of colony was gently washed with 3 ml sterile distilled water or saline. The suspension was allowed to stand for few minutes so that larger hyphal segments settle. The suspension was vortexed . Then the top homogenous layer was used to obtain desired inoculum density.
- Inoculum was adjusted using spectrophotometer with test inoculums in range 0.4×10^4 to 5×10^4 CFU/ml. Optical density was measured at 530nm
- For *Aspergillus* and *Sporothrix* OD – 0.09 -0.11
- For *Fusarium, Rhizopus and Scedosporium* OD – 0.15 – 0.17
- For *Bipolaris and Histoplasma* OD – 0.2
- 100µl of conidial suspension was added to 4.9ml RPMI (1:50 dilution) to get a viable conidial concentration of approx.0.4×10000 to 5×10000 CFU/ml
- RPMI 1640 medium supplemented with 0.2% glucose buffered to pH 7 was used.
- The following drug concentration ranges were used: Amphotericin B, 0.0313 to 16 µg/mL; Ketoconazole, 0.0313 to 16 µg/mL; Itraconazole,

0.0313 to 16 µg/mL; Fluconazole, 0.125 to 64 µg/mL; and new triazoles, 0.0313 to 16 µg/mL.

- Microdilution plates were incubated at 35°C and read at 24 hrs for Rhizopus and at 48 hours for *Aspergillus , Fusarium and Sporothrix* ; 78 hours for *Scedosporium.*
- Microdilution wells were visualised with reading mirror and growth compared to drug free growth control.
- Score :0 – Optically clear

  1 – Slight growth (25 % of growth control)

  2 – Prominent reduction in growth (50 % of growth control)

  3 – Slight reduction in growth (75 % of growth control)

  4 –No reduction in growth
- For Amphotericin B, newer azoles like Voriconazole, Itraconazole, Posaconazole, MIC = Score 0
- For Flucytosine , Fluconazole MIC = Score 2

Fungal isolates were considered susceptible to Amphotericin B, Itraconazole, Posaconazole, Voriconazole, and Caspofungin when the MIC/MEC was ≤1.0 mg/ml, intermediate when MIC/MEC was 2.0 mg/ml, and resistant when the MIC/MEC was ≥ 4.0 mg/ml.

Dose of Antifungal:

Weight (mg) = Volume (mL) X Concentration (µg/ML) /Assay Potency (µg/mg)

MIC Breakpoints:

Antifungal Agent	End Point	Sensitive (MIC)	Intermediate (MIC)	Resistant (MIC)
Amphotericin B	100% reduction in growth compared to the growth in the control well	≤1μg/mL	2 μg/mL	≥ 4 μg/mL
Fluconazole	50% or more reduction in growth compared to the growth in the control well	≤1 μg/mL	2 μg/mL	≥ 4 μg/mL
Clotrimoxazole	100% or more reduction in growth compared to the growth in the control well	≤1 μg/mL	2 μg/mL	≥ 4 μg/mL
Itraconazole	100% reduction in growth compared to the growth in the control well	≤1 μg/mL	2 μg/mL	≥ 4 μg/mL
Voriconazole	100% reduction in growth compared to the growth in the control well	≤1 μg/mL	2 μg/mL	≥ 4 μg/mL

DISC DIFFUSION TEST FOR YEASTS[66]:

- Antifungal sensitivity was done by disc diffusion test according to CLSI M27A2 for yeasts.
- Done on Mueller-Hinton Agar + 2% Glucose and 0.5 μg/mL Methylene Blue Dye (GMB) Medium with a pH of 7.2 – 7.4
- Preparation of the inoculum
    - The *Candida* colonies were subcultured on Sabouraud ‘s dextrose agar and incubated at 35 °C (±2 °C).
    - Inoculum was prepared by picking five distinct colonies of approximately 1 mm in diameter from a 24-hour-old culture of

Candida species. Colonies were suspended in 5 mL of sterile 0.85% saline.

- The resulting suspension was vortexed for 15 seconds and its turbidity was adjusted to 0.5 McFarland standard.
- This procedure will yield a yeast stock suspension of 1 x 10^6 to 5 x 10^6 cells per mL.

➢ Inoculation and Reading of Test Plates:

1. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension. The swab should be rotated several times and pressed firmly against the inside wall of the tube above the fluid level. This will remove excess fluid from the swab.
2. The dried surface of a sterile Mueller-Hinton + Glucose Methylene Blue agar plate was inoculated by evenly streaking the swab over the entire agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.
3. Antimicrobial disks were dispensed onto the surface of the inoculated agar plate.
4. The plates were inverted and placed in an incubator set to 35°C (± 2°C) within 15 minutes after the disks are applied.

5. Sensitivity was tested for AmphotericinB, Clotrimoxazole, Fluconazole, Itraconazole and Voriconazole.
6. The plates were read after 20 to 24 hours of incubation.
7. The zone diameter was measured to the nearest whole millimeter at the point at which there was prominent reduction in growth.

Interpretation of Disk Diffusion Test Results:

Drug	Potency	Zone Diameter in mm		
		S	I	R
Clotrimazole	10 μg	≥19	18-12	≤11
Amphotericin B	10 μg	≥15	14-10	≤10
Voriconazole	1 μg	≥17	16-14(DD)	≤13
Itraconazole	8 μg	≥15	14-10(DD)	<10
Fluconazole	25 μg	≥19	18-15(DD	≤14

MOLECULAR DIAGNOSIS:

Extraction of DNA:

1.) 500 µl TESS buffer + 1 loop of colony was added to aliquots in duplicate and boiled for 1 minute.
2.) 500 µl Phenol and 500 µl chloroform were added in the ratio 1:1 and vortexed.
3.) Centrifuge was done at 10000 rpm for 10 minutes
4.) The top aqueous layer was pipette.
5.) Equal volume of chloroform was added to remove traces of phenol present and mixed well
6.) Centrifuge was done at 10000rpm for 10 minutes.
7.) The top aqueous layer which containing the nucleic acid was pipette.
8.) Equal volume of isopropyl alcohol was added to precipitate the nucleic acid.
9.) The supernatant was discarded.
10.) Nucleic acid forms a pellet at the bottom of the tube.
11.) 200 µl of 70% ethanol was added and centrifuged at 10000rpm for 10 minutes
12.) The supernatant was discarded and air dried for 20 minutes.
13.) 40 µl nuclease free water was added.

Polymerase Chain Reaction (PCR)[75,76,98,99]:

1.) In a 0.2ml sterile PCR tube, 5 µl Enzyme buffer, 1 µl dNTPs, 0.5 µl Forward primer (ITS 1- 5′-GATACCGTCGTAGTCTTA-3′), 0.5ml Reverse primer (ITS 4- 5′-ATTCCTCGTTGAAGAGC-3′), 42.7 µl sterile nuclease free water, 0.3µl Taq polymerase and 5 µl template DNA were added.

2.) The tubes were placed in a thermocycler and PCR was performed.

Step	**Temperature (°C)**	**Time**
Initial denaturation	94	5min
Denaturation	94	30 seconds
Annealing	52	2 minutes
Extension	72	1 minute
Final extension	72	7 minutes

3.) Cycling was done for 30 cycles.

Electrophoresis:

1. Both ends of the gel tray were sealed with cellophane tape
2. 2g of agarose was dissolved in 100 ml buffer
3. The solution was microwaved for 2 minutes and cooled to 50°C
4. 1 µl ethidium bromide was added, mixed and poured into gel tray.
5. Combs were placed in appropriate position and the gel was allowed to set.
6. The gel was then placed in electrophoresis tank
7. Buffer was added to cover the gel upto 2 mm

8. 10 µl of DNA sample was mixed with 2 µl gel loading buffer and loaded into the lane.
9. 1 µl of DNA molecular weight marker, 4 µl water and 1 µl gel loading buffer were mixed and loaded into another lane
10. The tank lid was closed and current was applied at 1 to 5 volt/cm
11. The bands were then visualised under UV light. Pan fungal DNA is measured at 270 bp.

Gene Sequencing[77]:

The PCR products were purified with a Qiagen PCR purification kit. Both strands were sequenced by Sanger's dideoxynucleotide sequencing method. The sequences were obtained as overlapping runs of the two flanking primers (primers ITS-1 & NL-4), as well as runs of two internal primers (primers ITS-4 and NL-1).

Sequence analysis:

The sequence data were assembled and analyzed by the use of Sequence analysis software. Individual nucleotide-nucleotide searches are done with the BLASTn algorithm at the NCBI website(http://www.ncbi.nlm.nih.gov/BLAST/). The outputs from the BLAST searches are sorted on the basis of the maximum identity and were recorded as they appeared without modification of genus or species names that may have been synonyms or teleomorphs of other genus or species names in other GenBank records. Sequence-based identities with a cutoff of 97% or greater were considered significant in this study, and the best hit was defined as the sequence with the highest maximum identity to the query sequence.

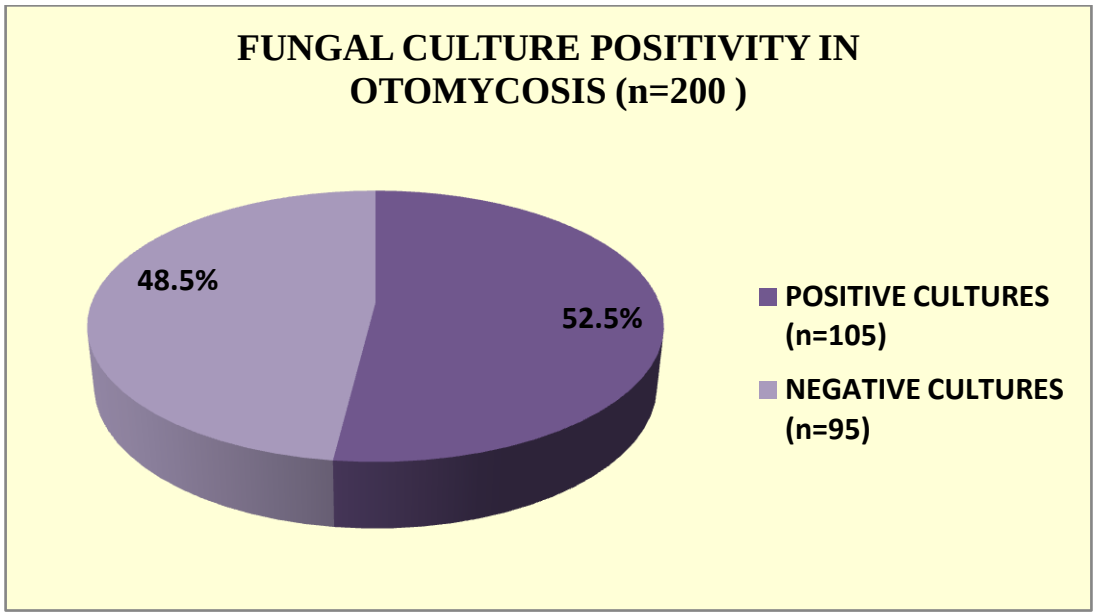
STATISTICAL ANALYSIS

The outcome of this study was noted, documented and analysed. The analysed data was presented in the form of statistical tables and represented as pie charts, bar diagrams or histograms wherever necessary. The p-values were calculated by Chi –Square test to compare the proportion between categorical variables. If expected cell frequency was less than five in more than 20% of cells, Fisher's exact Chi –Square test was applied. SPSS (Stastical package for the social science) version 22.0 was used to analyse the data. Significance level was fixed as 5% (α=0.005) with a p value of <0.05.The observationsof this study was compared and discussed with similar studies published in reputed scientific journals for simlarities and contrasts in the results.

RESULTS

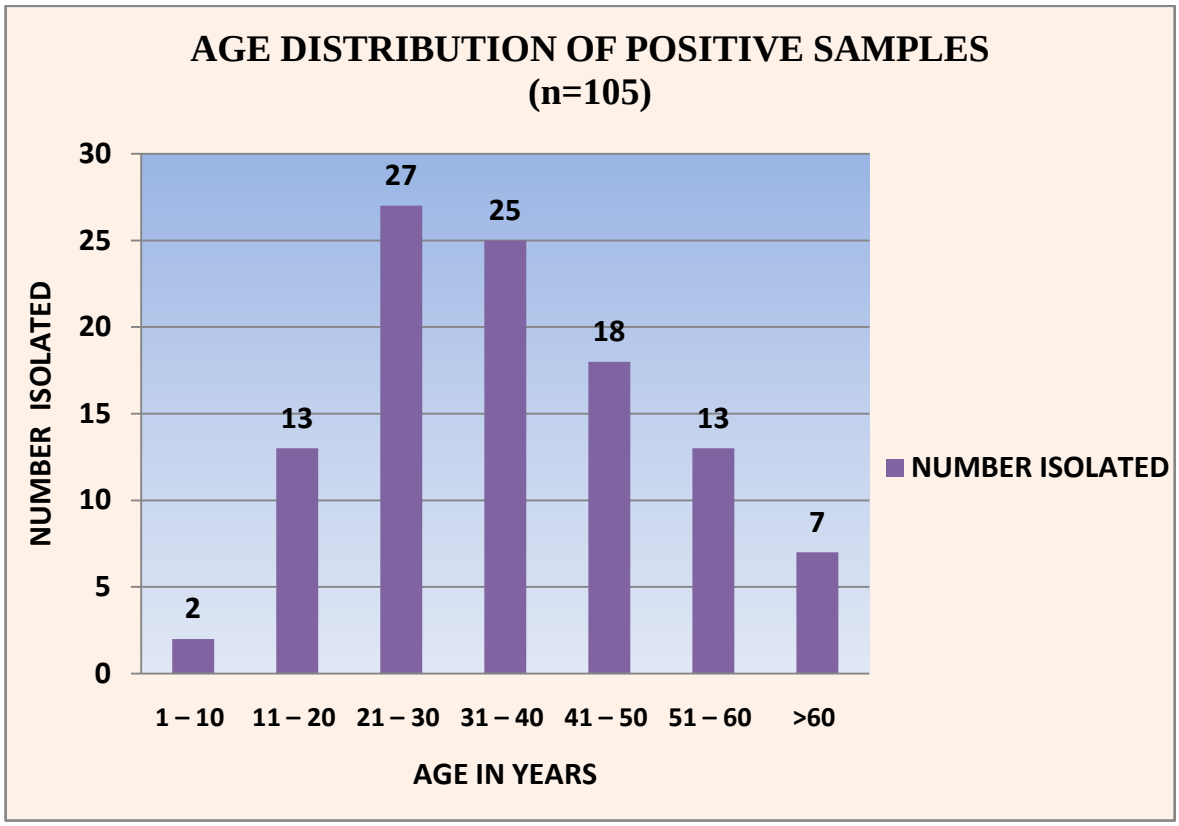
This study was carried out in the department of Microbiology, Government Kilpauk Medical College, over a period of 12 months from January 2016 to December 2016. The results were analyzed as follows.

CHART 1



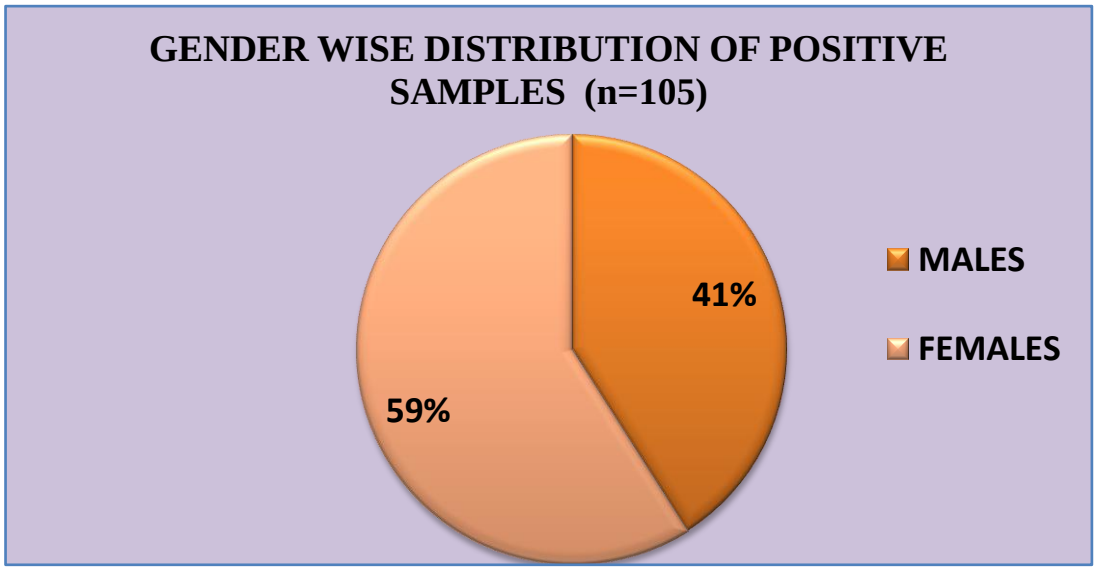
200 cases of clinically suspected otomycosis were analysed out of which 105 samples showed growth of fungi, accounting for 52.5%.

CHART 2



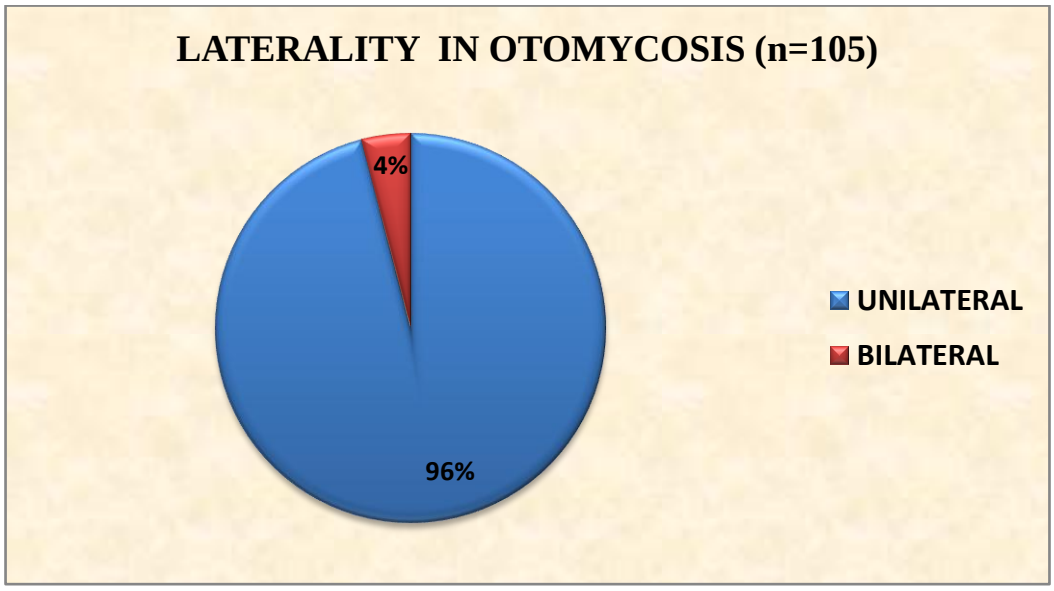
Fungus was isolated from all age groups ranging from 1 year to >60years of age. Maximum number of cases were observed in the age group 21-30 years with 27 cases (25.71%) followed by 31 – 40 years with 25 cases (23.81%) which showed a significant statistical p value of <0.001. Least number of cases were in the age group 1-10 years with 2 cases (1.9%).

CHART 3



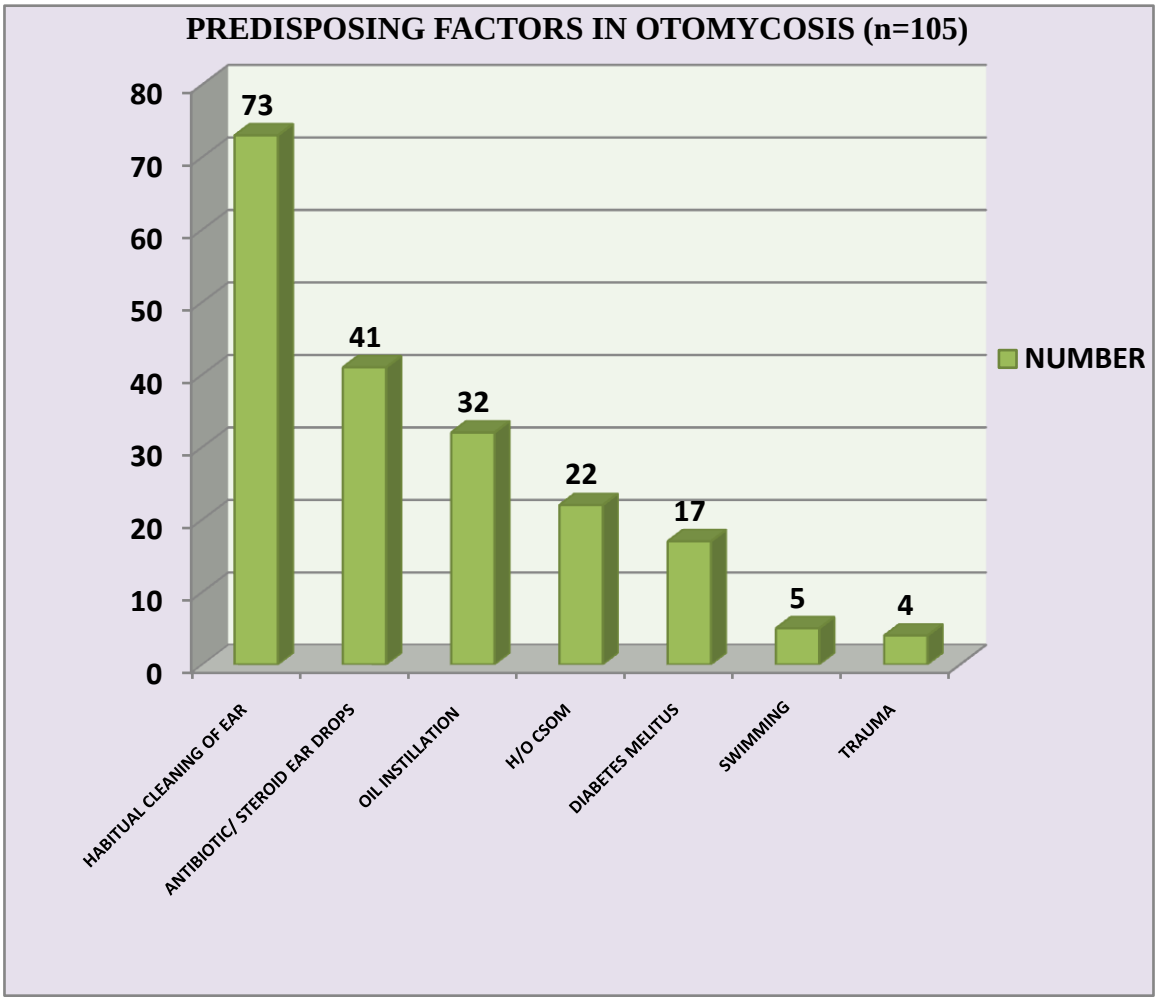
Among the positive samples 62 were from females accounting for 59% and 43were from males with 41%. The female: male ratio was 1.44:1.

CHART 4



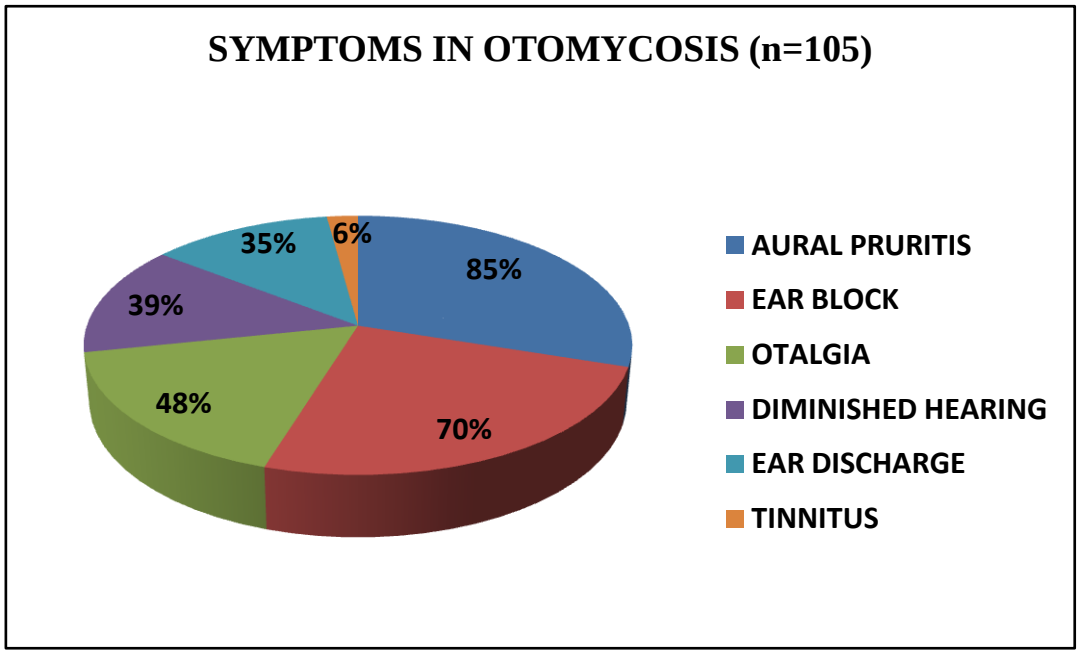
Otomycosis was unilateral in 101 cases (96%) and Bilateral in 4 cases (4%) with a significant value of $p < 0.001$.

CHART 5



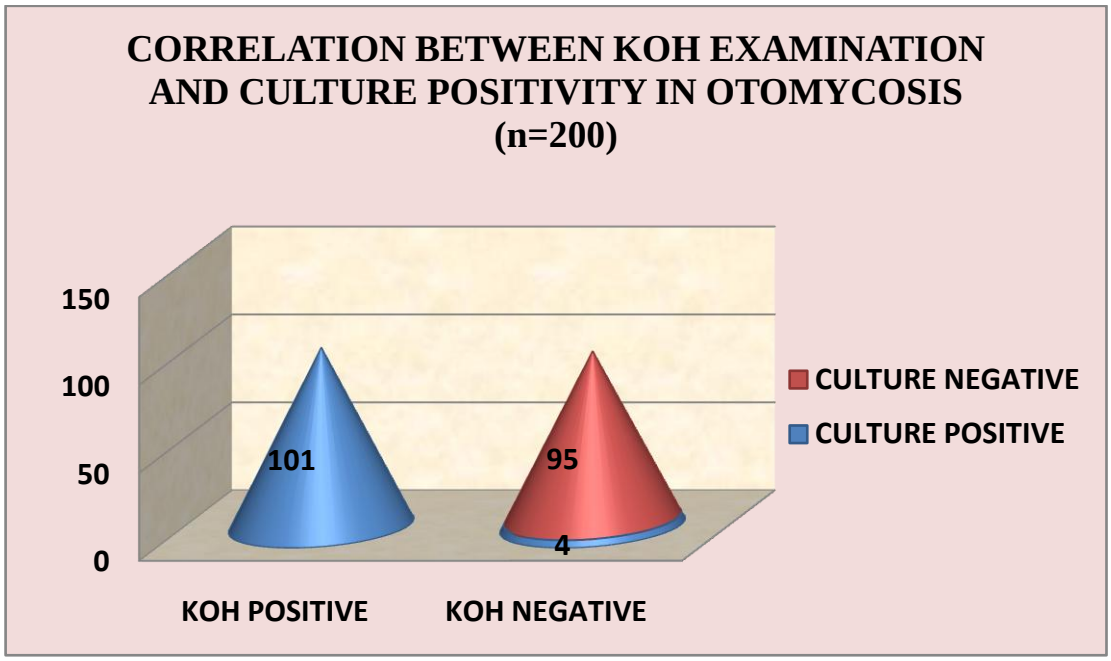
Habitual cleaning of ears using unsterile materials like ear buds, safety pins, match sticks was the common predisposing factor seen in 73 cases (70%). It was found to be statistically significant with a p value of <0.001. Use of ear drops was observed in 41 cases (39%) while Oil instillation in ears was noted in 32 cases (31%). Diabetes mellitus as a comorbid factor was noted in 17 patients (16%).

CHART 6



The presenting symptoms in decreasing order were Aural pruritis-89 (85%), Ear block/ Fullness of ear – 73(70%), Otalgia- 50 (48%), Diminished hearing – 41(39%), Ear discharge – 37(35%) and Tinnitus- 6 (6%). The p value by Pearson Chi Square was found to be <0.001 which was significant.

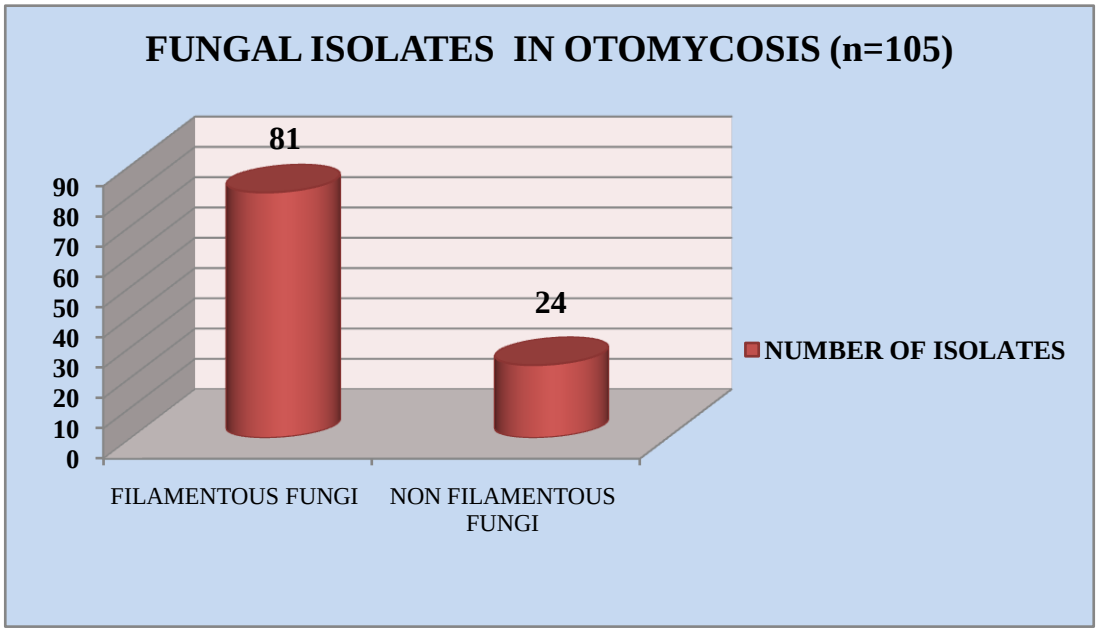
CHART 7



OF the 105 culture positives, 101 were KOH positive. The analysis was significant with a p value of <0.001. The parameters derived using statistical analysis for diagnosis by culture were.

Parameter	**Estimate (%)**	**Lower – Upper 95% CIs**
Sensitivity	100.0	96.34 – 100.0
Specificity	95.96	90.07 – 98.42
Positive Predictive Value	96.19	90.61 – 98.51
Negative Predictive Value	100.0	96.11 – 100.0
Diagnostic Accuracy	98.00	94.97 – 99.22

CHART 8



Out of the 105 fungi isolated, 81 were filamentous fungi (77%) and 24 were *Candida* species (23%). The p value was significant with <0.001.

TABLE 1

TABLE OF DIFFERENT SPECIES ISOLATED IN THE STUDY (n=105)

FUNGUS ISOLATED	NUMBER	PERCENTAGE
FILAMENTOUS FUNGI (n=81)		
Aspergillus niger	51	49%
Aspergillus flavus	14	13%
Aspergillus terreus	4	4%
Aspergillus fumigatus	2	2%
Rhizopus oryzae	2	2%
Cladosporium cladosporoides	2	2%
Aspergillus nidulans	1	1%
Aureobasidium pullulans	1	1%
Fonsaceae pedrosoi	1	1%
Rhizomucor pusillus	1	1%
Mucor circinelloides	1	1%
Curvularia lunata	1	1%
NON FILAMENTOUS FUNGI (n=24)		
Candida albicans	20	19%
Candida parapsilosis	2	2%
Candida glabrata	1	1%
Candida krusei	1	1%

Among the fungal species identified, *Aspergillus niger* was the most common isolate in 51 cultures (49%), followed by *Aspergillus flavus* in 14 cultures (13%). *Aspergillus terreus* was isolated in 4 cultures. There were 2 isolates each of *Aspergillus fumigatus* and *Rhizopus*. The rare causative fungi isolated were *Cladosporium* (2), *Aspergillus nidulans* (1), *Mucor* (1), *Aureobasidium pullulans*(1), *Fonsaceae pedrosoi* (1), *Curvularia lunata* (1) and *Rhizomucor* (1).

Of the 24 *Candida* species isolated, 20 were *Candida albicans*, 2 were *Candida parapsilosis*, 1 was *Candida glabrata* and 1 isolate was *Candida krusei*.

The results were statistically significant with a p value of <0.001

TESTS FOR IDENTIFICATION OF *CANDIDA* SPECIES

TABLE 2: GERM TUBE TEST (n=24)

CANDIDA SPECIES	GERM TUBE TEST POSITIVE	GERM TUBE TEST NEGATIVE
Candida albicans (n=20)	20	0
Candida parapsilosis (n=2)	0	2
Candida glabrata (n=1)	0	1
Candida krusei (n=1)	0	1

Of the 24 *Candida* isolates, 20 were Germ Tube Test positive and 4 were negative

TABLE 3: CHARACTERSTICS ON CHROMAGAR (n=24)

CHARACTERSTICS ON CHROMAGAR	NUMBER	PERCENTAGE	ORGANISM IDENTIFIED
Apple green colonies	20	83.33%	*Candida albicans*
White to pale pink	2	8.3%	*Candida parapsilosis*
White, large, glossy, pale pink to violet	1	4.2%	*Candida glabrata*
Large, flat, spreading, pale pink colonies with matt surface	1	4.2%	*Candida krusei*

Based on the colour of colonies on CHROMagar, 20 isolates of *Candida albicans,* 2 isolates of *Candida parapsilosis*, 1 isolate of *Candida glabrata* and 1 isolate of *Candida krusei* were identified .

TABLE 4: MORPHOLOGY ON CORN MEAL AGAR (N=24)

MORPHOLOGY ON CORN MEAL AGAR	NUMBER	PERCENTAGE	ORGANISM IDENTIFIED
Abundant pseudohyphae. Budding cells, spherical chlamydospores mostly terminal often on a swollen subtending cell.	20	83.33%	*Candida albicans*
Clusters of blastospores with occasional giant cells.	2	8.3%	*Candida parapsilosis*
Pseudohyphae absent. Budding unipolar. Cells ellipsoidal, typically arranged in dense groups. Chlamydospore absent.	1	4.2%	*Candida glabrata*
Branched pseudomycellium. Budding cells ellipsoidal to cylindrical. Cells liberated and arranged parallel to the main axis.	1	4.2%	*Candida krusei*

Morphology and chlamydospore formation on Corn Meal agar by Dalmau technique was used to identify and speciate *Candida* isolates. 20 isolates of *Candida albicans*, 2 of *Candida parapsilosis*, 1 of *Candida glabrata* and 1 isolate of *Candida krusei* were identified.

TABLE 5: SUGAR ASSIMILATION TEST FOR *CANDIDA* (n=24)

SPECIES	SUGAR ASSIMILATION						
	GLUCOSE	SUCROSE	LACTOSE	MALTOSE	GALACTOSE	RAFFINOSE	TREHALOSE
Candida albicans (n=20)	20 (100%)	18 (83%)	0	20 (100%)	20 (75%)	0	19 (92%)
Candida parapsilosis (n=2)	2 (100%)	1 (50%)	0	1 (50%)	2 (100%)	0	2 (100%)
Candida glabrata (n=1)	1 (100%)	0	0	1 (100%)	0	0	1 (100%)
Candida krusei (n=1)	1(100%)	0	0	0	0	0	0

On performing Sugar Assimilation test, all 20 isolates of *Candida albicans* assimilated glucose and maltose, 92% assimilated trehalose, 83% sucrose and 75% assimilated galactose. Both isolates of *Candida parapsilosis* assimilated glucose, galactose and trehalose while only 1 isolate assimilated sucrose and maltose. *Candida glabrata* assimilated glucose, maltose and trehalose. *Candida krusei* assimilated only glucose.

TABLE 6: SUGAR FERMENTATION TEST FOR *CANDIDA* (n=24)

SPECIES	SUGAR FERMENTATION				
	GLUCOSE	**SUCROSE**	**LACTOSE**	**MALTOSE**	**TREHALOSE**
Candida albicans (n=20)	20 (100%)	0	0	20 (100%)	18 (75%)
Candida parapsilosis (n=2)	2 (100%)	0	0	0	0
Candida glabrata (n=1)	1 (100%)	0	0	0	1 (100%)
Candida krusei (n=1)	1 (100%)	0	0	0	0

All 20 *Candida albicans* isolates fermented glucose and maltose while 18 fermented trehalose(75%). *Candida parapsilosis* was able to ferment only glucose. *Candida glabrata* fermented glucose and trehalose and *Candida krusei* fermented only glucose.

VIRULENCE TEST FOR *CANDIDA*

TABLE 7: BIOFILM FORMATION (n=24)

CANDIDA SPECIES	BIOFILM
***Candida albicans* (n=20)**	**6 (29%)**
***Candida parapsilosis* (n=2)**	**1 (50%)**
***Candida glabrata* (n=1)**	**0**
***Candida krusei* (n=1)**	**0**

6 isolates of *Candida albicans* (29%) and 1 isolate of *Candida parapsilosis* were biofilm producers.

VIRULENCE TESTS FOR *ASPERGILLUS* SPECIES

TABLE 8: UREASE TEST

Aspergillus species	**Number tested**	**Urease positive**	**Percentage**
Aspergillus niger	51	0	0
Aspergillus flavus	14	11	78.57%
Aspergillus terreus	4	4	100%
Aspergillus fumigatus	2	0	0
Aspergillus nidulans	1	0	0

Urease production was seen in 78.57% of the *Aspergillus flavus* isolates and all 4 *Aspergillus terreus* isolates. Urease production was not seen with *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus nidulans* .

TABLE 9: HEMOLYSIN PRODUCTION

***Aspergillus* species**	**Number tested**	**Hemolysin positive**	**Percentage**
Aspergillus niger	51	12	23.53%
Aspergillus flavus	14	4	28.57%
Aspergillus terreus	4	3	75%%
Aspergillus fumigates	2	2	100
Aspergillus nidulans	1	0	0

Hemolysin as a virulence factor was produced in 4 isolates of *Aspergillus niger* (7.8%), 2 isolates of *Aspergillus flavus*(1.4%), and 1 isolate of *Aspergillus terreus* (25%).

CHART -9

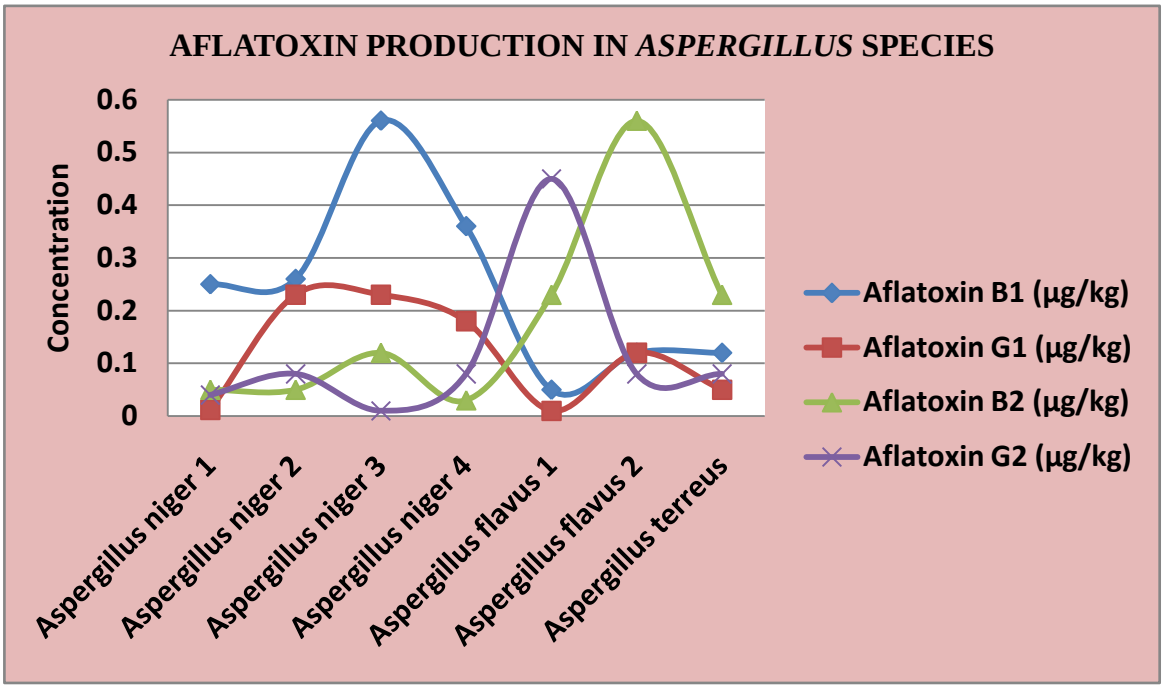


TABLE 10: AFLATOXIN PRODUCTION BY HPLC

	Aflatoxin B1 (µg/kg)	Aflatoxin G1 (µg/kg)	Aflatoxin B2 (µg/kg)	Aflatoxin G2 (µg/kg)
Aspergillus niger 1	0.25	0.012	0.05	0.04
Aspergillus niger 2	0.26	0.23	0.05	0.08
Aspergillus niger 3	0.56	0.23	0.12	0.01
Aspergillus niger 4	0.36	0.18	0.03	0.08
Aspergillus flavus 1	0.05	0.01	0.23	0.45
Aspergillus flavus 2	0.12	0.12	0.56	0.08
Aspergillus terreus	0.12	0.05	0.23	0.08

Aspergillus isolates producing hemolysin were tested for aflatoxin production by High Performance Liquid Chromatography (HPLC). All the isolates produced Aflatoxins. Higher production of Aflatoxins B1 and G1 was seen with *Aspergillus niger* while *Aspergillus flavus* and *Aspergillus terreus* produced more amounts of Aflatoxins B2 and G2.

MOLECULAR METHODS

TABLE 11: SPECIATION OF RARE FILAMENTOUS FUNGI:

Number Isolated	Pan Fungal PCR	Gene Sequencing	Species Identified
2	**POSITIVE**	**AAATCTAAAACTAAGGAAGGCCCTATATCGGG GGCATGCTGTTCGAGCGT CATTTCACCACTCAAGCCTCGCTTGGTATTGGG CAACGCGGTCCGCCGCG TGCCTCAAATCGACCGGCTGGGTCTTCTGTCCC CTAAGCGTTGTGGAAAC TATTCGCTAAAGGGTGCTCGGGGAGGCTACGC CGTAAAACAAACCCATTT CTAAGGTTGACCTCGGATCAGGTAGGGATACC CGCTGAACTTAAGCATAT CAATAAAGCGGGAGAAA**	***Cladosporium cladosporoides***

2 of the rare isolates were subjected to PCR followed by Sanger sequencing. 270 bp Pan-fungal genome was identified and sequenced by Sanger's dideoxynucleotide sequencing method. Individual nucleotide-nucleotide searches were done with the BLASTn algorithm at the NCBI website. Sequence-based identities with a cutoff of 97% were obtained for both isolates and the fungi were identified as *Cladosporidium cladosporoides.*

All the diagnosed cases were treated with Clotrimazole 1% ear drops or plugging of ear with Miconazole ointment applied in gauze. The patients were followed up at 1, 2, 4 weeks and 12 weeks. All the patients showed good response to treatment with negative culture by 2 weeks of treatment.

From a microbiologists perspective, antifungal susceptibility to commonly used antifungal drugs was done to determine the Minimum Inhibitory Concentration (MIC) of the antifungal agents to the fungal isolates.

ANTIFUNGAL SUSCEPTIBILTY PATTERN OF FILAMENTOUS FUNGI BY MICROBROTH DILUTION METHOD (CLSI M 38 A2)

TABLE 12: MINIMUM INHIBITORY CONCENTRATIONS (MIC) FOR CLOTRIMAZOLE

Drug		**Clotrimazole Minimum Inhibitory Concentration (mg/ml)**									
FUNGI	**n**	**0.03**	**0.0625**	**0.125**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**16**
A. niger	51	1	10	7	13	11	9	-	-	-	-
A. flavus	14	1	4	2	2	4	1	-	-	-	-
A. terreus	4	-	-	-	1	2	1	-	-	-	-
A. fumigatus	2	-	-	-	-	1	1	-	-	-	-
Rhizopus oryzae	2	-	-	-	-	-	2	-	-	-	-
Cladosporium cladosporoides	2	-	-	-	-	1	1	-	-	-	-
Aspergillus nidulans	1	-	-	-	-	-	1	-	-	-	-
Aureobasidium pullulans	1	-	-	1	-	-		-	-	-	-
Fonsaceae pedrosoi	1	-	-	-	-	-	1	-	-	-	-
Rhizomucor pusillus	1	-	-	-	-	1	-	-	-	-	-
Mucor circinelloides	1	-	-	-	-		1	-	-	-	-
Curvularia lunata	1	-	-	-	-	1		-	-	-	-

MIC of Clotrimazole to *Aspergillus niger* was in the range 0.03 – 1μg/ml. MIC50 was 0.25μg/ml and MIC90 was 1 μg/ml. For *Aspergillus flavus* the MIC range was 0.03 – 1 μg/ml. MIC 50 was 0.125 μg/ml and MIC90 was 0.5 μg/ml

TABLE 13: MINIMUM INHIBITORY CONCENTRATIONS (MIC) FOR AMPHOTERICIN B

Drug		**Amphotericin B Minimum Inhibitory Concentration (mg/ml)**									
FUNGI	**n**	**0.03**	**0.0625**	**0.125**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**16**
A. niger	51	2	2	3	11	24	9	-	-	-	-
A. flavus	14	-	1	1	1	7	4	-	-	-	-
A. terreus	2	-	-	-	-	1	1	-	-	-	-
A. fumigatus	2	-	-	-	-	1	1	-	-	-	-
Rhizopus oryzae	2	-	-	-	-	1	1	-	-	-	-
Cladosporium cladosporoides	1	-	-	-	-	-	1	-	-	-	-
Aspergillus nidulans	1	-	-	-	-	-	1	-	-	-	-
Aureobasidium pullulans	1	-	-	-	-	-	1	-	-	-	-
Fonsaceae pedrosoi	1	-	-	-	-	-	1	-	-	-	-
Rhizomucor pusillus	1	-	-	-	-	1	-	-	-	-	-
Mucor circinelloides	1	-	-	-	-	-	1	-	-	-	-
Curvularia lunata	1	-	-	-	-	-	1	-	-	-	-

MIC of Amphotericin B to *Aspergillus niger* was in the range 0.03 – 1μg/ml. MIC 50 was 0.5μg/ml and MIC90 was 1 μg/ml. For *Aspergillus flavus* the MIC range was 0.0625 – 1 μg/ml. MIC 50 was 0.5 μg/ml and MIC90 was 1μg/ml.

TABLE 14: MINIMUM INHIBITORY CONCENTRATIONS (MIC) FOR VORICONAZOLE

DRUG		**VORICONAZOLE MINIMUM INHIBITORY CONCENTRATION (µg/ml)**									
FUNGI	**n**	**0.03**	**0.0625**	**0.125**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**16**
A. niger	51	1	3	19	12	11	5	-	-	-	-
A. flavus	14	1	1	1	6	3	2	-	-	-	-
A. terreus	4	-	-	1	-	1	2	-	-	-	-
A. fumigatus	2	-	-	-	-	1	1	-	-	-	-
Rhizopus oryzae	2	-	-	-	-	1	1	-	-	-	-
Cladosporium cladosporoides	2	-	-	-	1	-	1	-	-	-	-
Aspergillus nidulans	1	-	-	-	-	-	1	-	-	-	-
Aureobasidium pullulans	1	-	-	-	-	1	-	-	-	-	-
Fonsaceae pedrosoi	1	-	-	-	-	1	-	-	-	-	-
Rhizomucor pusillus	1	-	-	-	-	-	1	-	-	-	-
Mucor circinelloides	1	-	-	-	-	1	-	-	-	-	-
Curvularia lunata	1	-	-	-	-	-	1	-	-	-	-

MIC of Voriconazole to *Aspergillus niger* was in the range 0.03 –1 µg/ml. MIC 50 was 0.25µg/ml and MIC90 was 0.5 µg/ml. For *Aspergillus flavus* the MIC range was 0.03 – 1 µg/ml. MIC 50 was 0.25 µg/ml and MIC90 was 1µg/ml.

TABLE 15: MINIMUM INHIBITORY CONCENTRATIONS (MIC) FOR ITRACONAZOLE

DRUG		**ITRACONAZOLE MINIMUM INHIBITORY CONCENTRATION (µg/ml)**									
FUNGI	**n**	**0.03**	**0.0625**	**0.125**	**0.25**	**0.5**	**1.0**	**2.0**	**4.0**	**8.0**	**16**
A. niger	51	2	7	8	11	13	10	-	-	-	-
A. flavus	14	1	5	3	1	2	2	-	-	-	-
A. terreus	4	-	-	-	-	2	2	-	-	-	-
A. fumigatus	2	-	-	-	-	1	1	-	-	-	-
Rhizopus oryzae	2	-	-	-	1	-	1	-	-	-	-
Cladosporium cladosporoides	2	-	-	-	-	1	1	-	-	-	-
Aspergillus nidulans	1	-	-	-	-	-	1	-	-	-	-
Aureobasidium pullulans	1	-	-	-	-	1	-	-	-	-	-
Fonsaceae pedrosoi	1	-	-	-	-	-	1	-	-	-	-
Rhizomucor pusillus	1	-	-	-	-	-	1	-	-	-	-
Mucor circinelloides	1	-	-	-	-	1	-	-	-	-	-
Curvularia lunata	1	-	-	-	-	1	-	-	-	-	-

MIC of Itraconazole to *Aspergillus niger* in the range 0.03 – 1 µg/ml. MIC 50 was 0.25µg/ml and MIC90 was 1 µg/ml. For *Aspergillus flavus* the MIC range was 0.03 – 1 µg/ml. MIC 50 was 0.125 µg/ml and MIC90 was 1µg/ml.

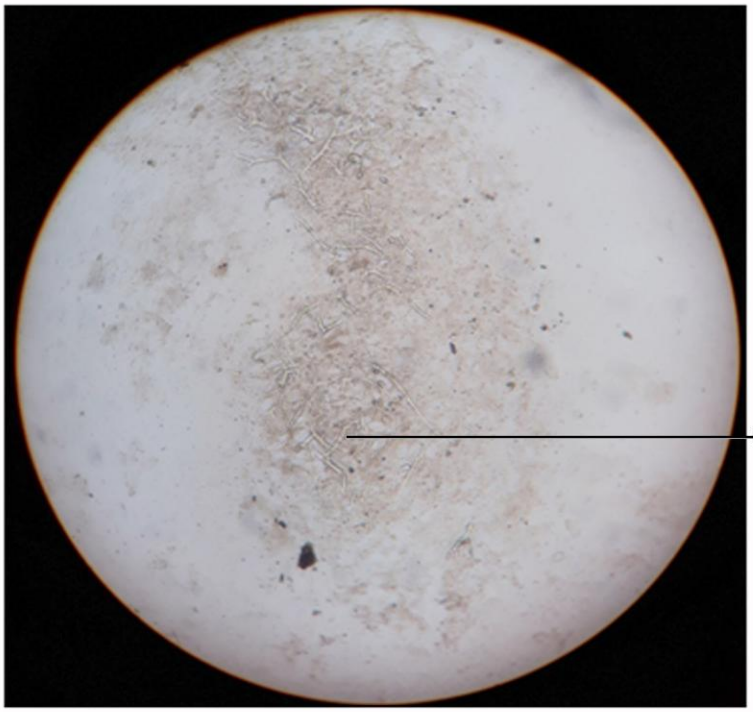
ANTIFUNGAL SUSCEPTIBILITY TESTING FOR *CANDIDA* SPECIES BY DISC DIFFUSION METHOD (CLSI M44)

TABLE 16: SENSITIVITY PATTERN OF *CANDIDA* SPECIES TO ANTIFUNGALS

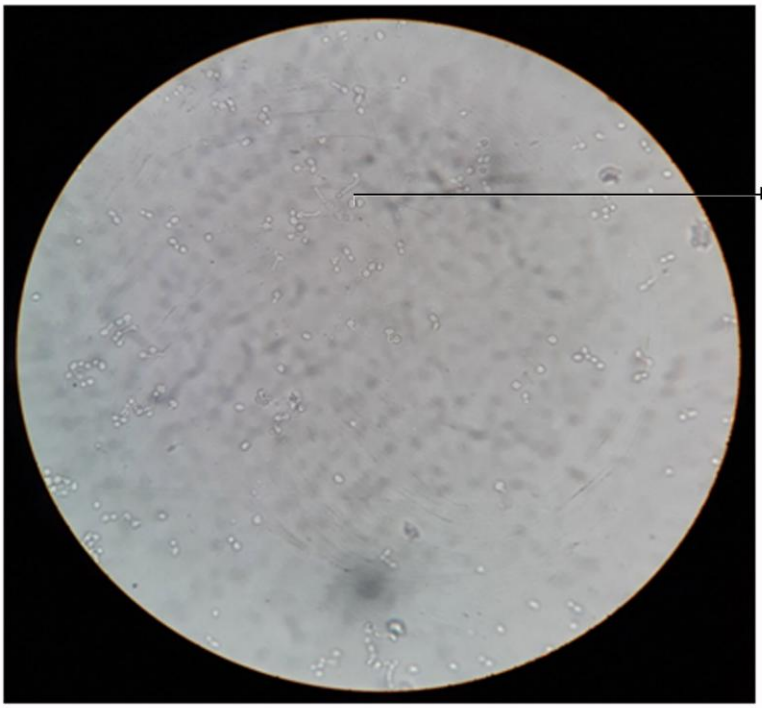
Drug	**Amphotericin B (100U)**		**Itraconazole (10µg)**		**Voriconazole (1µg)**		**Fluconazole (25µg)**		**Clotrimoxazole (10µg)**	
***Candida* Species**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**
***Candida albicans* (n=20)**	**20 (100%)**	**0**	**19 (95%)**	**1 (5%)**	**20 (100%)**	**0**	**18 (90%)**	**2 (10%)**	**20 (100%)**	**0**
***Candida parapsilosis* (n=2)**	**2 (100%)**	**0**	**2 (100%)**	**0**	**2 (100%)**	**0**	**1 (50%)**	**1 (50%)**	**2 (100%)**	**0**
***Candida glabrata* (n=1)**	**-**	**-**	**1 (100%)**	**0**	**1 (100%)**	**0**	**-**	**-**	**1 (100%)**	**0**
***Candida krusei* (n=1)**	**-**	**-**	**1 (100%)**	**0**	**1 (100%)**	**0**	**-**	**-**	**1 (100%)**	**0**

All isolates of *Candida albicans* were susceptible to Ampotericin B, Voriconazole and Itraconazole. 1 isolate was resistant to Itraconazole and 2 isolates were resistant to Fluconazole. Among the 2 isolates of *Candida parapsilosis*, both were susceptible to Amphotericin B, Itraconazole, Voriconazole and Clotrimazole while 1 isolate was resistant to Fluconazole. The isolate of *Candida glabrata* was susceptible to Voriconazole, Itraconazole and Clotrimoxazole. The isolate of *Candida krusei* was susceptible to Voriconazole, Itraconazole and Clotrimazole.

KOH MOUNT

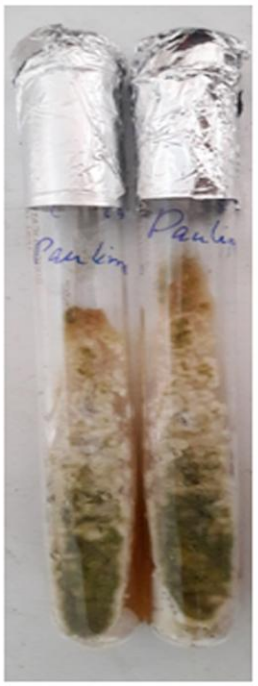


GERM TUBE TEST



GERM TUBE FORMATION BY *Candida albicans*

Aspergillus flavus



Aspergillus terreus

Aspergillus nidulans

Aspergillus niger

Aureobasidium pullulans

OBVERSE

REVERSE

Rhizomucor pusillus

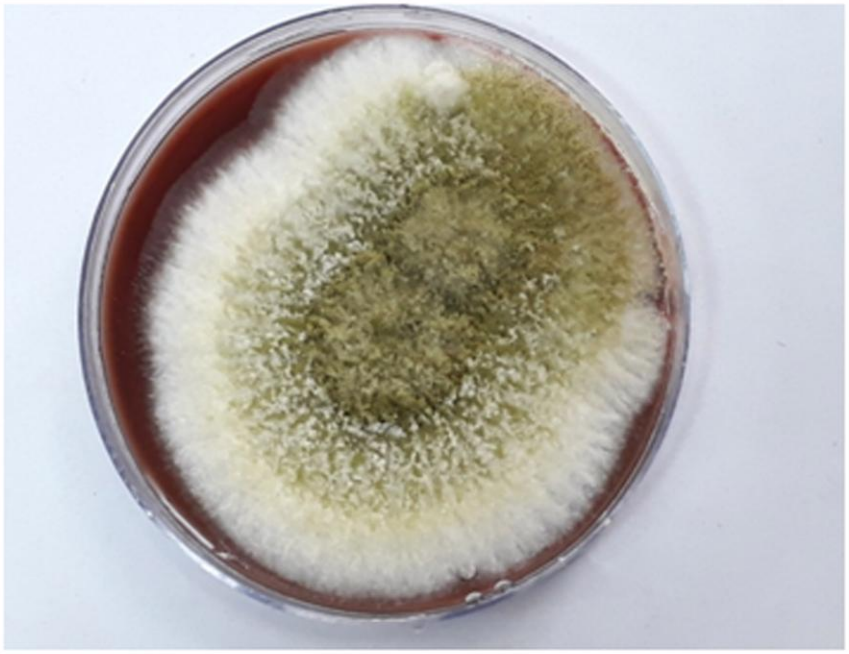
Cladosporium cladosporoides

1. REVERSE
2. OBVERSE

CULTURE ON SDA

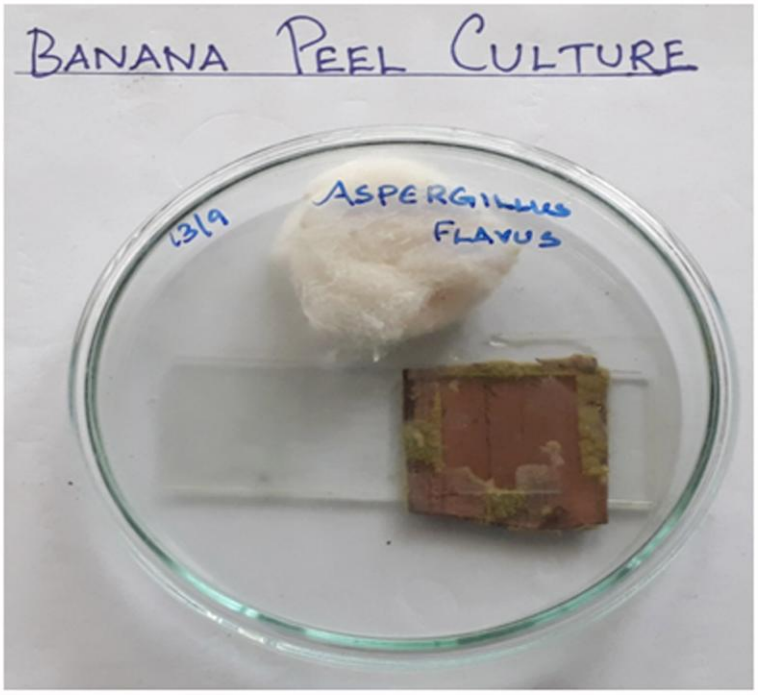
CULTURE ON PDA

CULTURE ON 7% SHEEP BLOOD AGAR



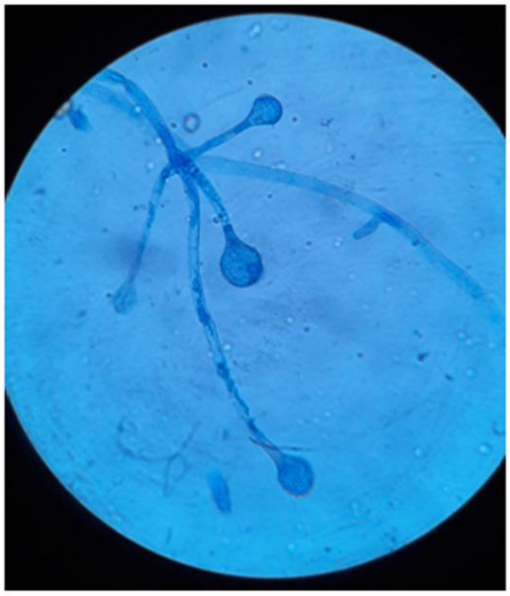
RIDDLE'S SLIDE CULTURE

BANANA PEEL CULTURE

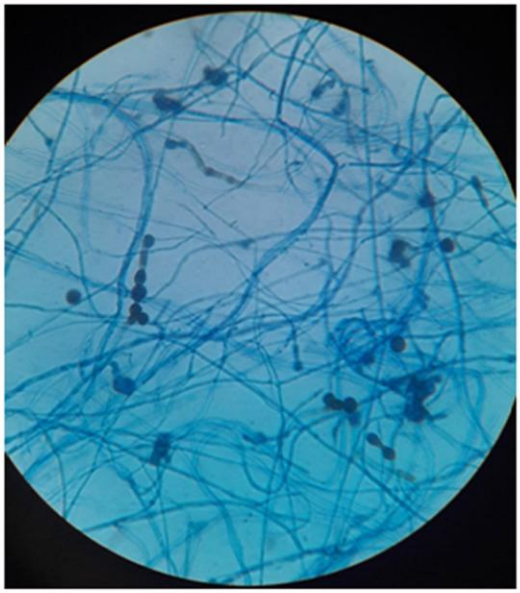


LPCB MOUNT

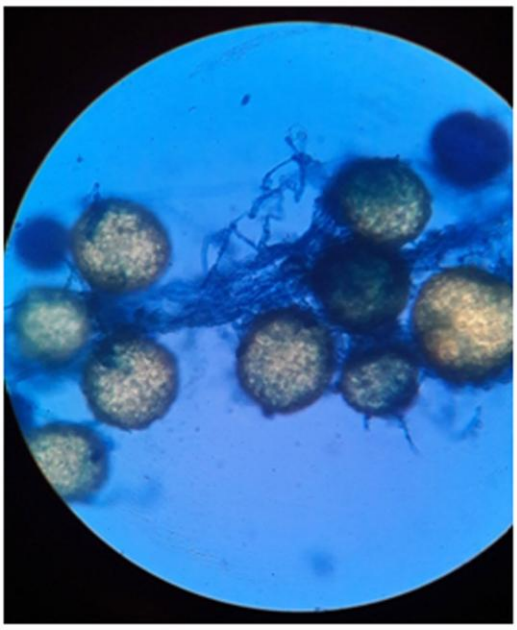
Rhizomucor pussilus



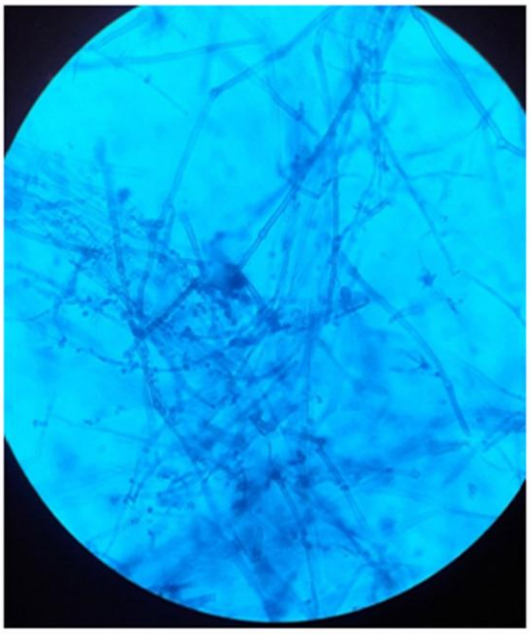
Aureobasidium pullulans



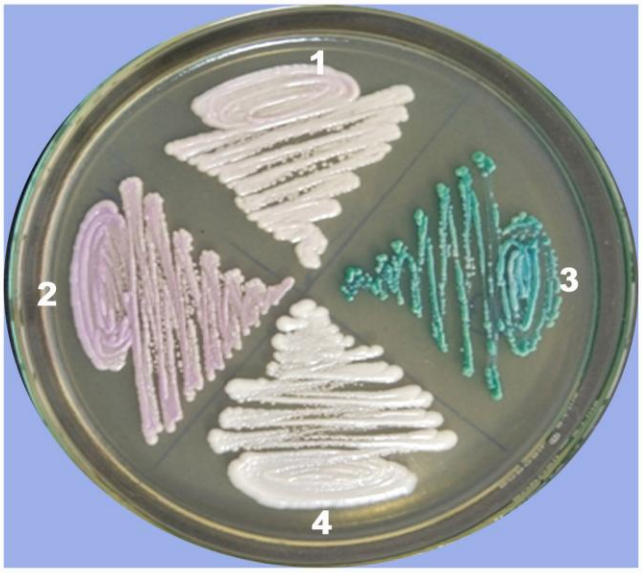
Aspergillus nidulans



Cladosporium cladosporoides

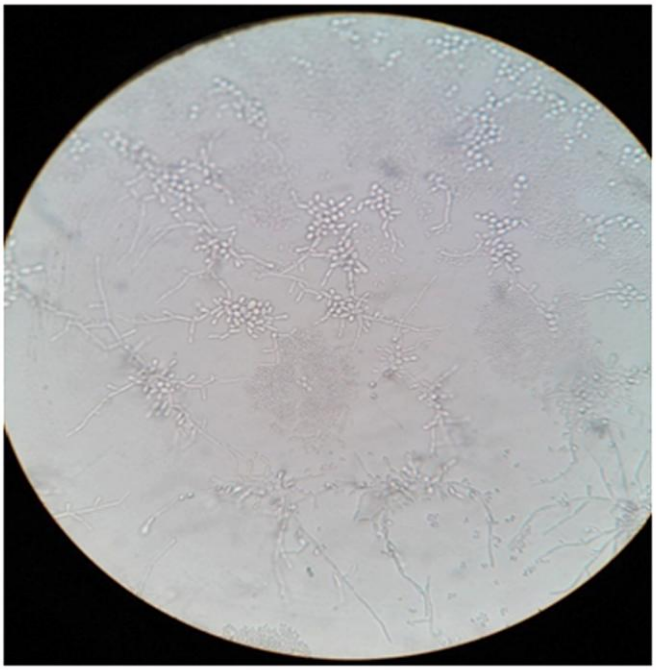


CANDIDA COLONIES ON CHROMAGAR



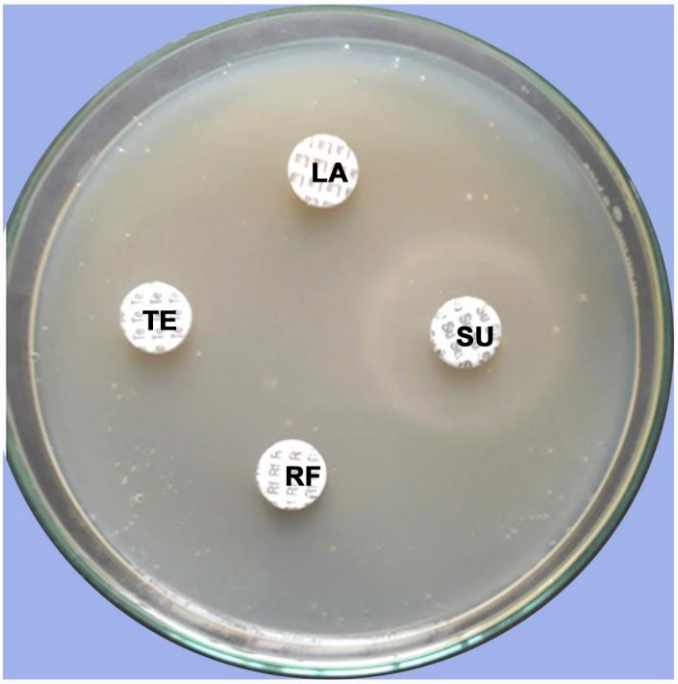
1-*Candida parapsilosis.* 2- *Candida krusei* .
3-*Candida albicans.* 4- *Candida glabrata*

MORPHOLOGY – DALMAU PLATE TECHNIQUE



Branched pseudomycellium with ellipsoidal to cylindrical budding cells of *Candida krusei*

SUGAR ASSIMILATION TEST FOR *CANDIDA*



LA - LACTOSE.
TE - TREHALOSE.
SU - SUCROSE.
RF - RAFFINOSE

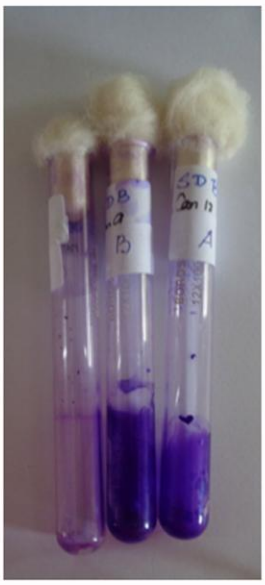
Assimilation of sucrose by *Candida albicans*

SUGAR FERMENTATION TEST FOR *CANDIDA*

VIRULENCE TESTS

UREASE TEST

CANDIDA-BIOFILM

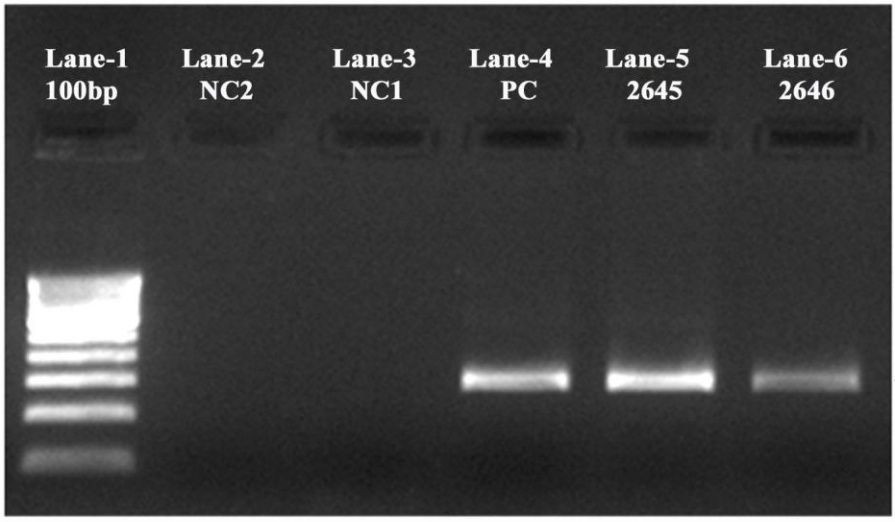


HEMOLYSIS ON BLOOD AGAR

HEMOLYTIC COLONIES OF *Aspergillus niger.*

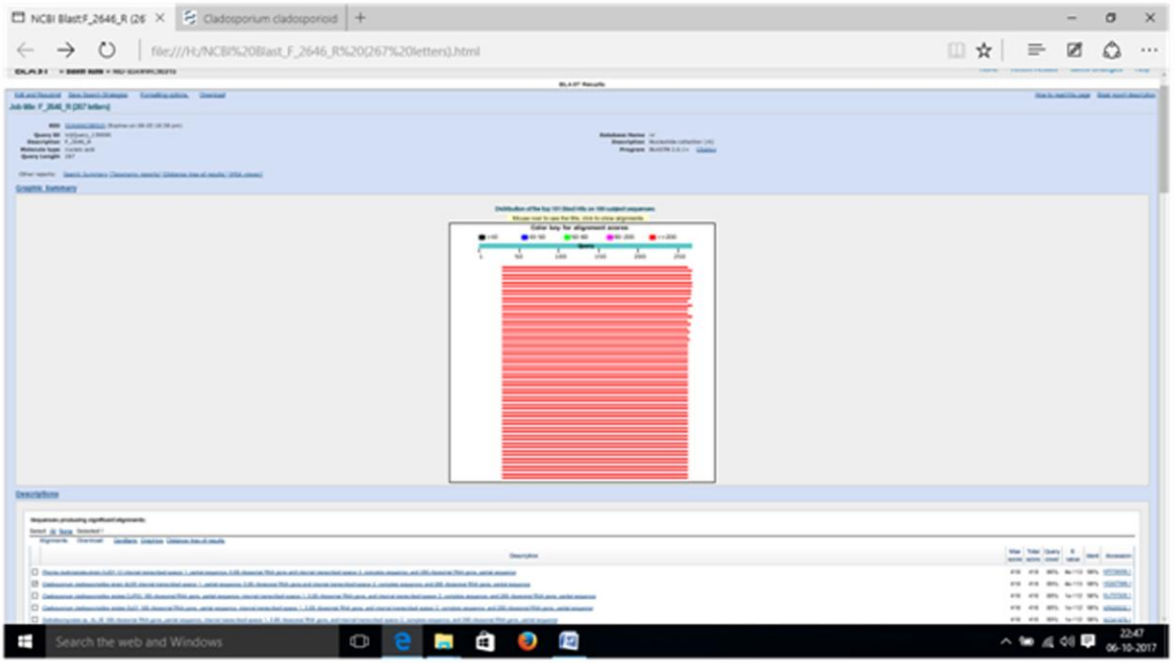
TESTING FOR AFLATOXIN BY HPLC

PCR – PAN FUNGAL GENOME



LANE 1: 100 bp Ladder.
LANE 2 And 3: Negative Controls.
LANE 4: Positive Control for ITS Primer.
LANE 5 And 6: Samples Positive with ITS Primer.

FUNGAL GENE SEQUENCING



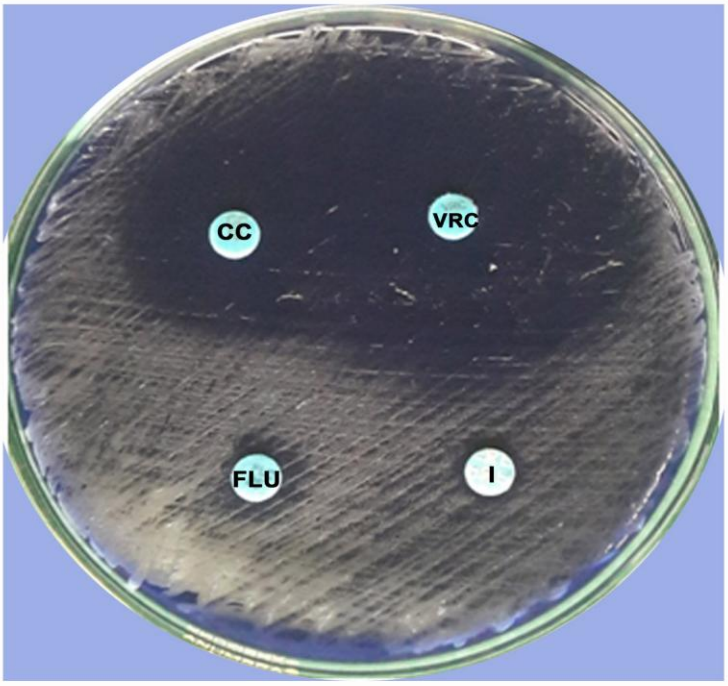
DETERMINATION OF MIC FOR MOULDS BY MICROBROTH DILUTION METHOD

16µg/ml, 8, 4, 2, 1, 0.5, 0.25, 1.125, 0.065, 0.31 GC

A.niger	1
A.flavus	2
A.terreus	3
A.niger	4
A.fumigatus	5
A.niger	6
A.terreus	7
Fonsaceae	8
Aureobasidium	9
A.flavus	10
Rhizomucor	11
A.niger	12
Drug control	DC

CLOTRIMAZOLE

ANTIFUNGAL SUSCEPTIBILITY TESTING FOR *CANDIDA* BY DISC DIFFUSION METHOD



CC- CLOTRIMAZOLE 10µg. **VRC- VORICONAZOLE 1µg.**
FLU- FLUCONAZOLE 25µg. **I- ITRACONAZOLE 10µg**

DISCUSSION

Otomycosis is one of the most frequently encountered clinical condition in otorhinology. It is a superficial, acute, subacute or chronic fungal infection of the external auditory canal. The etiologic agents are saprophytic fungi, commonest being *Aspergillus niger* and *Candida* species. Although not life threatening, otomycosis can be a frustrating condition for both the patient and the treating doctor due to the requirement for a long term treatment, regular follow up and its tendency for recurrence.

Of the 200 clinically suspected cases of otomycosis analysed, 105 samples showed fungal growth on culture accounting for 52.5%. This matches studies by Pankti Panchal et al[100] which showed positive growth in 46% and Nowrozi et al[54] which showed a positive growth in 57%. It is in contrast to study by Agarwal et al[3] where the positivity rate was 88.6% .

Fungus was isolated from all age groups ranging from 1 year to >60years. Among the positive cases, the youngest was a 5 year old male child, whose mother gave history of regular instillation of coconut oil in the ear. The eldest was a 72 year old male patient who gave h/o uncontrolled Diabetes mellitus.

Age distribution shows majority of the positive samples in the age group of 21-30 years, with 27 cases (25.71%) followed by 31-40 years with 25 cases (23.81%). Least number of positive cultures were in the age group 1-10 years with

2 cases. These findings are in concordance with studies by Channabasawaraj et al[65], Yadav et al[102] and Shilpa.K.Gokale et al[11] which showed maximum number of cases in the age group 21-30 years with 28.3%, 35.36% and 33% respectively. Studies by Praveen Kumar Karn et al[103] showed contrast to this study, where the maximum number of cases were in the age group 31-40 years followed by 21-30 years.

There was a female predominance observed in this study. Among the positive samples 62 were from females accounting for 59% and 43were from males with 41%. The female: male ratio was 1.44:1. These findings correlate with studies by Jyothi Swarup et al[50] and J Fasunla et al[13] which showed more number of cases among females than males. Sampath Chandra Prasad et al[12] and Kaur et al[105] reported more cases among males than females.

Otomycosis was observed to be unilateral in 101 cases (96%) and Bilateral in 4 cases (4%). Standard ENT reference books say that otomycosis is predominantly unilateral among immunocompetant individuals[4]. Likewise, Studies by Ho et al[83] and Nandyal et al[112] showed that majority of cases were unilateral (93%).

Among those with Unilateral otomycosis, 19 Males (18.1%) and 34 Females (32.4%) with a total of 53 cases (50.5%) showed a side predilection for

the Right side. 23 Males (21.9%) and 25 Females (23.8%) with a total of 48 cases (45.7 %) showed predilection for the Left side.

The same observation was seen in study by Paulose et al[80] where most of the cases were unilateral with a slight predominance in the right side. As opposed to this, study by J Chander et al[82] noted more cases in the left side while Yehia et al[106] found no preference for either side.

Habitual cleaning of ears with unsterile materials like ear buds, safety pins, and match sticks was the common predisposing factor seen in 73 cases (70%). It matches ENT publications which relate use of unsterile materials with increased incidence of otomycosis due to microtraumas and inoculation of fungal spores[34,107,108].

Use of ear drops was observed in 41 cases(39%) as in studies by H S Sathish et al (48%)[118]. Oil instillation in ears was noted in 32 cases (31%) like in study by Rao et al (35.1%)[45]. Previous history of CSOM was present in 22 cases (21%). Pus in CSOM along with the humid environment promotes growth of fungi[16,31].Fungal culture positivity was identified in 26.15% cases of CSOM in study by Arun Ghosh et al[41]. Swimming/ cleaning ears with water during bathing was seen in 5 cases (5%) correlating with study by Surinder Singh et al[111] (5%) as against other studies which showed higher percentage[109,110]. H/o trauma or

instrumentation of ears was present in 4 cases (4%) agreeing with findings by Deshmukh et al(4%)[17].

Diabetes mellitus as a co-morbid factor has been implicated in otomycosis. In our study, 17 patients had Diabetes mellitus as co-morbid illness (16%). This is in concordance with studies by Pankti Panchal et al[100], Deshmukh et al[17] and Senthil Kumaran et al[104] where Diabetes mellitus as a co-morbid factor was observed in 10%,13% and 6% respectively.

Aural pruritis was the most frequent symptom reported by 89 patients(85%), which is in agreement with studies by Nandyal et al (93.37%)[112] and Sheik et al (90.38%)[113]. Fullness of the ear/ Ear block was the second common complaint seen in 73 cases (70%) as against observations by Mathur et al[36] who found Ear block as a symptom in 93% of the patients. In this study, Otalgia was seen in 50 cases (48%), like in study by Viswanatha et al (40%)[10]. 41 patients had Diminished hearing(39%) as a symptom while 37 had Ear Discharge (35%) as presenting complaint. This is in disagreement to study by Reena Ray et al[114] where otorrhoea was the frequent complaint (58%). 6 patients presented with Tinnitus (6%), matching observations by Prasad et al[12] and Dhingra et al[47] who noted Tinnitus in only 8% and 6% of cases respectively.

On correlating KOH examination and culture positivity, it was observed that of the 105 culture positives, 101 were KOH positive. Sensitivity for diagnosis

by culture was 100% and Sensitivity by KOH mount was 96%. Similar correlation between KOH examination and fungal culture was noted in studies by Kondity et al[115], and Shilpa K Gokale et al[11]. Both KOH examination and fungal culture provide sensitive and reliable modalities of diagnosis.

Literature search shows filamentous fungi as the commonest causative agent of otomycosis correlating with this study[1,2,19]. Out of the105 fungi isolated, 81 were filamentous fungi (77%) and 24 were *Candida* species (23%). This is in accordance with studies by C Rodrigues et al[8], Barati et al[116] and Aneja et al[110] where the predominant isolates were filamentous fungi as opposed to studies by Zelia da Silva Pontes et al[117] and Kumar H et al[69] where *Candida* isolates were more than the filamentous isolates.

Among the fungi isolated *Aspergillus niger* was the most common isolate in 51 cultures (49%), followed by *Aspergillus flavus* in 14 cultures (13%). *Aspergillus terreus* was isolated in 4 cultures. There were 2 isolates each of *Aspergillus fumigatus* and *Rhizopus.* The rare causative fungi isolated were *Cladosporium*(2), *Aspergillus nidulans* (1),*Aureobasidium pullulans*(1), *Fonsaceae pedrosoi* (1), *Curvularia lunata* (1), *Mucor* (1), and *Rhizomucor* (1).

Of the 24 *Candida* species isolated, 20 were *Candida albicans*, 2 were *Candida parapsilosis*, 1 was *Candida glabrata* and 1 isolate was *Candida krusei*. Studies by Vrutika V Gandhi et al[44], Aneja et al[110], and Nagendran et al[84]

identified *Aspergillus niger* as the common isolate followed by *Aspergillus flavus*. *Candida* was the 2nd most common isolate in studies by Kondity et al[115], Sathish et al[118] and Prakash et al[40]. The results are in contrast with findings of Mathur et al[36] where *Aspergillus fumigatus* was isolated in 41.4% of the cases. Isolates of *Aspergillus terreus, Cladosporium, Mucor, Rhizopus* have been identified as etiological agents of otomycosis by A M Moharram et al[22], Munguia et al[122], Mugliston et al[15].

On performing Germ Tube test for identification of Candida, Germ tube test was positive for 20 out of the 24 Candida isolates. The 20 GTT positive isolates formed Germ Tube at 42°C. Formation of Germ Tube at 42°C provides a 100% sensitive test for identifying *Candida albicans* as shown in studies by Saranya et al[101].

Based on the colour of colonies on CHROMagar, 20 isolates of *Candida albicans*, 2 isolates of *Candida parapsilosis*, 1 isolate of *Candida glabrata* and 1 isolate of *Candida krusei* were identified[94,95,97] .

Morphology and chlamydospore formation on Corn Meal agar by Dalmau technique was used to identify and speciate *Candida* isolates. 20 isolates of *Candida albicans*, 2 of *Candida parapsilosis*, 1 of *Candida glabrata* and 1 isolate of *Candida krusei* were identified[92,93].

On performing Sugar Assimilation test, all 20 isolates of *Candida albicans* assimilated glucose and maltose, 92% assimilated trehalose, 83% sucrose and 75% assimilated galactose. Both isolates of *Candida parapsilosis* assimilated glucose, galactose and trehalose while only 1 isolate assimilated sucrose and maltose. *Candida glabrata* assimilated glucose, maltose and trehalose. *Candida krusei* assimilated only glucose. This result correlates with the Standard Operating Procedures for identification of yeasts released by the Indian Council of Medical Research[92].

All 20 *Candida albicans* isolates fermented glucose and maltose while 18 fermented trehalose(75%). *Candida parapsilosis* was able to ferment only glucose. *Candida glabrata* fermented glucose and trehalose and *Candida krusei* fermented only glucose matching the results of Standard Operating Procedures for identification of yeasts released by the Indian Council of Medical Research[92].

Virulence factors in fungi, determine the severity of the disease and its response to antifungal treatment. So testing for fungal virulence factors is an integral part of the laboratory diagnosis of fungi.

6 isolates of *Candida albicans* (29%) and 1 isolate of *Candida parapsilosis* were biofilm producers. This is similar to studies by Analy S Melo et al[121] where biofilm formation was more among *Candida albicans* than *Candida parapsilosis*.

Urease production was seen in 78.57% of the *Aspergillus flavus* isolates and all 4 *Aspergillus terreus* isolates (100%). Urease production was not seen with

Aspergillus niger, Aspergillus fumigatus and *Aspergillus nidulans* which matches findings by A.M.Moharram et al[22].

Hemolysin as a virulence factor was produced in 12 isolates of *Aspergillus niger* (23.53%), 4 isolates of *Aspergillus flavus* (28.57%), and 3 isolates of *Aspergillus terreus* (75%). In study by Raksha et al[23] hemolysin was produced by 83% of *Aspergillus niger*, 57% of *A.flavus*, 77% of *Aspergillus fumigatus* and 100% of *Aspergillus terreus*.

Aspergillus niger species showed higher production of Aflatoxins B1 and G1 while *Aspergillus flavus* and *Aspergillus terreus* produced more amounts of Aflatoxins B2 and G2. Aflatoxins induce teratogenicity and mutagenicity usually on ingestion or inhalation. Studies by Boonen et al[29] and Rastogi et al[30] have documented permeation of aflatoxin through the skin. In depth studies are needed to identify the mutagenic and teratogenic potential of fungi involved in otomycosis.

Molecular Diagnosis:

2 of the rare isolates were subjected to PCR followed by Sanger sequencing for speciation. 270 bp pan fungal genome was identified and sequenced by Sanger's dideoxynucleotide sequencing method. Individual nucleotide-nucleotide searches were done with the BLASTn algorithm at the NCBI website. Sequence-based identities with a cut-off of 97% was obtained for both isolates and the fungi were identified as *Cladosporidium cladosporoides*.

All the diagnosed cases were treated with Clotrimazole 1% ear drops or plugging of ear with Miconazole ointment applied in gauze. The patients were followed up at 1, 2, 4 and 12 weeks. All the patients showed good response to treatment with negative culture by 2 weeks of treatment. Antifungal susceptibility testing to commonly used antifungal drugs was done by Microbroth dilution method to determine the Minimum Inhibitory Concentration (MIC) of the antifungal agents to the fungal isolates.

MIC of Clotrimazole to *Aspergillus niger* was in the range 0.03 – 1μg/ml. MIC50 was 0.25μg/ml and MIC90 was 1 μg/ml. For *Aspergillus flavus* the MIC range was 0.03 – 1 μg/ml. MIC 50 was 0.125 μg/ml and MIC90 was 0.5 μg/ml.

MIC of Amphotericin B to *Aspergillus niger* was in the range 0.03 – 1μg/ml. The MIC 50 was 0.5μg/ml and MIC90 was 1 μg/ml. For *Aspergillus flavus* the MIC range was 0.0625 – 1 μg/ml. The MIC 50 was 0.5 μg/ml and MIC90 was 1 μg/ml.

MIC of Voriconazole to *Aspergillus niger* was in the range 0.03 –1 μg/ml. MIC 50 was 0.25μg/ml and MIC90 was 0.5 μg/ml. For *Aspergillus flavus* the MIC range was 0.03 – 1 μg/ml. MIC 50 was 0.25 μg/ml and MIC90 was 1μg/ml.

MIC of Itraconazole to *Aspergillus niger* in the range 0.03 – 1 μg/ml. The MIC 50 was 0.25μg/ml and MIC90 was 1 μg/ml. For *Aspergillus flavus* the MIC range was 0.03 – 1 μg/ml. MIC 50 was 0.125 μg/ml and MIC90 was 1 μg/ml.

Among the 20 isolates of *Candida albicans* tested, all isolates were susceptible to Ampotericin B, Voriconazole and Itraconazole. 1 isolate was resistant to Itraconazole and 2 isolates were resistant to Fluconazole. Among the 2 isolates of *Candida parapsilosis*, both were susceptible to Amphotericin B, Itraconazole, Voriconazole and Clotrimazole while 1 isolate was resistant to Fluconazole. The isolate of *Candida glabrata* was susceptible to Voriconazole, Itraconazole and Clotrimoxazole. The isolate of *Candida krusei* was susceptible to Voriconazole, Itraconazole and Clotrimazole.

SUMMARY

- Otomycosis is one of the common infections of the ear prevalent in the tropical and subtropical regions.
- In this study, fungi was isolated from 105 cases (52.5%) of clinically suspected otomycosis. High prevalence could be due to the hot and humid climatic conditions prevalent in this region.
- Otomycosis was seen in all age groups with the highest number of cases in the age group 21-30 years (25.71%) followed by 31-40 years (23.81%). This could be due to maximum exposure in these age groups to environmental spores.
- There was a female predominance with 62 cases in females (59%) and 43 cases in males (41%). Majority of the cases were unilateral (96%) while only 4 were bilateral (4%).
- Among predisposing factors, habitual cleaning of ear with unsterile materials (70%) was found to be the commonest followed by injudicious use of antibiotic/ steroid ear drops (39%).
- Uncontrolled diabetes was the most frequent comorbid condition associated with otomycosis (16%).
- On correlation of diagnosis by microscopy and culture, culture showed a sensitivity of 100%, specificity of 95.96%, positive predictive value of 96.19%, negative predictive value of 100% with a diagnostic accuracy of 98% validating culture as a reliable test for diagnosis.

- Among fungi causing otomycosis, filamentous fungi were isolated from 81 samples (77%). *Aspergillus niger* was the commonest etiological agent in otomycosis(49%) followed by *Candida albicans* (19.1%), *Aspergillus flavus* (13%), *Aspergillus terreus*(4%), *Aspergillus fumigatus*(2%), *Rhizopus*(2%).
- Rare isolates were *Cladosporium cladosporoides* (2), *Aspergillus nidulans* (1), *Aureobasidium pullulans*(1), *Fonsaceae pedrosoi* (1), *Curvularia lunata* (1), *Mucor* (1), *Rhizomucor* (1), *Candida parapsilosis* (2), *Candida glabrata* (1) and *Candida krusei*(1).
- *Candida* species can be reliably speciated based on germ tube test, colour of colonies on CHROMagar, morphology on Dalmau plating, sugar assimilation and sugar fermentation tests as shown in this study.
- Identification of virulence factors is an important part of diagnosis in fungal infections as it determines the severity of infections as well as its response to treatment. In this study, biofilm production was seen with 6 isolates of *Candida albicans* (29%) and 1 isolate of *Candida parapsilosis.*
- Hemolysin as a virulence factor was produced in 12 isolates of *Aspergillus niger* (23.53%), 4 isolates of *Aspergillus flavus*(28.57%), and 3 isolates of *Aspergillus terreus* (75%).
- *Aspergillus niger* species showed higher production of Aflatoxins B1 and G1 while *Aspergillus flavus* and *Aspergillus terreus* produced more amounts of Aflatoxins B2 and G2. Aflatoxin B1 has been shown to

permeate the skin. In depth studies are needed to identify the mutagenic and carcinogenic potential of *Aspergillus* species involved in otomycosis

- 2 of the rare isolates were subjected to PCR followed by Sanger sequencing for speciation and identified as *Cladosporidium cladosporoides*. Sequence-based identities serves as a reliable method for identification of rare fungi as well for estimating drug resistance.
- The diagnosed cases were treated with Clotrimazole 1% ear drops or plugging of ear with Miconazole ointment applied in gauze. The patients were followed up at 1, 2, 4 and 12 weeks. All the patients showed good response to treatment with negative culture by 2 weeks of treatment. There was no recurrence on follow up for 3 months.
- All the filamentous fungi were susceptible to commonly used antifungals like Clotrimoxazole, Amphotericin B, Voriconazole and Itraconazole tested by microbroth dilution method, with MIC ranging between 0.03 –1µg/ml.
- Among the 20 isolates of *Candida albicans* tested, 1 isolate was resistant to Itraconazole and 2 isolates were resistant to Fluconazole. Among the 2 isolates of *Candida parapsilosis*, 1 isolate was resistant to Fluconazole. The isolate of *Candida glabrata* was susceptible to Voriconazole, Itraconazole and Clotrimoxazole. The isolate of *Candida krusei* was susceptible to Voriconazole, Itraconazole and Clotrimazole.

Otomycosis is challenging for both patients and otolaryngologist as it frequently requires long term treatment and follow up . In recent years there has

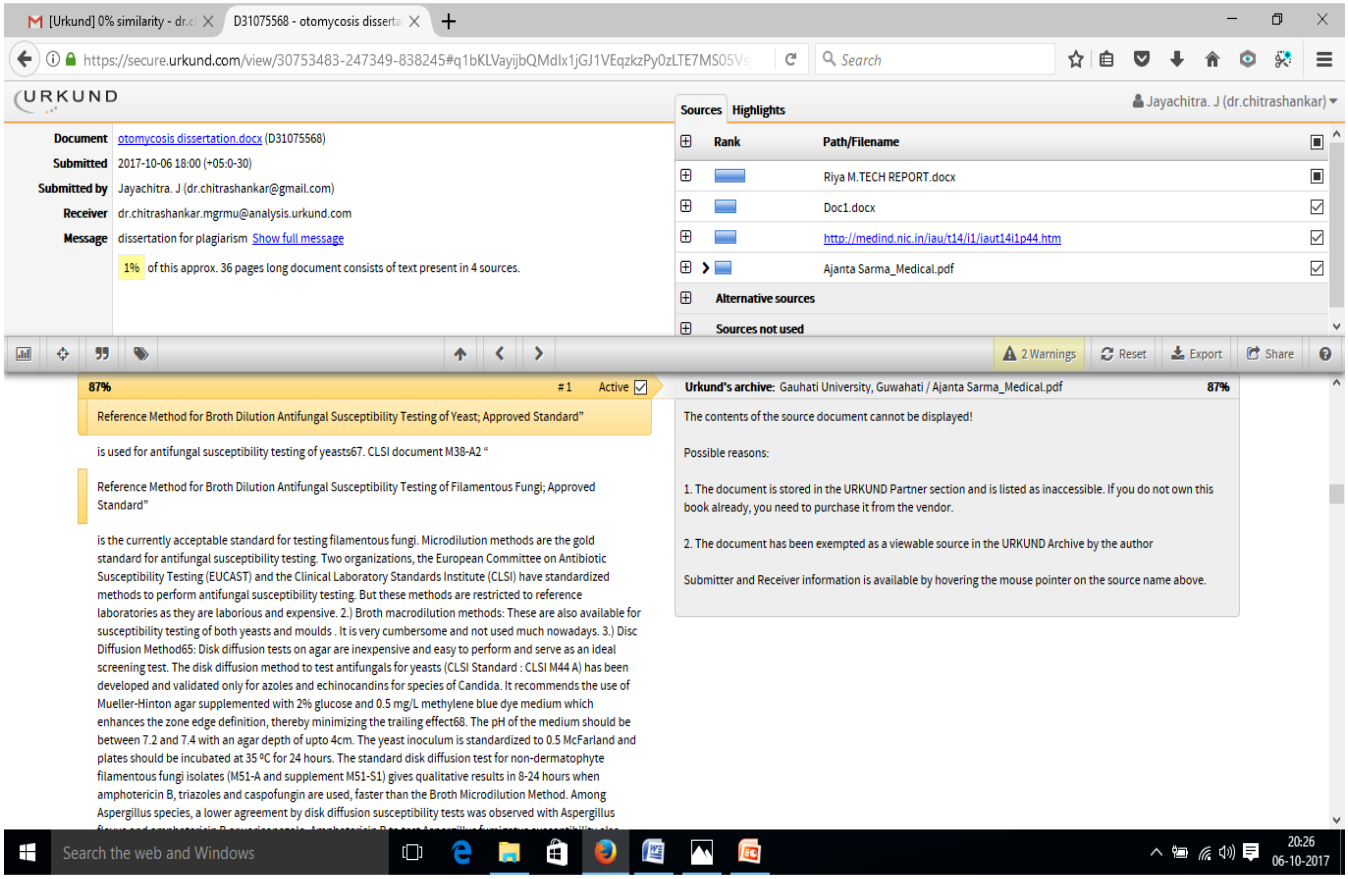
been an increased awareness about fungal infections and a high degree of clinical suspicion among doctors in diagnosing otomycosis. Removal of predisposing factors is the first step in treating otomycosis which can be achieved by proper counselling of the patients.

More in-depth studies on the various otomycosis causing fungi and their degree of sensitivity to currently available antifungal drugs will help the clinician in diagnosing and refining treatment of otomycosis.

CONCLUSION

- Otomycosis is a frequently encountered infection of the ear in our region.
- Habitual cleaning of ears with unsterile materials is the major predisposing factor and should be avoided.
- Injudicious use of antibiotic or steroid ear drops can lead to worsening as well as persistence of otomycosis.
- *Aspergillus* and *Candida* are the most common etiological agents of otomycosis.
- Fungi like *Cladosporium, Aureobasidium, Fonsaceae, Mucor, Rhizopus, Rhizomucor* and *Curvularia* are also isolated in otomycosis.
- Analysis of virulence factors showed biofilm formation in *Candida*.
- Production of Urease and Hemolysin were identified as virulence factors among *Aspergillus* species.
- On testing production of **Aflatoxins** among the *Aspergillus* species , *Aspergillus niger* species showed higher production of Aflatoxins B1 and G1 while *Aspergillus flavus* and *Aspergillus terreus* produced more amounts of Aflatoxins B2 and G2.
- **PCR** and **Gene sequencing** are reliable methods for identification and speciation of rare fungi.
- All the patients showed good response to treatment with topical clotrimoxazole drops or miconazole ointment .

- Otomycosis is a benign condition which responds well to topical antifungals.
- Diagnosis of otomycosis at the earliest, confirmation with various tests for virulence and identification of antifungal susceptibility pattern is mandatory to effectively manage otomycosis without complications or recurrence.


[Urkund] 0% similarity - dr.c
D31075568 - otomycosis disserta
https://secure.urkund.com/view/30753483-247349-838245#q1bKLVayijbQMdlx1jGJ1VEqzkzPy0zLTE7MS05Vs
Search
URKUND
Jayachitra. J (dr.chitrashankar)
Document otomycosis dissertation.docx (D31075568)
Submitted 2017-10-06 18:00 (+05:0-30)
Submitted by Jayachitra. J (dr.chitrashankar@gmail.com)
Receiver dr.chitrashankar.mgrmu@analysis.urkund.com
Message dissertation for plagiarism Show full message
1% of this approx. 36 pages long document consists of text present in 4 sources.
Sources
Highlights
Rank
Path/Filename
Riya M.TECH REPORT.docx
Doc1.docx
http://medind.nic.in/iau/t14/i1/iaut14i1p44.htm
Ajanta Sarma_Medical.pdf
Alternative sources
Sources not used
2 Warnings
Reset
Export
Share
87%
#1
Active
Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard"
is used for antifungal susceptibility testing of yeasts67. CLSI document M38-A2 "
Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard"
is the currently acceptable standard for testing filamentous fungi. Microdilution methods are the gold standard for antifungal susceptibility testing. Two organizations, the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI) have standardized methods to perform antifungal susceptibility testing. But these methods are restricted to reference laboratories as they are laborious and expensive. 2.) Broth macrodilution methods: These are also available for susceptibility testing of both yeasts and moulds . It is very cumbersome and not used much nowadays. 3.) Disc Diffusion Method65: Disk diffusion tests on agar are inexpensive and easy to perform and serve as an ideal screening test. The disk diffusion method to test antifungals for yeasts (CLSI Standard : CLSI M44 A) has been developed and validated only for azoles and echinocandins for species of Candida. It recommends the use of Mueller-Hinton agar supplemented with 2% glucose and 0.5 mg/L methylene blue dye medium which enhances the zone edge definition, thereby minimizing the trailing effect68. The pH of the medium should be between 7.2 and 7.4 with an agar depth of upto 4cm. The yeast inoculum is standardized to 0.5 McFarland and plates should be incubated at 35 ºC for 24 hours. The standard disk diffusion test for non-dermatophyte filamentous fungi isolates (M51-A and supplement M51-S1) gives qualitative results in 8-24 hours when amphotericin B, triazoles and caspofungin are used, faster than the Broth Microdilution Method. Among Aspergillus species, a lower agreement by disk diffusion susceptibility tests was observed with Aspergillus
Urkund's archive: Gauhati University, Guwahati / Ajanta Sarma_Medical.pdf
87%
The contents of the source document cannot be displayed!
Possible reasons:
1. The document is stored in the URKUND Partner section and is listed as inaccessible. If you do not own this book already, you need to purchase it from the vendor.
2. The document has been exempted as a viewable source in the URKUND Archive by the author
Submitter and Receiver information is available by hovering the mouse pointer on the source name above.
Search the web and Windows
20:26
06-10-2017

INSTITUTIONAL ETHICS COMMITTEE
GOVT.KILPAUK MEDICAL COLLEGE,
CHENNAI-10
Protocol ID. No. 10/2015 Dt: 22.12.2015
CERTIFICATE OF APPROVAL

The Institutional Ethical Committee of Govt. Kilpauk Medical College, Chennai reviewed and discussed the application for approval "Isolation, characterization and Antifungal sensitivity pattern of fungi causing otomycosis in patients reporting in a tertiary care hospital" - For Project Work submitted by Dr.J.Jayachitra, Post Graduate in MD (Micro), Govt. Kilpauk Medical College, Chennai.

The Proposal is APPROVED.

The Institutional Ethical Committee expects to be informed about the progress of the study any Adverse Drug Reaction Occurring in the Course of the study any change in the protocol and patient information /informed consent and asks to be provided a copy of the final report.

DEAN,
Govt.Kilpauk Medical College,
Chennai – 10.

PROFORMA

NAME :

AGE/SEX :

ADDRESS :

OCCUPATION :

OP /IP No :

LAB No :

SYMPTOMS :

1. **AURAL PRURITIS -**
2. **OTALGIA -**
3. **EAR DISCHARGE -**
4. **EAR BLOCK -**
5. **HARD OF HEARING -**
6. **TINNITUS -**

PREDISPOSING FACTORS/ COMORBID CONDITIONS:

a. **EXCESSIVE EXPOSURE TO WATER**
b. **USE OF EAR DROPS**
c. **USE OF OIL**
d. **TRAUMA**
e. **HABITUAL CLEANING OF EAR CANAL WITH BUDS, FINGER TIPS, SAFETY PINS, ETC.**
f. **DIABETES**
g. **CSOM**
h. **POST RADIOTHERAPY**
i. **IMMUNOCOMPROMISED**

CLINICAL DIAGNOSIS :

SAMPLE COLLECTED:

LAB DIAGNOSIS :

- **EXAMINATION UNDER MICROSCOPE:**
  1.GRAM's STAIN
  2.KOH MOUNT
- **CULTURE CHARACTERSTICS :** On Emmons modified Sabouraud dextrose agar,
- **LPCB STAIN:**
- **SPECIES IDENTIFICATION AND CHARACTERISATION :**
- **ANTIFUNGAL SUSCEPTIBILITY TESTING :**

SPECIES IDENTIFIED:

Signature of Investigator **Signature of the Guide**

PATIENT CONSENT FORM

ISOLATION, CHARACTERISATION AND ANTIFUNGAL SENSITIVITY PATTERN OF FUNGI CAUSING OTOMYCOSIS IN PATIENTS REPORTING IN A TERTIARY CARE HOSPITAL

STUDY CENTER : KILPAUK MEDICAL COLLEGE AND HOSPITAL, CHENNAI.
PATIENT NAME : **PATIENT AGE:**
IDENTIFICATION NUMBER:
PATIENT TO TICK ( )THESE BOXES

I confirm that I have understood the purpose of procedure for the above study.
I have the opportunity to ask the question and all my questions and doubts have been answered to my satisfaction. ( )

I understand that my participation in the study is voluntary and that I am free to withdraw at anytime without giving any reasons, without my legal rights being affected.
( )

I understand that the investigator, regulatory authorities and the ethics committee will not need my permission to look at my health records both in respect to the current study and any further research that may be conducted in relation to it, even if withdraw from the study, I understand that my identity will not be revealed in any information released to third parties or published, unless as required under the law. I agree not to restrict the use of any data or results that arise from the study.
( )

I agree to take part in the above study and to comply with the instructions given during the study and faithfully cooperative with the study team and to immediately inform the study staff if I suffer from any deterioration in my health or wellbeing or any unexpected or unusual symptoms.
( )

I hereby give consent to participate in this study.
I hereby give permission to undergo complete clinical examination and diagnostic test.

Signature/Thumb impression: Place: Date:

Patient name and address:

Signature of the investigator: Place: Date:

Study investigator's name:

சுய ஒப்புதல் படிவம்

ஆய்வு செய்யப்படும் தலைப்பு: **ISOLATION, CHARACTERISATION AND ANTIFUNGAL SENSITIVITY PATTERN OF FUNGI CAUSING OTOMYCOSIS IN PATIENTS REPORTING IN A TERTIARY CARE HOSPITAL**

ஆய்வு செய்யப்படும் இடம்: கீழ்பாக்கம் அரசு மருத்துவக்கல்லூரி மற்றும் மருத்துவமனை, சென்னை-10

பங்கு பெறுபவரின் பெயர்:
பங்கு பெறுபவரின் வயது: பங்குபெறுபவரின் எண் :

மேலே குறிப்பிட்டுள்ள மருத்துவ ஆய்வின் விவரங்கள் எனக்கு விளக்கபட்டுள்ளது. நான் இவ்வாய்வில் தன்னிச்சையாக பங்கேற்கின்றேன். எந்த காரணத்தினாலோ, எந்த சட்ட சிக்கலுக்கும் உட்படாமல் நான் இவ்வாய்வில் இருந்து விலகிக் கொள்ளலாம் என்றும் அறிந்துகொண்டேன்.

இந்த ஆய்வு சம்பந்தமாகவோ, இதை சார்ந்து மேலும் ஆய்வு மேற்கொள்ளும் போதும் இந்த ஆய்வில் பங்குபெறும் மருத்துவர் என்னுடைய மருத்துவர் என்னுடைய மருத்துவ அறிக்கைகளை பார்ப்பதற்கு என் அனுமதி தேவை இல்லை என அறிந்து கொள்கிறேன். இந்த ஆய்வின் மூலம் கிடைக்கும் தகவலையோ,முடிவையோ பயன்படுத்திக் கொள்ள மறுக்கமாட்டேன்.

இந்த ஆய்வில் பங்கு கொள்ள ஒப்புக் கொள்கிறேன். இந்த ஆய்வை மேற்கொள்ளும் மருத்துவ அணிக்கு உண்மையுடன் இருப்பேன் என்று உறுதியளிக்கிறேன்.

பங்கேற்பவரின் கையொப்பம்: சாட்சியாளரின் கையொப்பம்

இடம் : இடம் :
தேதி : தேதி :
பங்கேற்பவரின் பெயர் மற்றும் விலாசம்:

ஆய்வாளரின் கையொப்பம்:
இடம் :
தேதி :

APPENDIX

POTASSIUM HYDROXIDE MOUNTS (10% KOH):

The aqueous Potassium hydroxide (KOH) acts as a clearing agent, dissolving the cellular debris without affecting the fibrillar glucan and chitin structure of the fungal cell wall.

Composition:

Potassium hydroxide	10gm
Glycerol	20ml
Distilled Water	80ml

- To solution of 10% KOH, 10% glycerol is added to prevent drying.
- Mix these ingredients properly.
- Store solution at room temperature.

LACTOPHENOL COTTON BLUE STAIN

The Lactophenol Cotton Blue (LPCB) mount is used to study morphological features of fungal isolates.

Composition:

Melted Phenol	: 20ml
Lactic Acid	: 20ml
Glycerol	: 40ml
Cotton Blue	: 0.05gm
Distilled Water	: 20ml

- Dissolve 0.05gm of Cotton Blue stain in distilled water before mixing with remaining reagents.
- Mix all the reagents thoroughly.
- Store at room temperature.
- The phenol acts as disinfectant, lactic acid preserves morphology of fungi and glycerol is hygroscopic agent which prevents drying. The Cotton Blue stains outer wall of fungus.

SABOURAUD'S DEXTROSE AGAR (SDA):

Composition:

Dextrose	: 40g
Peptone	: 10g
Agar	: 20g
Distilled water	: 1 litre

- Mix reagents by boiling
- Dispense in tubes
- Autoclave at 121 ºC for 15 minutes
- Adjust pH of 5.5 to 5.6
- Add Gentamicin 50 µg/ml or Chloramphenicol 50 µg/ml and mix well
- Allow tubes to cool at slanted position
- Store at 4 ºC
- Shelf life: 30 days for tubes and 14 days for petridishes.

EMMON'S MODIFICATION OF SABOURAUD'S DEXTROSE AGAR:

Composition:

Dextrose	: 20g
Peptone	: 10g
Agar	: 20g
Distilled water	: 1 litre

- Mix reagents by boiling
- Dispense in tubes
- Autoclave at 121 ºC for 15 minutes
- Adjust pH to 6.9±0.2
- Add Gentamicin 50 μg/ml or Chloramphenicol 50 μg/ml and mix well
- Allow tubes to cool in slanted position
- Store at 4 ºC
- Shelf life: 30 days for tubes and 14 days for petridishes.

POTATO DEXTROSE AGAR (PDA):

Composition:

Potatoes	: 250g
Dextrose	: 20g
Agar	: 20g
Distilled water	: 1000ml

- Peel, slice and boil potatoes in 100ml distilled water for 1 hour
- Filter with gauze
- Add dextrose and agar.
- Add distilled water to make final volume upto 1000ml.
- Bring to boil to dissolve agar.
- Autoclave at 121 ºC for 15 minutes.

CORN MEAL AGAR:

Composition:

Corn meal	: 40g
Distilled water	: 1litre
Agar	: 20g

- Heat Corn meal in a water bath at 60ºC for one hour. Filter through gauze.
- Make up filtrate to 1000 ml with distilled water.
- Add 2% agar
- Autoclave at 121 ºC for 15 minutes.
- Dispense in sterile petridishes.
- Shelf life: 14 days

CHROMAGAR- CANDIDA DIFFERENTIAL AGAR:

Available as a readymade powder with Himedia. Used for rapid isolation, identification and speciation of *Candida* species.

Composition:

Peptone	: 15 g/litre
Yeast extract	: 4 g/litre
Dipotassium hydrogen phosphate	: 1 g/litre
Chromogenic mixture	: 7.22 g/litre
Chloramphenicol	: 0.5g /litre
Agar	: 15 g/litre
Distilled water	: 1000 ml

- Suspend 42.72 gms in 1000ml of distilled water.

- Heat to boiling to dissolve the medium completely.
- Do not autoclave.
- Cool to 45 – 50 ºC
- Adjust pH to 6.3±0.2
- Mix well and pour into sterile petridishes.

YEAST NITROGEN BASE:

Composition:

PART A:

Noble agar : 25.0g

Distilled water : 1 litre

PART B:

Yeast Nitrogen Base : 6.7g/100 ml distilled water

- Suspend40 gms in 900 ml distilled water.
- Heat to boiling to dissolve the medium completely.
- Autoclave at 121 ºC for 15 minutes.
- Cool to 50 ºC and aseptically mix sterile Part B solution inoculated with the yeast colonies.
- Add 3 ml of sterile 5% Tartaric acid for 100 ml of the mixture just before pouring the plate.

MCFARLAND TURBIDITY STANDARD FOR INOCULUM PREPARATION

A Barium sulphate 0.5 McFarland standard was prepared as follows

1. A 0.5 ml of 0.048mol/L of Barium chloride was added to 99.5 ml of 0.18 mol/L of H_2SO_4 with constant stirring to maintain a suspension.

2. Correct density of the turbidity standard was verified by using a spectrophotometer. The absorbance of 625nm should be 0.08 to 0.10 for the 0.5 McFarland standards.

3. The Barium sulphate suspension was transferred in 4-6 ml to a screw capped tube of the same size as those used in growing or diluting the bacterial inoculum.

4. These tubes were tightly sealed and stored in the dark at room temperature.

5. The Barium sulphate turbidity standard was vigorously agitated before each use and inspected for a uniform turbid appearance

Candida Speciation On ChromAgar And Corn Meal Agar:

SPECIES	CHROMAGAR	CORN MEAL AGAR
Candida albicans	Apple green colonies	Abundant pseudohyphae. Budding cells, spherical chlamydospores mostly terminal often on a swollen subtending cell.
Candida tropicalis	Metallic blue to purple infused into surrounding agar with pale pink edges	Abundant pseudohyphae with blastoconidia. Budding cells ellipsoidal. Long poorly branched elements often narrowed towards a sterile apex. Conidia arranged in small groups around middle of each cellular element.
Candida parapsilosis	White to pale pink	Clusters of blastospores with occasional giant cells.
Candida krusei	Large, flat, spreading, pale pink colonies with matt surface	Branched pseudomycellium. Budding cells ellipsoidal to cylindrical. Cells liberated and arranged parallel to the main axis.
Candida glabrata	White, large, glossy, pale pink to violet	Pseudohyphae not present. Budding unipolar cells ellipsoidal, typically arranged in dense groups. Chlamydospore absent.
Candida guillermondii	Small, pink to purple colonies	Pseudohyphae with clusters of blastospores radiating from the centre of masses of spherical to broadly ellipsoidal budding cells.
Candida lusitaniae	Pink, gray purple colonies	Branched pseudohyphae present.
Candida famata	White to light pink colonies	Pseudohyphae absent.
Candida haemuloni		Pseudohyphae absent. Spherical budding cells.
Candida auris		Pseudohyphae absent. Ovoid to ellipsoidal budding cells.

SPECIATION OF *CANDIDA* BY SUGAR ASSIMILATION TEST

SUGAR ASSIMILATION	Candida albicans	Candida tropicalis	Candida dubliniensis	Candida glabrata	Candida guillermondii	Candida krusei	Candida parapsilosis	Candida haemulonii
Galactose	+	+	+	-	+	-	+	V
Sucrose	V	V	+	-	+	-	+	+
Maltose	+	+	+	-	+	-	+	+
Cellobiose	-	+	-	-	+	-	-	-
Trehalose	+	+	+	-	+	-	+	+
Lactose	-	-	-	V	+	-	-	-
Melibiose	-	-	V	-	-	-	-	-

SPECIATION OF *CANDIDA* BY SUGAR FERMENTATION TEST:

SUGAR FERMENTATION	Candida albicans	Candida tropicalis	Candida dubliniensis	Candida glabrata	Candida guillermondii	Candida krusei	Candida parapsilosis	Candida haemulonii
Glucose	+	+	+	+	+	+	+	+
Galactose	V	+	V	-	V	-	V	-
Sucrose	-	V	-	-	+	-	-	+
Maltose	+	+	+	-	-	-	-	-
Raffinose	-	-	-	-	+	-	-	-
Trehalose	V	+	V	V	+	-	-	+

ANTIFUNGAL SUSCEPTIBILITY TEST BY MICROBROTH DILUTION METHOD

Scheme for Preparing Dilution of Water Insoluble Antifungal Agents:

Antimicrobial Solution						
Step	**Concentration (µg/ml)**	**Source**	**Volume (ml)**	**Solvent (ml)**	**Intermediate Concentration (µg/ml)**	**Final Concentration At 1:50 (µg/ml)**
1	1600	Stock	0.5		1600	32
2	1600	Stock	0.5	0.5	800	16
3	1600	Stock	0.5	1.5	400	8
4	1600	Stock	0.5	3.5	200	4
5	200	Step 4	0.5	0.5	100	2
6	200	Step 4	0.5	1.5	50	1
7	200	Step 4	0.5	3.5	25	0.5
8	25	Step 7	0.5	0.5	12.5	0.25
9	25	Step 7	0.5	1.5	6.25	0.125
10	25	Step 7	0.5	3.5	3.13	0.0625

Scheme for Preparing Dilution of Water Soluble Antifungal Agents:

Antimicrobial Solution						
Step	**Concentration (µg/ml)**	**Source**	**Volume (ml)**	**Solvent (ml)**	**Intermediate Concentration (µg/ml)**	**Final Concentration At 1:50 (µg/ml)**
1	5120	Stock	1	7	640	128
2	640	Step1	1	1	320	64
3	640	Step1	1	3	160	32
4	160	Step3	1	1	80	16
5	160	Step3	0.5	1.5	40	8
6	160	Step3	0.5	3.5	20	4
7	20	Step6	1	1	10	2
8	20	Step6	0.5	1.5	5	1
9	20	Step6	0.5	3.5	2.5	0.5
10	2.5	Step9	1	1	1.25	0.25

ABBREVIATION

BLAST	-	Basic Local Alignment Search Tool
CLSI	-	Clinical Laboratory Standards Institute
CSOM	-	Chronic Suppurative Otitis Media
DNA	-	Deoxyribonucleic acid
ELISA	-	Enzyme Linked Immunosorbent Assay
E- TEST	-	Epsilometer Test
EUCAST	-	European Committee on Antibiotic Susceptibility Testing
GTT	-	Germ Tube Test
HPLC	-	High Performance Liquid Chromatography
IMC	-	Isothermal Micro Calorimetry
ITS	-	Internal Transcribed Spacer
KOH	-	Potassium hydroxide
LPCB	-	Lactophenol Cotton Blue
MALDI –TOF	-	Matrix Assisted Laser Desorption/Ionisation – Time of Flight.
MEC	-	Minimal Effective Concentration
MIC	-	Minimum Inhibition Concentration
NCBI	-	National Center for Biotechnology Information

OD	-	Optical density
OPD	-	Out Patient Department
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction
PDA	-	Potato Dextrose Agar
rDNA	-	ribosomal DNA
RPMI-1640	-	Roswell Park Memorial Institute-1640 medium
SDA	-	Sabouraud Dextrose Agar
SSPS	-	Statistical Package for the Social Sciences

KEY TO MASTER CHART

M	-	Male
F	-	Female
1	-	Aural Pruritis
2	-	Otalgia
3	-	Ear Discharge
4	-	Ear Block/ Feeling of Fullness
5	-	Diminished Hearing
6	-	Tinnitus
a	-	Swimming
b	-	Use of Antibiotics/ Steroid Ear Drops
c	-	Oil Instillation in Ears
d	-	Trauma/ Previous Ear Surgery
e	-	Habitual Cleaning of Ear with Unsterile materials
f	-	Diabetes melitus
g	-	H/o CSOM
POS	-	Positive
NEG	-	Negative

CHROM	-	Colour on CHROM agar
A	-	Apple green colonies
B	-	White,pale pink to violet
C	-	White to pale pink
D	-	Large, flat, spreading, pale pink colonies with matt surface
CMA	-	Morphology on Corn Meal Agar
7	-	Abundant pseudohyphae. Budding cells, terminal spherical chlamydospores
8	-	Pseudohyphae absent. Budding unipolar. Cells ellipsoidal
9	-	Clusters of blastospores
10	-	Branched pseudomycellium. Budding cells ellipsoidal and parallel to the main axis
P	-	Produced
A.niger	-	*Aspergillus niger*
A.flavus	-	*Aspergillus flavus*
A.fumigatus	-	*Aspergillus fumigatus*
A.terreus	-	*Aspergillus terreus*
A.nidulans	-	*Aspergillus nidulans*
C.albicans	-	*Candida albicans*
C.parapsilosis	-	*Candida parapsilosis*
C.glabrata	-	*Candida glabrata*
C.krusei	-	*Candida krusei*

NG	-	No Growth
NP	-	Not Produced
H	-	Hydrolysed
NH	-	Not Hydrolysed
NA	-	Not Applicable
HE	-	Hemolytic
NHE	-	Non Hemolytic
CLO	-	Clotrimoxazole
ITR	-	Itraconazole
VOR	-	Voriconazole
FLU	-	Fluconazole
AMP	-	Amphotericin B
S	-	Susceptible
R	-	Resistant

BIBLIOGRAPHY

1. C. J. Alexopoulos, C.W. Mims, M. Blackwell Introductory Mycology. 4th Edition.
2. Jagdish Chander, Textbook of Medical Mycology. 4th Edition. Chapter 30: pg 669-81.
3. Priti Agarwal, Leimapopam Sumitra Devi. Otomycosis in a rural community attending a tertiary care hospital: Assessment of risk factors and identification of Fungal and bacterial Agents. *J Clin Diagn Res*; Jun 2017; 11(6): 14-8
4. Scott Brown's Otorhinolaryngology, Head and Neck Surgery. 7th Edition. Pg:213- 4
5. Topley & Wilson's Medical Mycology 10th Edition. Part I, Part II, Part VI.
6. Logan Turner's Diseases of the nose , ear and throat, head and neck surgery. 11th Edition
7. PL Dhingra. Diseases of Ear, Nose and Throat & Head and Neck Surgery. 6th Edition.
8. C Rodrigues, V C Ohri, D Raghunath. Antifungal Susceptibility testing in Otomycosis. *Indian J Med Microbiol.* 1998; 6 (4): 337-41
9. Jonathan Cohen, William G Powderly. Infectious Diseases. 11nd Edition
10. Vishwanatha Borlingegowda, Sumantha, Dadarao, Vijayashree, Maliyappanahalli Siddappa. Otomycosis in immunocompetent and

immunocompromised patients: Comparative study and literature review. *Ear, Nose and Throat J*. 2012; 91(3):114-21

11. Shilpa.K.Gokale, Shashidhar.S.Suligavi, Mahesh Baragundi, Anushka D, Manjula R. Otomycosis : A Clinico Mycological Study. *Int J Med Health Sci*. 2013;2(2): 218 -23
12. Sampath Chandra Prasad, Nikhil Dinaker Thada, Prashanth Prabhu, Tina D' Souza, and Kishore Chandra Prasad. Primary Otomycosis in the Indian Subcontinent: Predisposing Factors, Microbiology, and Classification. *Int J of Microbiol* ; 2014 (2014): 1-9
13. J. Fasunla, T. Ibekwe, and P. Onakoya. Otomycosis in western Nigeria. *Mycoses* . 2008; 51(1) 67–70
14. Than KM, Naing KS and Min M (1980): Otomycosis in Burma, and its treatment. *Am J Trop Med Hyg* ; 29: 620-3.
15. Mugliston T and O'Donoghue G (1985): Otomycosis: A continuing problem. *J Laryngol Otol* ;99: 327-33.
16. Juyal D, Negi V, Sharma M, Adekhandi S, Prakash R, Sharma N. Significance of fungal flora in chronic suppurative otitis media. *Annals of Tropical Medicine & Public Health.* 2014;7:120-3
17. Jayant Deshmukh, Rajendra Surpam, Asmita Band. Mycological Study of *Aspergillus* infections in Otomycosis in Easter part of Maharashtra. *Int J Health Sci Res* 2014; 4(10): 77-82
18. Elias J Anaissie, Michael R. McGinnis, Michael A. Pfaller Clinical Mycology. 2nd Edition, Chapter 2, 7,11

19. Chester W Emmons, Chapman H Binford, John P. Utz. Medical Mycology. 2nd Edition.
20. Sumit Gupta, Rohit Singh, Kranthi Kosaraju, Indira Bairy, Balakrishnan Ramaswamy. A study of antibacterial and antifungal properties of human cerumen. *Ind J of Otology* .2012 ; 18 (4): 189-92
21. Rudolf Probst, Gerhard Grevers, Heinrich Iro. Basic Otorhinolaryngology. 2nd Edition.
22. A.M.Moharram, H E Ahmed and Salma Nasr. Otomycosis in Assiut, Egypt. J Basic Appl Mycol; (2013); 4: 1-11
23. Raksha, Gurjeet Singh, A D Urhekar. Virulence Factors Detection in *Aspergillus* Isolates from Clinical and Environmental Samples. *J Clin Diagn Res*;2017; 11(7) : 13-8
24. Aufauvre-Brown A, Brown J S and Holden D W (1998), Comparison of virulence between clinical and environmental isolates of *Aspergillus fumigatus. Eur. J. Clin. Microbiol. Infect. Dis.;*17: 778-80.
25. Kupfahl C, Michalka A, Lass-Florl C, Fischer G, Haase G, Ruppert T, Geginat G and Hof H. Gliotoxin production by clinical and environmental *Aspergillus fumigatus* strains. *Int J Med Microbiol*; 298: 319-27.
26. Lisker, J., Michaeli, R., Frank, Z.R., 1993. Mycotoxigenic potential of *Aspergillus flavus* strains isolated from groundnuts growing in Israel. *Mycopathologia* ; 122: 177–83.

27. Miguel, A.M.R., Guillermo, S.F., 1986. Aflatoxin-producing potential of *Aspergillus flavus* strains isolated from Spanish poultry feeds. *Mycopathologia;* 95: 129–32

28. K. Kamei & A. Watanabe. *Aspergillus* mycotoxins and their effect on the host. *Medical Mycology*; 2005; 43: S95-9

29. *Boonen Jente, Malysheva Svetlana V, Taevernier Lien, Diana Di Mavungu José, De Saeger Sarah, De Spiegeleer Bart (2012). Human skin penetration of selected model mycotoxins. Toxicology; 301: 21–32*

30. Rastogi S, Dogra RK, Khanna SK, Das M, Skin tumorigenic potential of aflatoxin B1 in mice. *Food Chem Toxicol.* 2006 ;44(5):670-7.

31. Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases. 7th Edition. Volume II, Chapter 258: Pg 3241-7

32. A Tuzcu, M Bahceci, M K Celen, N Kilinc, S Ozmen. Necrotizing Malignant Otitis Externa: An Unusual Localization of Mucormycosis. *Indian J Med Microbiol.* 2006; 24 (4):289-91

33. Borlingegowda Viswanatha and Khaja Naseeruddin. Fungal Infections of the Ear in Immunocompromised Host: a Review. *Mediterr J Hematol Infect Dis*. 2011; 3

34. Haruna S, Haruna Y, Schachern PA, Morizono T, Paparella MM. Histopathogy update: Otomycosis. *Am J Otolaryngol*. 1994; 15:74 -8.

35. Mathew J. Carfrae, MD, Bradley W. Kesser, MD. Malignant Otitis Externa. *Otolaryngol Clin N Am* .2008; 41 : 537–49

36. Mathur, Maheshwar Dayal. Otomycosis: a clinicomycological study. *Ear, Nose and Throat J;* Aug 2000

37. Rama Kumar K. Silent perforation of tympanic membrane and otomycosis. *Indian J Otolaryngol Head Neck Surg* 1984;36(4);161-2.

38. Ozkan M, Ozcan KM, Karaarslan A. Concomitamt Otomycosis and Dermatomycosis: a Clinical and Microbiological Study. *Eur Arch Otorhinology.* 2003; 260: 24-7

39. Vadisha Srinivas Bhat, Shubha P Bhat, Harish Rao. External Ear infections in diabetics – Challenges in Management. *Otolaryngology J.* 2015; 5 (4)

40. Prakash S.B, Leelatejaswini R, Deekshita V. A clinical and microbial study of Otomycosis : An original study. *J of Evolution of Med Dent Sci.* 2015; 4 (71):12376-84

41. Arun Ghosh, Amit Rana, Surendra Prasad. Prevalence of Fungal Infection in Chronic Suppurative Otitis Media – A Study at Tertiary Care Hospital in Western Uttar Pradesh. *Indian J Microbiol Res.* 2015;2(3):159-62

42. Harrison's Principles of Internal Medicine 19th edition.

43. Gyongyi Szigeti, Janos Varga, Sandor Kocsube. Species assignment and Antifungal susceptibilities of black *Aspergilli* recovered from otomycosis cases in Iran. *Mycoses* ; 2012; 55(4): 333-8

44. Vrutika V. Gandhi, P. Neelu Sree, M Kalyani. A Study on Etiological Agents with Special Reference to Fungal Isolates causing Chronic Suppurative Otitis Media in a Tertiary Care Hospital. *Int.J.Curr.Microbiol.App.Sci*. 2016;5(11): 508-14

45. Rajeshwari Prabhakar Rao, Rishmitha Rao. A Mycologic Study of Otomycosis in a Tertiary Care Teaching Hospital in Karnataka, India. *International Journal of Clinical Medicine Research;* 2016; 3 (7) : 1918-20
46. Alexis Jackman, Robert Ward, Max April, John Bent. Topical antibiotic induced otomycosis. *Int J Ped Otorhinolaryngol*; 2008; 72(4): 453-59
47. Rachna Dhingra, Shamim Monga , Mandeep Kaur. Role of fungal infections in CSOM: Prospective study. *Ind J Basic Appl Med Res;* 2014;3(2) :598-608
48. Ali Zarei Mahmoudabadi, Saied Masoomi. Clinical and Mmycological Studies of Otomycosis. *Pak J Med Sci* . 2010; 26(1) : 187-90
49. Metwally Abdelazeem, Ahmed Gamea, Hanan Mubarak, Nessma Elzawawy. Epidemiology, causative agents, and risk factors affecting human otomycosis infections. *Turk J Med Sci*. 2015; 45: 820-6
50. Jyothi Swarup R. Wasim Ahmed Shah, Mohan M. Otomycosis- Mycological Spectrum Aetio- Pathological Factors and Management. *J Evol Med Dent Sci.* 2014; 3 (18): 4850-8.
51. R.B.Chapparbandi, Farha Naaz Kazi, Kazim Ali. Otomycosis: An Overview in Hyderabad Karnataka region. *Sci* .2014;l 3(47): 11213-5
52. Channabasawaraj, Nandyal, Archana S Choudhari. Evaluation of Topical Clotrimazole and Topical Miconazole in the Treatment of Otomycosis- A Prospective Study. *Naional Journal of Medical Research*. 2015; 5(2):14550
53. Errol Reiss, H. Jean Shadomy, G. Marshall Lyon Fundamental Medical Mycology. Chapter 2, 14

54. Hossein Nowrozi, Fatemeh Doustdar Arabi, Hamid Ghaffarnejad Mehraban, Ahmad Tavakoli. Mycological and clinical study of Otomycosis in Tehran, Iran. *Bull Environ Pharmacol Life Sci*; 2014; 3(2) : 29-31

55. Fran Fisher, Norma B. Cook Fundamentals of Diagnostic Mycology "Some opportunistic fungi and yeasts and yeast-like fungi," pg. 35–225.

56. Patrick R Murray, Ellen Jo Baron, James H. Jorgensen, Marie Louise Landry, Michael A.Pfaller. Manual of Clinical Microbiology 9th edition. Chapter 121 Pg:1802-18

57. AJ Kindo, A Tupaki‐Sreepurna, M Yuvaraj. Banana peel culture as an indigenous medium for easy identification of late‐sporulation human fungal pathogens. *Indian J Med Microbiol.*2016; 34(4): 457-61

58. Vijaya D, Harsha T.R, Nagaratnamma T. *Candida* Speciation Using Chrom Agar. *J Clin Diagn Res;* 2011; 5(4): 755-7

59. Laura H. Hogan, Bruce S. Klein, Stuart M. Levitz. Virulence Factors Of Medically Important Fungi *Clinical Microbiology Reviews*.1996;9(4): 469–88

60. Arturo Casadevall. Determinants of virulence in the pathogenic fungi. *Fungal Biol Rev*. 2007; 21(4): 130–2.

61. Hassan Yazdanpanaha, Afshin Zarghia, Ali Reza Shafaatia. Analysis of Aflatoxin B1 in Iranian Foods Using HPLC and a Monolithic Column and Estimation of its Dietary Intake. *Iran J Pharm Res;*2013; 12(S): 83-9.

62. Elena Traistaru, Raul Ciprian Moldovan. Comparative Study Regarding the Application of HPLC and ELISA Methods for Aflatoxins Detection in Peanuts. *J of Agroalimentary Processes Technologies.*2012;18(1): 81-5

63. Evaluation of Glucose-Methylene-Blue-Mueller-Hinton Agar for E-Test Minimum Inhibitory Concentration Determination in *Candida* spp. *Indian J Med Microbiol.*2007; 25(4)

64. Vivek Agwan, Ruchika Butola, Molly Madan. Agwan, *et al.*: Biofilm formation in clinical isolates of *Candida* species. *Indian J Pathol Microbiol* 2015;58(4): 475-8

65. Channabasawaraj B Nandyal, Archana S Choudhari and Netravati B Sajjan. A Cross sectional study for Clinico mycological Profile of Otomycosis in North Karnataka. *Int J Med Health Sci*. 2015;4(1): 64-9

66. National Committee for Clinical Laboratory Standards (2002) Reference method for antifungal disc diffusion susceptibility testing of Yeasts. Approved standard M44A. Wayne (Pennsylvania): National Committee for Clinical Laboratory Standards. Volume 24; Number 15.

67. National Committee for Clinical Laboratory Standards (2002) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard M38A2. Wayne (Pennsylvania): National Committee for Clinical Laboratory Standards Volume 28; Number 16

68. A. Espinel-Ingroff, A. Fothergill, M. Ghannoum, E. Manavathu, L. Ostrosky-Zeichner, M. Pfaller. Quality Control and Reference Guidelines for CLSI Broth Microdilution Susceptibility Method (M38-A Document) for

Amphotericin B, Itraconazole, Posaconazole, and Voriconazole. *J. Clin. Microbiol*.; 2005; 43(10): 5243–6

69. Kumar H, Seth S. Bacterial and Fungal study of 100 cases of CSOM. *J Clin Diagn Res*; 2011; 5 :1224-7
70. A. Espinel-Ingroff, B. Arthington-Skaggs, N. Iqbal, D. Ellis, M. A. Pfaller. Multicenter Evaluation of a New Disk Agar Diffusion Method For Susceptibility Testing Of Filamentous Fungi With Voriconazole, Posaconazole, Itraconazole, Amphotericin B, And Caspofungin. *J. Clin. Microbiol*. June 2007; 45(6) : 1811–20
71. Brunella Posteraro, Riccardo Torelli, Elena De Carolis, Patrizia Posteraro and Maurizio Sanguinetti. Antifungal Susceptibility Testing: Current Role from the Clinical Laboratory Perspective. *Mediterr J Hematol Infect Dis* 2014; 6.
72. Alastruey-Izquierdo, A.; Melhem, M.S.C.; Bonfietti, L.X. & Rodriguez-Tudela, J.L.–Susceptibility test for fungi clinical and laboratorial correlations in medical mycology. *Rev. Inst. Med. Trop.* Sao Paulo; 57(S19): 57-64.
73. Jesu´s Guinea,Sandra Recio, Pilar Escribano, Marta Torres-Narbona. Rapid Antifungal Susceptibility Determination for Yeast Isolates by Use of Etest Performed Directly on Blood Samples from Patients with Fungemia. *J. Clin. Microbiol*. 2010; 48(6): 2205–12

74. Jo-Anne Van Burik, Randall W. Schreckhise. Panfungal PCR Assay for Detection of Fungal Infection in Human Blood Specimens. *J. Clin. Microbiol*. 1998; 36(5): 1169-75

75. Cornelia Lass-Flörl, Wolfgang Mutschlechner, Maria Aigner. Utility of PCR in Diagnosis of Invasive Fungal Infections: Real-Life Data from a Multicenter Study . *J. Clin. Microbiol.* 2013; 51(3): 863-8.

76. Dr K. Lily Therese and Dr H.N. Madhavan. Optimization and Application of Molecular Techniques for Rapid Detection of Fungal Genome from Ocular Specimens: Our Experience in a Tertiary Care Centre in India. *Sci J Med & Vis Res Foun*; 2016 ; 34(2) :57-62

77. Anna M. Romanelli, Deanna A. Sutton, Elizabeth H. Thompson. Sequence-Based Identification of Filamentous Basidiomycetous Fungi from Clinical Specimens: a Cautionary Note. *J. Clin. Microbiol*. 2010; 48(3): 741-52

78. Jan W. M. van der Linden, Eveline Snelders, Jan P. Arends. Rapid Diagnosis of Azole- Resistant Aspergillosis by Direct PCR Using Tissue Specimens. *J. Clin. Microbiol*. Apr 2010; 48(4): 1478-80

79. Diana E. Ciardo, Katja Lucke, Alex Imhof, Guido V. Bloemberg, and Erik C. Bottger. Systematic Internal Transcribed Spacer Sequence Analysis for Identification of Clinical Mold Isolates in Diagnostic Mycology: a 5-Year Study. *J. Clin. Microbiol*.; 2010; 48(8): 2809–13

80. K. O. Paulose , S. Al Khalifa , P. Shenoy, R. K. Sharma. Mycotic infection of the ear (otomycosis): A prospective study. *J Laryngol Otol*; Jan 1989; 103 (1): 30-5

81. Aju Ravindran, Sagesh M. Effectiveness of oral itraconazole in the management of otomycosis with tympanic membrane perforation. *Int J Res Med Sci.* 2017 ;5(4):1373-6

82. Chander J Maini S, Subramanyan S. Otomycosis- a clinico-mycological study and efficacy of mercurochrome in its treatment.*Mycopathologica.*1996;135:9-12

83. Tang Ho, MD, MSc, Jeffrey T. Vrabec, MD, Donald Yoo, BS,Otomycosis: Clinical features and treatment Implications. *Otolaryngology–Head and Neck Surgery* .2006; 135: 787-91

84. Nagendran Navaneethan, Raj Prakash , Yaadhava Krishnan. Type of Antifungals: Does it matter in empirical treatment of otomycosis. *Indian J otolaryngol Head & Neck Surgery.* 2015; 67 (1) :64-7

85. Sofia Perea and Thomas F. Patterson. Antifungal Resistance in Pathogenic Fungi. *Clinical Infectious Diseases;* 2002; 35:1073–80.

86. AD Kaya et al. In Vitro Susceptibilities of *Aspergillus* Spp. Causing Otomycosis to Amphotericin B, Voriconazole and Itraconazole. *Mycoses;* 50 (6), 447-50

87. M. Pfaller, L. Boyken, R. Hollis, J. Kroeger, S. Messer, S. Tendolkar, and D. Diekema . Use of Epidemiological Cutoff Values to Examine 9-Year Trends in Susceptibility of *Aspergillus* Species to the Triazoles. *J. Clin. Microbiol*. 2011; 49(2): 586–90

88. Dannaoui E, Borel E, Monier MF, Piens MA, Picot S, et al. (2001) Acquired itraconazole resistance in *Aspergillus fumigatus*. *J Antimicrob Chemother* 47: 333–40

89. Verweij PE, Mellado E, Melchers WJ (2007) Multiple-triazole-resistant aspergillosis. *N Engl J Med* 356: 1481–3.

90. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, et al. (2007) A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of cyp51A alterations. *Antimicrob Agents Chemother* 51: 1897–904.

91. N Pahwa, R Kumar, S Nirkhiwale, A Bandi. Species distribution and drug susceptibility of *Candida* in clinical isolates from a tertiary care centre at Indore. *Indian J Med Microbiol.* 2011; 29(3): 309-11.

92. Standard Operating Procedures for identification of yeasts released by the Indian Council of Medical Research

93. S Agarwal, V Manchanda, N Verma, P Bhalla. Yeast Identification In Routine Clinical Microbiology Laboratory And Its Clinical Relevance. *Indian J Med Microbiol.* 2011; 29(2): 172-7

94. Jabra-Rizk, M. A., T. M. Brenner, M. Romagnoli, A. A. M. A. Baqui, W. G. Merz, W. A. Falkler, Jr., and T. F. Meiller. 2001. Evaluation of reformulated CHROMagar *Candida*. *J. Clin. Microbiol*.. 39:2015–6.

95. Powell, H. L., C. A. Sand, and R. P. Rennie. 1998. Evaluation of CHROMagar *Candida* for presumptive identification of clinically important Candida species. *Diagn. Microbiol. Infect. Dis.* 32:201–4.

96. Pfaller MA, Houston A, Coffmann S. Application of CHROMagar Candida for rapid screening of clinical specimens for *Candida albicans, Candida tropicalis, Candida krusei,* and *Candida (Torulopsis) glabrata*. *J. Clin. Microbiol*. 1996;34:58-61

97. B. Madhumati and R. Rajendran. Evaluation of Chrom Agar in Speciation of *Candida* Species from Various Clinical Samples in a Tertiary Care Hospital. *Int.J.Curr.Microbiol.App.Sci* .2015; 4(9): 463-72

98. P. Lewis White, Carlo Mengoli, Stephane Bretagne. Evaluation of *Aspergillus* PCR Protocols for Testing Serum Specimens. *J. Clin. Microbiol*. 2011; 49(11): 3842-8

99. RS Iyer, RB Pal, RY Patel, DD Banker. Polymerase Chain Reaction Based Diagnosis Of Systemic Fungal Infections And Sensitivity Testing Of The Fungal Isolates. *Indian J Med Microbiol.* 2002; 20 (3):132-6

100. Dr. Pankti Panchal, Dr. Jayshree Pethani, Dr. Dipti Patel, Dr. Sanjay Rathod, Dr. Parul Shah. Analysis of various fungal agents in clinically suspected cases of Otomycosis. *Ind J Basic Appl Med Res*; 2013;2 (8):865-9

101. Selvam Saranya, Kannaiyan Moorthy, Siva Sakthivel, Arul Sheeba Malar. Prevalence and Antifungal Susceptibility pattern of *Candida albicans* from low socio-economic group. *Int J Pharm Sci.* 6(2) :158-62

102. Rajesh Kumar Yadav, Kumar Gaurav, Megha Bansal, Abhishek Jaiswal. Fungal Profiling in Patients with Chronic Suppurative Otitis Media.*IJCMR*; 2016; 3 (8):2271-4

103. Praveen Kumar Karn, Anandhi Lakshmanan, Hemamalini M, Radha Madhavan. Otomycosis: A study from a tertiary care center. *J Pharm Res* 2014,8(3): 266-8

104. S. Senthil Kumaran, M. Kavitha. A Prospective Non-Randomised Observational Case Study- The Study Of Otomycosis. *J. Evolution Med. Dent. Sci.* 2017; 6 (12):931-5

105. Ravinder Kaur, Nalini Mittal, Manish Kakkar, Arun Kumar Agarwal. Otomycosis: A clinicomycological study. *Ear, Nose & Throat J*.2000:606-8

106. Yehia MM, Al Habib HM, Shehab NM. Otomycosis : A common problem in north Iraq. *J Laryngol Otol* ; 1990; 104: 387-9

107. Mohanty JC, Mohanty SK, Sahoo RC. Clinico-microbial profile of otomycosis in Berhampur. *Indian J of Otology* ; 1999; 5(2): 81-3

108. Prasanna V, V M Hemlata katiyar, I. Kannan. Study of Etiological factors, mycological profile and treatment outcome of Otomycosis. *International Journal of Medical Research and Review*. 2014; 2 (4)

109. Ozkan MK, Ozkan M, Karaarsalan. Otomycosis in Turkey: predisposing factors, etiology and therapy. *J Laryngolo Otol*.2003; 117: 39-42.

110. Aneja KR, Sharma C, Joshi R. Fungal infection of the ear: a common problem in the north eastern part of Haryana. *Int J Pediatr Otorhinolaryngol*. 2010;74(6):604-7.

111. Dr.Surinder Singh, Dr.Harsimrat Singh,Ms Amandeep Kaur. Otomycosis : A Clinical and Mycological Study. *Ind J Basic Appl Med Res*; 2017;(6)2:271-5

112. Nandyal C, Choudhari A S, Sajjan N B. A cross sectional study for mycological profile of Otomycosis in North Karnataka. *Int J Med Health Sci.* 2015,Vol-4(2): 64-9

113. Mohd. S. Sheikh Q B. Y. Qazi Q B. Rameen. Otomycosis In Khozistan. *Ind J Otolaryngol And Head And Neck Surgery;*1993; 2(2):73-5

114. Reena Ray (Ghosh), Suranjan Pal , Mallika Ghosh , Debapriyo Samaddar and Manas Banerjee. Prevalence of Fungal infection in chronic otitis media-A study at a tertiary care hospital in Eastern India. *Int.J.Curr.Microbiol.App.Sci* 2015; 4(3): 684-90

115. Vara Prasad Kondity, Suguna Shobha rani Murahari and Geethanjali Anke. A Study of Mycotic Ear Infections in a Tertiary Care Hospital *Int.J.Curr.Microbiol.App.Sci* ; 2015; 4(11): 511-6

116. B Barati, SAR Okhovvat, A Goljanian1, MR Omrani. Otomycosis in Central Iran: A Clinical and Mycological Study. *Iran Red Crescent Med J* 2011; 13(12):873-6

117. Zélia Braz Vieira da Silva Pontes, Anna Débora Ferreira Silva, Edeltrudes de Oliveira Lima. Otomycosis: a retrospective Study. *Braz J Otorhinolaryngol*. 2009;75 (3):367-70.

118. H.S.Sathish, Viswanatha.B, Manjuladevi. A Clinical Study of Otomycosis. *IOSR J Den Med Sci* . 2013; 5(2):57-62

119. Kurnatowski P, Filipiak A. Otomycosis: prevalence, clinical symptoms, therapeutic procedure; *Mycoses* 2001; 44: 472–9

120. Tapan Majumdar, Jhinuk Basu Malik . Determinants of virulence factors and biofilm formation among isolates of vulvovaginal candidiasis. *J Med Sci*; 2016; 36(2); 53-8

121. Analy S Melo, Fernando C Bizerra, Edna Freymuller, Beth A Arthington-Skaggs, Arnaldo L Colombo. Biofim production and evaluation of antifungal susceptibility amongst clinical *Candida* spp. isolates, including strains of the *Candida parapsilosis* complex. *Medical Mycology* . Apr 2011; 49: 253–62.

122. Raymundo Munguia, Sam J.Daniel. *Int J Pediatr Otorhinolaryngol*. 2008;72(4): 453-9

S.No	IP/OP No	Age	Sex	Lab No:	Predisposing factors	Co morbid conditions	Symptoms	Side involved	GTT	CHROM	CMA	*Organisms identified*	VIRULENCE FACTORS				ANTIFUNGAL SUSCEPTIBILITY				
													Candida - Biofilm	Urease test	Hemolysin	Aflatoxin	CLO	ITR	FLU	AMP	VOR
1	291	25	F	2	b,c,e		1,3	left	POS	A	7	*C. albicans*	NP				S	S	S	S	S
2	18	26	M	3	b		1,2	left				NG									
3	669	19	F	6	b,e		2,3,4	right				*A.niger*		NH	NHE		S	S	S	S	S
4	985	41	M	7	b,e	f	1,3	left	NEG	B	8	*C. glabrata*	NP				S	S	NA	NA	S
5	607	45	M	9	c,e		1,2,3	right				*A.niger*		NH	HE	B1,G1,B2,G2	S	S	S	S	S
6	1725	48	F	10	e	f	1,2,4,6	bilateral	POS	A	7	*C. albicans*	P				S	R	R	S	S
7	1855	22	F	12	b,c		1,2,3	right	POS	A	7	*C. albicans*	NP				S	S	S	S	S
8	1514	23	M	13	a,c,e		1,3,4	left				*A.niger*		NH	NHE		S	S	S	S	S
9	2927	73	F	14	c		1,2,4,5	right				*A.niger*		NH	HE		S	S	S	S	S
10	2003	21	M	15	a		1,4,5	right				NG									
11	2052	70	M	20	b,e	f	1,5	left				*A.niger*		NH	HE		S	S	S	S	S
12	2256	24	M	21	e		1,4	right				NG									
13	2160	17	M	34	e		1,2,4	right				*Fonsaceae*		NA	NA		S	S	S	S	S
14	2322	29	M	39	c		1,2,5	left				*A.niger*		NH	NHE		S	S	S	S	S
15	476	41	F	40	i,j		1,2	left				NG									
16	2361	31	F	49		f	1,4,5	right				*A.flavus*		H	HE	B1,G1,B2,G2	S	S	S	S	S
17	4252	72	M	60	b	f	1,3,4,5	right	NEG	C	9	*C. parapsilosis*	NP				S	S	S	S	S
18	4267	32	M	65	b		1,2,4,5	left				*A.niger*		NH	NHE		S	S	S	S	S
19	5089	20	M	66	a,g		1,2,5	right	POS	A	7	*C. albicans*	NP				S	S	S	S	S
20	5162	30	M	67	b		1,5	right				*A.niger*		NH	NHE		S	S	S	S	S
21	6671	16	M	90	a,g		3,4	left				*A.flavus*		NH	NHE		S	S	S	S	S
22	837	50	F	91	b,c,e		1,2,3,4	right				NG									
23	8303	16	M	92	b,d		2,3,4	left				NG									
24	5365	15	M	93	a,e		2,3,4	left				NG									
25	4947	42	F	94	e		1,3,4	right				*A.flavus*		H	NHE		S	S	S	S	S

26	978	50	M	95	e	f	1,2,4,5	left				*A.fumigatus*		NH	HE		S	S	S	S	S
27	8663	18	F	96	e		1,2,4	left				*A.niger*		NH	NHE		S	S	S	S	S
28	9589	60	M	97		f	1,5	bilateral				*A.niger*		NH	HE	B1,G1,B2,G2	S	S	S	S	S
29	3168	46	M	99	e		1,3	right				NG									
30	4354	57	F	100	c,e		1,2,3	right				NG									
31	8345	51	M	101	b,c	f	2,3,4	left				NG									
32	8981	32	F	102	e		2,3	left				NG									
33	10252	28	F	103	e		2,3,4	right				NG									
34	10526	38	F	104	c,e		1,4	right				*A.niger*		NH	NHE		S	S	S	S	S
35	10312	30	M	105	e,g		1,2,3	right	POS	A	7	*C. albicans*	P				S	S	S	S	S
36	10152	20	M	106	b		1,2	left				NG									
37	11032	9	M	107	b,c		1,4	right				*A.niger*		NH	NHE		S	S	S	S	S
38	8576	29	F	108	e		1,2,3,4	left	POS	A	7	*C. albicans*	NP				S	S	S	S	S
39	10032	35	F	109	b,c,e		1,4	left				*Rhizomucor*		NA	NA		S	S	S	S	S
40	8242	38	F	110	e		1,4	left				*A.niger*		NH	HE		S	S	S	S	S
41	11279	52	F	111	b		1,2,3,4	right				*A.flavus*		H	HE		S	S	S	S	S
42	14246	25	M	112	c,e		1,4,5	right				*Aureobasidium*		NA	NA		S	S	S	S	S
43	13169	65	F	113	c,e		2,3	bilateral				NG									
44	13377	70	F	114	e		1,4,5	right				*Cladosporium*		NA	NA		S	S	S	S	S
45	13673	25	F	115	c,e		1,2,4	left				*A.terreus*		H	HE		S	S	S	NA	S
46	1444	39	M	116	b,c		1,3,4	left				*A.niger*		NH	NHE		S	S	S	S	S
47	20316	48	M	117	c,e,j		1,2,3,5	right				NG									
48	11865	64	M	118	e	f	1,2	left				NG									
49	19096	54	M	119	e	f	1,2,3	bilateral				NG									
50	20230	46	M	120	b,c	f	1,4	right				NG									
51	23046	77	F	121	c,e	f	2,4,5	left				NG									
52	23321	32	M	122	e	f	1,2,3,5	right				*A.terreus*		H	HE		S	S	S	NA	S
53	27021	40	M	123	a		1,3,5	left				NG									
54	13793	39	F	124	c,e		2,5	right				NG									
55	9218	90	M	125	b,e	f	1,2,4,5	right				NG									

56	32609	45	M	126	e		1,5	right				NG									
57	20316	35	F	127	c,e		1,4	left				*A.niger*		NH	NHE		S	S	S	S	S
58	17010	64	M	128	b,e,g	f	1,2,4	right				*A.niger*		NH	HE		S	S	S	S	S
59	25246	36	F	129	e		1,2,4,5	right				*A.niger*		NH	NHE		S	S	S	S	S
60	21632	60	M	130	e		2,3	bilateral				NG									
61	34647	25	M	132	e		4,5	left				NG									
62	11652	14	F	133	c		1,2,3	left				NG									
63	35676	25	F	134	c		1,2,3	right				NG									
64	1425	47	M	136	b,c		2,3	right				NG									
65	22555	16	F	137	e		2,3,5	right				NG									
66	38911	22	F	138	e,g		1,2,3,4	right				*A.terreus*		H	NHE		S	S	S	NA	S
67	1435	46	F	139	e		2,4,5	left				*A.flavus*		H	HE	B1,G1,B2,G2	S	S	S	S	S
68	41632	66	M	140	e,g		1,3	right	NEG	C	9	*C. parapsilosis*	P				S	S	R	S	S
69	1412	30	F	142	b,e		1,2,4	right				*A.niger*		NH	NHE		S	S	S	S	S
70	41905	39	F	143	b,c		1,2,3,4	left				NG									
71	42968	36	F	144	b,e		1,4,5	left				NG									
72	17127	58	M	145	e	f	1,4,6	right				*A.niger*		NH	HE		S	S	S	S	S
73	30798	38	F	146	a,c,e		3,4	left				NG									
74	42857	42	F	147	c,g		1,2,4,5	left				*A.nidulans*		NH	NHE		S	S	S	S	S
75	1487	75	F	148	g	f	1,2,3,G12 24	right				NG									
76	41058	17	M	155	b		1,2,5	right				NG									
77	43428	47	M	156	b,e	f	2	left				NG									
78	2133	34	M	158	b		1,2,5	right				*A.flavus*		H	HE		S	S	S	S	S
79	51815	25	M	159	b,c,e		3	left				NG									
80	52590	35	F	160	e		2,4,5	right				*A.niger*		NH	NHE		S	S	S	S	S
81	42027	28	F	166	c,e		1,4,5	left				*A.niger*		NH	NHE		S	S	S	S	S
82	1841	41	F	170	b,c,e		1,4	left				*A.niger*		NH	NHE		S	S	S	S	S
83	40808	20	F	171	b,e,g		1,6	right				*A.niger*		NH	NHE		S	S	S	S	S
84	27391	46	M	172	d		1,2,3	left				NG									
85	13686	28	F	173	d,e		1,2,3	right	POS	A	7	*C. albicans*	NP				S	S	S	S	S

86	13563	60	F	201	e		1,4,5	right				*Cladosporium*		NA	NA		S	S	S	S	S
87	19738	40	F	205	c,e		1,4	right				*A.niger*		NH	NHE		S	S	S	S	S
88	41120	21	F	206	b,c,e		1,2,4	left				*A.niger*		NH	NHE		S	S	S	S	S
89	56798	19	F	207	c,d,e		3	right				NG									
90	47427	17	F	210	g		2,3,4	right				NG									
91	47247	28	F	213	e		2,4,5	right	NEG	D	10	*C. krusei*	NP				S	S	NA	NA	S
92	56597	13	M	215	d,g		2,4	left				NG									
93	47124	37	M	222	e	f	1,3,4	right				*A.niger*		NH	HE		S	S	S	S	S
94	40697	47	F	224	b,c,e		1,4,5	left				NG									
95	50766	21	F	225	e,g		1,2	left				*A.niger*		NH	NHE		S	S	S	S	S
96	46246	19	F	226	b,d,g		1,2,3,5	left				NG									
97	48624	5	M	227	c		1,3,4	left				*A.niger*		NH	NHE		S	S	S	S	S
98	40639	58	F	228		f	2,3,5,6	right	POS	A	7	*C. albicans*	P				S	S	R	S	S
99	10562	28	M	229	d		1,2,3	left				NG									
100	6016	35	F	230	e		1,4	left				*A.niger*		NH	NHE		S	S	S	S	S
101	46375	27		233	b,g		3,4,5	bilateral				NG									
102	41843	20	F	234	e		3,4	right				NG									
103	18607	50	F	235	a,c,e		1,3,5	right				NG									
104	9024	8	F	236	b,c,e		3,5	right				NG									
105	19106	14	F	237			2,3,4	left				NG									
106	40630	56	M	238	e,g		1,3,5	right				*A.terreus*		H	HE	B1,B2,G2	S	S	S	NA	S
107	48935	40	M	239	a	f	3,4,5	left	POS	A	7	*C. albicans*	P				S	S	S	S	S
108	19339	54	F	240	e		2,3	left				NG									
109	19028	34	F	241	c,e		2,3,4	right				NG									
110	891	37	F	242	e,g	f	2,3,4	bilateral	POS	A	7	*C. albicans*	NP				S	S	S	S	S
111	43590	34	F	243	b		1,2	right				NG									
112	38910	24	M	246	b,c		2	right				NG									
113	47103	21	M	247	d		1,2,3,4	left				*A.fumigatus*		NH	HE		S	S	S	S	S
114	21020	54	M	248	c,e		1,4,5	left				*A.niger*		NH	NHE		S	S	S	S	S
115	37554	40	F	250	b,c		1,4	left				NG									

116	6172	43	F	251	e		2,4	right				NG									
117	50155	26	F	252	e		2,3	left				NG									
118	41443	55	F	254	e,g		1,2,5	right				*A.flavus*		H	NHE		S	S	S	S	S
119	43556	24	M	256	e		1,2,5	left				NG									
120	50906	34	M	257	b,e	f	2,3,5	bilateral				NG									
121	20893	58	M	258	a	f	2,4	left				NG									
122	50428	13	M	259	b,g		1,3,4	left				*A.niger*		NH	NHE		S	S	S	S	S
123	8191	58	M	262	b,e,g	f	2,4,5	right				*A.niger*		NH	NHE		S	S	S	S	S
124	21433	22	F	266	c,e		3	right				NG									
125	13978	19	F	267	c,e		1,4	left				*Curvularia*		NA	NA		S	S	S	S	S
126	43541	34	F	268	e,g		2,3,4	left	POS	A	7	*C. albicans*	NP				S	S	S	S	S
127	51116	17	M	269	e		2,3,4	right	POS	A	7	*C. albicans*	NP				S	S	S	S	S
128	25871	30	F	270			2,3,4	left				NG									
129	46391	50	F	271	b,e		1,4	right				*Rhizopus*		NA	NA		S	S	S	S	S
130	35333	59	M	273	a,b		2,3	right				NG									
131	35925	42	F	274	b,e		1,4,5	left	POS	A	7	*C. albicans*	P				S	S	S	S	S
132	34261	47	F	276	e		1,4	right				*A.niger*		NH	NHE		S	S	S	S	S
133	32482	30	F	277	c		4	left				NG									
134	50131	56	F	280	b,e	f	1,4	right				NG									
135	51507	34	F	281	c,e		2,3	right				NG									
136	7286	49	F	284	b,c		2,3,4	left				NG									
137	47887	26	F	287	a,c		2,3	right				NG									
138	52253	64	M	290	b		1,4,5	right				NG									
139	38737	17	F	291	a,c,g		3	left				NG									
140	2060	42	M	292	e,g		1,4	left				*A.niger*		NH	NHE		S	S	S	S	S
141	51177	28	M	295	b,e		1,4	left				*A.flavus*		H	NHE		S	S	S	S	S
142	52837	31	F	296	e		1,5	right				*A.niger*		NH	NHE		S	S	S	S	S
143	53007	38	M	298	b,e		1,3	left	POS	A	7	*C. albicans*	NP				S	S	S	S	S
144	7549	60	F	301	b,g	f	3,4	bilateral				*A.niger*		NH	HE	B1,G1,B2,G2	S	S	S	S	S
145	52046	17	F	303	e		2,3,4	left				NG									

146	13417	7	F	304	e		2,3	right				NG									
147	48918	23	F	312	b,e		1,3	right	POS	A	7	*C. albicans*	NP				S	S	S	S	S
148	1163	40	M	321	b,e,g		1,4,5	left				*A.niger*		NH	NHE		S	S	S	S	S
149	44940	31	F	322	b,e		2,5	left				NG									
150	52920	27	F	324	b,c,e,g		1,2,4	left				*A.niger*		NH	NHE		S	S	S	S	S
151	40654	17	M	328	b,g		1	left				*A.niger*		NH	NHE		S	S	S	S	S
152	55030	50	F	330	b,e		1,4	right	POS	A	7	*C. albicans*	NP				S	S	S	S	S
153	54138	21	M	333			3,4	right				NG									
154	54326	14	F	334	b,e		1,4,5	left				*A.flavus*		H	NHE		S	S	S	S	S
155	38361	60	M	335	c,e,g		2,4,5	right				*A.niger*		NH	NHE		S	S	S	S	S
156	54923	30	F	337	d,e		1,3,4	left				*A.niger*		NH	NHE		S	S	S	S	S
157	45012	40	F	341	b,c,e		1,2,3,5	right				*Rhizopus*		NA	NA		S	S	S	S	S
158	49756	16	M	342	b,c		2,4	left				NG									
159	54935	50	M	345	e		1,2,3	left				NG									
160	57948	16	F	346			2,3,4	right				NG									
161	61006	28	M	347	a,e		2,3,4,5	left	POS	A	7	*C. albicans*	NP				S	S	S	S	S
162	41476	27	F	349			2,3,4	right				NG									
163	4934	35	M	350	c		1,2,3,4	left				NG									
164	48237	45	F	351			3,4	right				NG									
165	61496	60	F	352	e		1,4,5	left				*A.niger*		NH	HE	B1,G1,B2,G2	S	S	S	S	S
166	10898	28	F	353	g		1,4	left				*A.flavus*		H	NHE		S	S	S	S	S
167	54569	45	F	354	e,g		3,4,5	bilateral				NG									
168	24827	42	F	355	b,e		1,4	left				NG									
169	52197	15	F	356	b,e		1,2	right				*A.flavus*		NH	NHE		S	S	S	S	S
170	4643	38	F	361	e		4	right				NG									
171	65164	48	M	362	a		1,2,4	left				NG									
172	14347	32	F	367			4	right				NG									
173	31693	25	M	368	b,e		1,3	right				NG									
174	65053	51	F	369	b,c		1,4	left				NG									
175	41979	21	F	370			3,4	left				NG									

176	65332	15	F	371	b		1,2	right				NG									
177	65823	27	M	372			2,3	right				NG									
178	9812	67	F	375	b,e		2,3,4	right				NG									
179	56569	45	F	382	b,e		1,2,5	left	POS	A	7	*C. albicans*	P				S	S	S	S	S
180	69330	36	F	394	c,e		2,	right				NG									
181	25571	43	F	395	e		1,2,3,6	right				*A.niger*		NH	NHE		S	S	S	S	S
182	1738	52	M	396	e	f	2,4	left				*A.flavus*		H	NHE		S	S	S	S	S
183	16546	19	F	397	c		2,3	left	POS	A	7	*C. albicans*	NP				S	S	S	S	S
184	42415	32	M	398	b,c		1,2,3,5	right				*Mucor*		NA	NA		S	S	S	S	S
185	34824	32	F	399	b,d		1, 4	right				*A.flavus*		H	NHE		S	S	S	S	S
186	9478	23	F	400	b,e		1,4	right				*A.flavus*		NH	NHE		S	S	S	S	S
187	6382	47	M	413	c		1,5	left				NG									
188	61006	50	F	414	b,c		1,2,4,5	right				*A.niger*		NH	HE		S	S	S	S	S
189	70312	65	F	415	a,b,c	f	1,5	left				NG									
190	1127	50	M	416	c		1,5	left				*A.niger*		NH	NHE		S	S	S	S	S
191	71952	26	F	417	b		1,6	right				*A.niger*		NH	NHE		S	S	S	S	S
192	72883	42	F	418	b,c		1,2,5	left				*A.niger*		NH	HE		S	S	S	S	S
193	1839	27	F	419	c,e		1,2,5	left				NG									
194	71004	40	F	421	e		1,2,4	right				*A.niger*		NH	NHE		S	S	S	S	S
195	21563	68	M	422	b,c		1,2,4	left				*A.niger*		NH	NHE		S	S	S	S	S
196	66943	80	M	423	b,c		1,5	right				NG									
197	1846	33	M	430	c		1,4,5	left				*A.niger*		NH	NHE		S	S	S	S	S
198	74385	18	F	435	a,c		2,3,5	right				NG									
199	72904	23	F	444	e		1,2,4	right				*A.niger*		NH	NHE		S	S	S	S	S
200	6848	25	F	479	c,e		1,3,4	right	POS	A	7	*C. albicans*	NP				S	S	S	S	S