

# CHAPTER ONE

## 1. INTRODUCTION

### 1.1. Introduction

Otomycosis is fungal infection of the external auditory canal and its associated complications sometimes involving the middle ear. It occurs because the protective lipid/acid balance of the ear is lost. Fungi cause 10% of all cases of otitis externa. In recent years, opportunistic fungal infections have gained greater importance in human medicine, perhaps because of the increasing number of immunocompromised patients. However, such fungi may also produce infection in immunocompetent hosts in immunocompromised patients; treatment of otomycosis should be vigorous to prevent complications such as hearing loss and invasive temporal bone infection. Its prevalence is greatest in hot humid and dusty areas of the tropics and subtropics. Andrall and Gaverret were the first to describe fungal infections of the ear; although a wide spectrum of fungi are involved, *Aspergillus* and *Candida* are the most common species encountered. In 1960's studies by Geaney and by Lakshmipathi and Murthy revealed that all cases observed by them had been caused by either *Aspergillus* or *Candida* species (Satish *et al.*, 2013).

The infection usually characterized by inflammation, pruritus, trauma to external auditory canal, scaling and severe discomfort such as suppuration and pain (Desai *et al.*, 2012).

*Aspergillus niger* and *Candida albicans* are by far the most common offenders, a wide spectrum of other fungi can cause otomycosis. Various factors have been proposed as predisposing factors for otomycosis, including a humid climate, presence of cerumen, instrumentation of the ear,

immunocompromised host, more recently, increased use of topical antibiotic/steroid preparations (Prasad *et al.*, 2014).

Fungi are abundant in soil or sand that contains decomposing vegetable matter. This material is desiccated rapidly in tropical sun and blown in the wind as small dust particles. The airborne fungal spores are carried by water vapours, a fact that correlates the higher rates of infection with the monsoon, during which the relative humidity rises to 80% (Satish *et al.*, 2013).

One of the most common predisposing factors is swimming, especially in fresh water. Other factors include skin conditions such as eczema and seborrhea, trauma from cerumen removal, use of external devices such as hearing aids, and cerumen buildup (Schaefer and Baugh, 2012).

In Sudan Hashim and his colleges (2010) conducted similar study among patients visiting Ear, Nose and Throat (ENT) Hospital, Khartoum. The isolates were identified as: *Aspergillus niger* (59.6%), followed by *A. flavus* (21.3%), *A. terreus* (12.8%), *Penicillium species* (2.1%), *Curvularia species* (2.1%) and *Scopulariopsis species* (2.1%). *Aspergillus species* constituted 93.6% of the total isolates and their isolation frequency from the total samples was 44%. Most of isolates (81%) were isolated from patients, especially males (Hashim *et al.*, 2010).

## **1.2. Rationale**

Because of distribution of otomycosis in the Sudan and increased uncontrolled usage of antibiotics this study will take place to explain the increase of evidence indicating misdiagnosed cases of otomycosis (Hashim *et al.*, 2010).

### **1.3. Objectives**

#### **1.3.1. General objective**

To determine the frequency and etiology of otomycosis among patients attending Khortoum ENT hospitals, during March to June 2014.

#### **1.3.2. Specific objectives**

**I.** To isolate and identify the fungal pathogens.

**II.** To study relation between otomycosis and different factors e.g. (age, gender and symptoms).

## **CHAPTER TWO**

### **2. LITREATURE REVIEW**

#### **2.1. Anatomy of auditory external canal**

The ear consists of three areas: the outer ear, the middle ear, and the inner ear. The ear contains the receptors for two senses: hearing and equilibrium. These receptors are all found in the inner ear (Scanlon and Sanders, 2007).

#### **2.2. Otomycosis**

##### **2.2.1. Definition of otomycosis**

Otomycosis is a fungal infection of the external auditory canal; middle ear and open mastoid cavity that is frequently encountered by otolaryngologists. It presents with nonspecific symptoms of itching, earache, ear discharge, hearing loss, aural fullness, and tinnitus. Otomycosis is seen more frequently in immunocompromised patients as compared to immunocompetent persons. In recent years, opportunistic fungal infections are gaining greater importance as a result of possibly increasing number of immunocompromised patients (Satish *et al.*, 2013).

Otomycosis is occurring because the protective lipid/acid balance of the ear is lost. Fungi cause 10% of all cases of otitis externa (Satish *et al.*, 2013).

Otomycosis is a fungal infection of external ear with troublesome symptoms like otorrhea, otalgia, pruritis etc. Fungal agents responsible for this clinical entity are commonly found to be saprophytes in environment which makes infection to occur frequently in humid climate (Panchal *et al.*, 2013).

##### **2.2.2. History**

Andral and Gavarret in 1843 and Mayer in 1844 first described fungal infections of the external auditory canal and subsequently Virchow suggested the term 'otomycosis'. In 1851, Pacini was the first to describe a preparation for the treatment of otomycosis. Additional reports were

published at the beginning of the twentieth century. Wolf described the relationship of various fungi to this clinical entity. It was postulated that cleansing and drying were essential aspects of the management and provided symptomatic relief but whether additional therapy was necessary remained controversial. Gregson and other coworker realised the significance of fungal infections in the aetiology of the otitis externa and labeled it as a neglected disease (Chander, 2009).

The predominant role of *A. niger* in otomycosis was established in the 1960's and 1970's. Damato also believed that recurrence was likely if cleansing and drying were the only management regimes and further stated the role of tolnaftate in its treatment. Stern *et al*, suggested that most cases of otomycosis would resolve with meticulous cleansing and drying. Otomycosis classically has been described as a fungal infection of the external auditory canal but Paulosee suggested that the term should be expanded and redefined to include fungal infections of the middle ear and open mastoid cavities (Chander, 2009).

### **2.2.3. Epidemiology**

Otitis externa is common and manifests as an acute or chronic form. The acute form affects four in 1,000 persons annually and the chronic form affects 3-5% of the population. Acute disease commonly results from bacterial (90%) or fungal (10%) overgrowth in an ear canal subjected to excess moisture or to local trauma. Fungi have been implicated overall in ~9% of cases of external otitis. In various studies, it is estimated that about 5-25% of total cases of otitis externa are due to otomycosis. The disease is worldwide in distribution. Otomycosis is more prevalent in warm, humid climates, particularly in the rainy season as compared to arid or cold climates. It is more frequent in individuals of lower socio-economic status

with poor hygienic conditions. It is most commonly seen between 2nd and 3rd decades of life. Workers in mouldy or dusty settings are usually more affected. The prevalence of otomycosis is higher in malnourished children as compared to the normal children (Chander, 2009).

Diseases caused by *Aspergillus spp.* are increasing in importance, especially among immunocompromised hosts. Clinical manifestations are variable, ranging from allergic to invasive disease, largely depending on the status of the host's immune system. *A. fumigatus* is the predominant etiological agent followed by *A. flavus* but the latter may be the principle cause in certain countries such as, Saudi Arabia, Sudan and Tunisia. *A. flavus* has been reported to be associated with outbreaks of mucocutaneous and subcutaneous aspergillosis (Hadrich *et al.*, 2013).

#### **2.2.4. Predisposing Factors**

Predisposing factors such as a failure in the ear's defense mechanisms (changes in the coating epithelium, changes in pH, quantitative and qualitative changes in ear wax), bacterial infection, hearing aid or a hearing prosthesis, self-inflicted trauma (use of q-tips to clean the ear), swimming, broad spectrum antibiotic agents, steroids and cytostatic medication, neoplasia and immune disorders, all of which can render the host susceptible to the development of otomycosis (Pontes *et al.*, 2009).

#### **2.2.5. Causative agents**

Many species of fungi have been identified to cause of otomycosis. *Aspergillus niger* and *Candida albicans* are the most common causative agents of otomycosis. *Aspergillus* is considered the predominant causal organism in tropical and subtropical regions. *Alternaria humicola* and *Aspergillus niveus* are added as new etiologic agents causing otomycosis. *Aspergillus terreus*, *Scolpuriopsis polyacillium*, *Candida albicans* and

species of *Mucor* and *Rizopus* were the main etiological agents and their origin was either from air or from soil (Srivastava and Gautam, 2013).

#### **2.2.5.1. *Aspergilli* species**

*Aspergillus* species are globally ubiquitous saprophytes found in a variety of ecological niches. Almost 200 species of aspergilli have been identified, less than 20 of which are known to cause human disease (Dagenais and Keller, 2009).

Aspergillosis is the most common invasive mold infection worldwide. Aspergilli are ubiquitous saprobes in nature and may be found in soil, potted plants, decaying vegetation, pepper, and construction sites. *Aspergillus* spp. can cause diseases in humans by airway colonization with subsequent allergic reactions, colonization of preexisting cavities (aspergilloma) or by tissue invasion. Aspergilli secrete various metabolic products, such as gliotoxins, and a variety of enzymes, including elastase, phospholipase, various proteases, and catalase, which may play a role in virulence (Murray *et al.*, 2009).

#### **2.2.5.2. *Candida* species**

*Candida albicans* is an opportunistic fungus causing various forms of candidiasis. However, under certain circumstances it is capable of becoming pathogenic (Nasutio, 2013). *C. albicans*, as most pathogens, has developed an effective battery of virulence factors and specific strategies to assist the ability to colonize host tissues, cause disease, and overcome host defenses. An outstanding attribute of *C. albicans* biology is its capacity to grow in a diversity of morphological forms, ranging from unicellular budding yeast (blastospores), pseudohyphae, to true hyphae with parallel-sided walls. The yeast-hyphae transition contributes to tissue invasion and to the escape from phagocyte cells after host internalization, and is therefore considered an

important virulence factor. Additionally, several other factors have been described in association with virulence, including the production of proteins that mediate adherence, the colonization and invasion of host tissues, the maintenance of cell wall integrity, phenotypic switching, and the avoidance of the host immune response (Ferreira *et al.*, 2010).

#### **2.2.5.3. *Alternaria* species**

*Alternaria* is a ubiquitous fungal genus that includes saprobic, endophytic and pathogenic species. It is associated with a wide variety of substrates including seeds, plants, agricultural products, animals, soil and the atmosphere. Species of *Alternaria* are known as serious plant pathogens, causing major losses on a wide range of crops. Several taxa are also important post harvest pathogens, causative agents of phaeohyphomycosis in immuno-compromised patients or airborne allergens (Woudenberg *et al.*, 2013).

#### **2.2.6. Clinical Features**

Otomycosis usually presents with a history of itching, irritation, discomfort, pain and scanty discharge from the affected ear. There is also a feeling of blockage in the ear due to collection of debris material in external auditory canal. Irritation is more marked in fungal as compared to bacterial otitis externa. Pruritus and discharge are the most common symptoms, with reddened epidermis and lining of the tympanic cavity being common. These manifestations are usually unilateral but rarely bilateral involvement has also been seen. If there is a concurrent perforation of the tympanic membrane and particularly otalgia is a prominent feature, suppurative otitis media caused by *Aspergillus* or other fungi should be considered (Chander, 2009). Fungal infection should be suspected in all cases of chronic otitis externa which do not respond to conventional topical antibacterial therapy. Otoscope



examination reveals infection confined to the ear canal. There is greenish or black fuzzy growth on cerumen or debris resembling wet ‘blotting paper’, which may fill up the entire meatus. There may be slight conduction deafness also due to mechanical obstruction of the external auditory canal. The local area may be hyperemic and sometimes bleeding may be observed. In immunocompromised patients especially diabetics, *Aspergillus* may invade locally to adjacent anatomical sites like mastoid bone or even brain. *Aspergillus* may cause invasive external otitis (necrotizing or malignant otitis externa) with local spread to bone and cartilage, which is a severe and potentially life-threatening disease. This may be associated with underlying immunocompromised situation, diabetes mellitus or patient receiving haemodialysis entailing high mortality. Invasive otitis externa is more frequently caused by *A. fumigatus* than *A. niger*. In addition, invasive *A. tympanomastoiditis* may be encountered in immunocompetent patients as well. Recently efficacy of antifungal therapy with voriconazole 200 mg twice a day in invasive otitis externa caused by *Aspergillus* has been reviewed (Chander, 2009).

### **2.2.7. Identification**

#### **2.2.7.1. Identification of *Aspergillus* species**

Identification methods for *Aspergillus* species in diagnostic criteria include the recognition of asexual or sexual structures and their characteristics such as shape, size, color, ornamentation and/or mode of attachment. Unfortunately, numerous difficulties exist in such a phenotype-based scheme largely because these characteristics are unstable and clinical *Aspergilli* sometimes manifest atypically with slow sporulation and aberrant conidiophore formation (McClenny, 2005).

Microscopic methods, such as wet mounts, Gram stains, and conventional histopathology, provide clues that suggest the presence of *Aspergillus spp.* in tissue. Blankophor or Calcofluor mixed with 10%-20% potassium hydroxide (KOH) stains fungal cell walls and improves detection of fungi. While Calcofluor crystallizes in an alkaline pH, Blankophor does not and it can be stored in a working solution for up to a year (McClenny, 2005).

Phenotypic markers detected by histopathologic stains, as well as by Gram stain or wet mounts, provide valuable information for clinically important fungi, especially in the absence of culture. However, confirmation of microscopic findings by culture is always desirable and, in most cases involving opportunistic molds, essential for definitive identification of the pathogen. The use of potato dextrose, potato flake, malt extract, inhibitory mold agar, or similar sporulation agars as primary isolation media for *Aspergillus spp.* may speed growth rate and the production of conidia. The addition of antibacterial agents to isolation media helps reduce time to identification by inhibiting bacterial overgrowth and reducing the need for subculture. The initial incubation of fungal media at 35°C/37 °C instead of, or in addition to, 30 °C may speed the growth of some aspergilla. Similarly, daily inspection of culture media ensures the earliest possible detection. By incubating culture plates in a microaerophilic environment at 35 °C. With a quick Scotch-tape or tease mount, conidial heads of *Aspergillus spp.* can typically be identified. However, a slide culture may be necessary when sporulation is slow or atypical. Riddell's classic slide culture method has been supplemented with other, less labor-intensive techniques. A quick method is simply to push an 18×18 mm coverslip at a 45 degree angle into a sporulation media, such as potato flake agar. When the mould sporulates, the coverslip is carefully withdrawn from the agar and mounted in adrop of

lacto-phenol blue or lacto-fuchsin on amicroscope slide. Another drop is placed on top of the small coverslip before completing the assembly with a 22×22 mm coverslip. Isolation in culture and phenotypic identification of common clinical isolates of *Aspergillus spp.* is usually quick and easy (McClenny, 2005).

*A. fumigatus* is a rapid grower. The typical velutinous, grey-blue-green colonies and uniseriate conidial heads develop within 24-48 hour on both fungal media and the sheep blood agar commonly used for bacterial culture. Other Aspergilli associated with invasive aspergillosis, specifically, *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus* have growth rates similar to that of *A. fumigatus* when colonies were measured on malt extract agar and Czapek yeast agar after incubation for seven days at both 25°C and 37°C. Hyphae of aspergilla is 2.5-8 mm wide, septate, hyaline, acute angle branching, tree- or fan-like branching, may resemble hyphae of zygomycetes under microscope. Conidial head uniseriate, columnar, conidiain chains or detached and dispersed. Single or paired conidia may resemble yeast cells. *A. niger* Conidial head biseriate, radiate, conidia in chains or detached and dispersed. Single or paired conidia may resemble yeast cells. *A. terreus* small, round, hyaline conidia ('accessory' conidia) attached to the vegetative hyphae (McClenny, 2005).

#### **2.2.7.2. Identification of *Candida species***

The identification of *Candida species* is very important in the diagnostic laboratory, because such identification shows prognostic and therapeutical significance, allowing the early and correct antifungal therapy. Identification of *Candida spp.* done by phenotypic and genotypic methods, discriminating *C. albicans* from other species of *Candida*. Samples were seeded on Sabouraud dextrose agar (SDA) with chloramphenicol (16

mg/mL). The tests used for phenotypic analysis were germ-tube and chlamydo conidia production, culture in CHROM agar *Candida*, carbohydrate assimilation test, and growth at 45 ° C and culture in Tween 80 agar. Genotypic confirmation was performed by PCR (Marinho *et al.*, 2010).

#### **2.2.7.3. Identification of *Alternaria species***

Identification of some *Alternaria* species still offers considerable difficulties, owing to their high variability and diversity, consequently, different opinions have still remained. The *Alternaria species* was identified according to the identification key proposed by Morphological studies were carried out on the strains grown at 25°C on malt extract agar (MEA) and potato carrot agar (PCA) media. Colony characters were observed using stereo light microscope as; color of culture and reverse, number of growth zones, diameter of colony (cm), presence of aerial and submerged mycelium type of conidial chains and abundance of conidia. The microscopic characteristics to identify the *Alternaria species* were; color, shape, number and position of septa (longitudinal, oblique or transverse) of conidia and their attachment with the conidiophores, ornamentation of conidial walls, presence, size and shape of conidial beak, presence of apical or basal pores (Bashir *et al.*, 2014).

#### **2.2.8. Background studies**

Many previous studies were planned to investigate the prevalence of otomycosis and etiological agents. A similar study conducted by Saki and another co-worker in Khuzestan province, south-west Iran, 2012. The study examined and cultured 881 swabs from suspected external otitis cases. Fungal agents were identified by slide culture and complementary tests when necessary. Results: The mean patient age was 37 years. The 20-39 year age group

had the highest prevalence of otomycosis: 293 cases, comprising 162 (55.3%) women and 131 (44.7%) men. The seasonal distribution of cases was: summer, 44.7%; autumn, 28.7%; winter, 14.7%; and spring, 11.9%. The fungal agents isolated were *Aspergillus niger* (67.2 %), *Aspergillus flavus* (13%), *Candida albicans* (11.6%), *Aspergillus fumigatus* (6.2%) and *penicillium species* (2%) (Saki *et al.*, 2012).

Another study achieved by Kenan Değerli and his colleges in Turkey, 2012 reported that 28% (638/2279) were found positive by direct microscopy and 24% (544/2279) by culture methods. Among culture-positive cases the isolation rates of mold-like and yeast-like fungi were 66% (359/544) and 34% (185/544), respectively. The number of distribution of the molds were as follows; *Aspergillus niger* (180), *Aspergillus fumigatus* (95), *Aspergillus terreus* (32), *Aspergillus flavus* (23), *Aspergillus spp.* (14), *Penicillium spp.* (13), *Trichophyton spp.* (*T. rubrum* 1, *T. mentagrophytes* 1); while this distribution was as follows for the yeasts; *Candida tropicalis* (97), *Candida albicans* (39), *Candida parapsilosis* (21), *Candida glabrata* (19), *Candida kefyr* (4), *C. guilliermondii* (2), *Candida krusei* (1), *Geotrichum candidum* (1) and *Trichosporon capitatum* (1). It was notable that 96% (344/359) of mold-like fungi were *Aspergillus spp.*, and 99% (183/185) of yeast-like fungi were *Candida spp.* The results of this study indicated that the most frequent agents of otomycosis were non-dermatophyte species such as *Aspergillus*, followed by *Candida*. Dermatophytes were isolated in a small number of otomycosis cases (Değerli *et al.*, 2012).

Another study achieved by Shadman Nemati and others in Iran, 2013 to determine the pathogens that caused otomycosis and also the efficacy of different antifungal agents. 43 % of patients were positive by culture. The

most prevalent fungal pathogen was *Aspergillus niger* which was sensitive to Clotrimazole, Fluconazole, Ketoconazole. *Candida albicans* was sensitive to all drugs, in which, the most sensitivity was due to fluconazole. The most frequent fungal pathogen in our otomycosis cases is *A. niger*, and most of fungi that caused otomycosis are sensitive to clotrimazole (Nemati *et al.*, 2013).

Other study in Iran, 2011 conducted that the black aspergilli are among the main causative agents of otomycosis worldwide. In this study, the species assignment of black aspergilli isolated from otomycosis cases in Iran was carried out using sequence analysis of part of the calmodulin gene. The results indicate that *Aspergillus niger* is not the only black *Aspergillus species* involved in otomycosis cases in Iran: *Aspergillus awamori* and *Aspergillus tubingensis* are also able to cause ear infections (Szigeti *et al.*, 2011).

Another study conducted that otomycosis was more common among females as compared to males & also common in age groups of 20-35 years. The most common fungal pathogens isolated were *Aspergillus niger* (75.82%) followed by *Aspergillus fumigatus*( 13.19 %), *Candida albicans* (7.69 %) & *Aspergillus flavus* ( 3.3 %) (Desai *et al.*, 2012).

Other study in India, 2009 showed that 82 (75.92%) samples were positive for the presence of fungal elements, of which 48(44.44%) specimens were smear and culture positive while 34(31.48%) were smear negative but culture positive. Males and females were almost equally affected, ratio being (1.1:1). Chief fungal isolates included *Aspergillus niger* (52.43%), *Aspergillus fumigatus* (34.14%), *Candida albicans* (11%), *Candida pseudotropicalis* (1.21%) and *Mucor spp.* (1.21%). Among 8 patients mixed

fungal growth was obtained, while bacterial coinfection/ super infection were detected in 14 cases (Kumar, 2009).

### **2.2.9. Immunity**

The saprophytic mold *Aspergillus fumigatus* and *Candida albicans*, a commensal of the mucocutaneous membranes, are the most common fungal pathogens among immunocompromised patients. Both fungi cause severe invasive infections and are responsible for substantial morbidity and mortality despite effective antifungal treatment. T-cell-mediated heterologous immunity to different pathogens is promising for the development of immunotherapeutic strategies. *Aspergillus fumigatus* and *Candida albicans*, the 2 most common fungal pathogens causing severe infections in immunocompromised patients, are controlled by CD4<sup>+</sup> type 1 helper T (TH1) cells in humans and mice, making induction of fungus-specific CD4<sup>+</sup> TH1 immunity an appealing strategy for antifungal therapy. *A. fumigatus* cell wall Crf1 glucanase can be presented by 3 common major histocompatibility complex class II alleles and that induces memory CD4<sup>+</sup> TH1 cells with a diverse T-cell receptor repertoire that is cross-reactive to *C. albicans* (Stuehler *et al.*, 2011).

*Aspergillus fumigatus* activate, suppress, or subvert host immune response during life cycle in vivo through dynamic changing of cell wall structure and secretion implicates discriminative immune sensing of distinct fungal components (Bozza *et al.*, 2009).

Cell wall component of *A. fumigatus* that is capable of modulating the immune response is galactosaminogalactan (GAG). GAG has been shown to serve as an adhesin of *Aspergillus*, and to shield  $\beta$ -glucan moieties on the cell wall. This polysaccharide that is shed into the host environment during *Aspergillus* vegetative growth induces immunosuppressive effects that

results in diminished neutrophil recruitment, which predisposes mice to *A. fumigatus* infection (Gresnigt *et al.*, 2014).

#### **2.2.10. Prevention**

A number of preventive measures have been recommended, including use of earplugs while swimming, use of hair dryers on the lowest settings and head tilting to remove water from the ear canal, and avoidance of self-cleaning or scratching the ear canal. Acetic acid 2% (Vosol) otic solutions are also used, either two drops twice daily or two to five drops after water exposure. However, no randomized trials have examined the effectiveness of any of these measures (Schaefer, and Baugh, 2012).

Targeting typical causal culprits of Acute Otitis Externa (AOE), such as moisture and trauma, seems prudent. Some experts recommend simple techniques for keeping water out of the ears (e.g., inserting a soft, malleable plug into the auricle to block entry to the ear canal) or removing water from the ears after swimming (by positioning or shaking the head,). Others advise avoiding cotton swabs because they might impact cerumen. Daily prophylaxis with alcohol or acidic drops during at-risk activities has also been suggested but not studied. Using hard earplugs should be avoided because they can cause trauma, and the use of custom ear canal molds and tight swim caps remains controversial (Hui, 2013).

#### **2.2.11. Treatment**

*Aspergillus species* have emerged as important causes of morbidity and mortality in immunocompromised patient (Walsh *et al.*, 2008). Treatment involves elimination of predisposing factors. Topical antibiotic solutions must be stopped. Patients' nails must be inspected to rule out onychomycosis. The ear canal must be thoroughly debrided of all visible debris. It is our practice to avoid syringing and clear the debris by suctioning



alone. Fungicidal drops are the most popular form of treatment. Clotrimazole has an antibacterial effect, and this is an added advantage when treating mixed bacterial-fungal infections. Fungicidal creams with ketoconazole or fluconazole may also be applied. A readily available and usually effective preparation for *Candida* is tolnaftate, available over the counter for the treatment of athlete's foot. Nystatin is another useful drug against *Candida*. The major advantage of nystatin is the fact that it is not absorbed across intact skin. Nystatin is not available as an otic preparation; however it can be prepared as a solution or a suspension for the treatment of otomycosis. Studies on animals with experimentally induced fungal infections have furnished evidence for the risk of the infections' spreading to the inner ear and causing serious damage to the organ of Corti; indirect damage to these structures by mycotoxins cannot be ruled out. In rare refractory cases of otomycosis due to HIV or other immunocompromised states, or in life threatening condition, parenteral antifungals like amphotericin B or tolnaftate may be used (Prasad *et al.*, 2014).

Treatment is involved administration of antifungal agents as local or systemic, local debridement and prohibition use of topical antibiotics or steroids (Nowrozi *et al.*, 2014).

## **CHAPTER THREE**

### **3. MATERIALS AND METHODS**

#### **3.1. Study design**

Hospital – analytical study.

#### **3.2. Study type**

Descriptive – cross sectional.

#### **3.3. Study approach**

Prospective and qualitative study.

#### **3.4. Study area**

Khartoum city ENT (Ear Nose and Throat) Hospital, Aldoha Specialized Hospital and Africa Specialized Hospital.

#### **3.5. Study population and duration**

All clinically suspected cases of otomycosis attended the unit of ENT during period of the study from March - June 2014.

##### **3.5.1. Inclusion criteria**

Patients who presented with signs and symptoms of otomycosis.

##### **3.5.2. Exclusion criteria**

Patients with other ear infections and who were taking antifungal treatment were excluded.

#### **3.6. Sampling**

##### **3.6.1. Sample type**

Non-probability sampling.

##### **3.6.2. Sample size**

One hundred ear swabs specimen were collected.

##### **3.6.3. Sampling technique**

Convenience.

### **3.7. Study variables**

Screen of otomycosis (dependent variable).

Etiological agents, age and sex as independent variables.

### **3.8. Data collection**

Data was collected by direct interviewing by questionnaire contain all study variables (appendix 1).

### **3.9. Ethical Clearance**

Permission of this study was obtained from College Ethical Board; the objectives of the study clearly and simply explained to all participants in the study, verbal inform consent was also obtained from them.

### **3.10. Experimental Work**

#### **3.10.1. Method of collection of specimens**

Ear swabs were collected from patients by using sterile cotton tipped swabs with help of otologist.

#### **3.10.2. Macroscopic examination**

Color and consistency of discharge for each specimen was observed and recorded.

#### **3.10.3. Microscopic examination**

##### **3.10.3.1. Wet preparation**

The swab was rolled on clean slide and a drops of 20% potassium hydroxide (appendix 3) was added, and covered with cover slip, then examined under microscope using X10 and X40 after 10 minutes for the detection of head of *Aspergilli*, hyphae and budding yeast (Aslam *et al.*, 2008).

##### **3.10.3.2. Gram's stain**

The swab was smeared on clean slide to make smear and let to air dry, then smear fixed by passing the slide through the flame three times gently,

covered with crystal violet stain (appendix 3) for one minute. Then washed by tap water, and then covered with lugol's iodine (appendix3) for one minute. Iodine was washed off, and smear was decolourized with acetone-ethanol (appendix 3) for few seconds and washed by tap water. Safranin (appendix 2) was added for two minutes, washed off with tap water and let to air dry and microscopically examined using oil immersion objective (X100) to observed yeast cell morphology, size, Gram positive reaction and presence of pus cells, epithelial cells (Bhavan *et al.*, 2010).

#### **3.10.4. Culture**

Swab was cultured on Sabouraud Dextrose Agar (SDA) (appendix 2) with 0.05mg/ml Chloramphenicol and incubated at 25 °C for 48-72 hour in case of yeast and up to two weeks in case of molds (Bhavan *et al.*, 2010).

#### **3.10.5. Identification of *Aspergillus species***

##### **3.10.5.1. Colonial Morphology**

Growth rate, texture and color of the colony at surface and reverse was observed.

##### **3.10.5.2. Needle Mount**

By an inoculation needle, small portion of the colony was taken and mounted in a drop of lactic acid and Lacto Phenol Cotton Blue (LPCB) on a clean microscope slide. The slides covered with a cover slip, squash the preparation with the butt of the inoculation needle and then the excess fluid blotted off. The mount examined using low Power Field (10X) and High Power Field (40X).

##### **3.10.5.3. Slide Culture technique**

By using a sterile blade an agar block (7 x 7 mm) was cutted out, small enough to fit under a cover slip. Flip the block up onto the surface of the agar plate. The four sides of the agar block were inoculated with spores or

mycelial fragments of the fungus to be grown. Flamed coverslip was placed centrally upon the agar block. The plate was Incubated at 26 °C until growth and sporulation have occurred. The cover slip was removed from the agar block. A drop of 95% alcohol as a wetting agent was applied. Gently lower the coverslip onto a small drop of lacto phenol cotton blue on a clean glass slide. The slide was left overnight to dry and later sealed with fingernail polish. When sealing with nail polish use a coat of clear polish followed by one coat of red colored polish. Then examined under microscope by using 10X and 40X for detection of hyphae.

### **3.10.6. Identification of *Candida species***

#### **3.10.6.1. Gram's stain**

Indirect Gram's stain was performed for yeast suspected colonies which revealed Gram positive yeast cells

#### **3.10.6.2. Germ tube test (GTT)**

Test proves yeast germination, and its characteristic for the detection of *Candida albicans*

This is rapid test for presumptive identification of *C.albicans*. Three drops of serum were put into small tube by using a Pasteur pipette, by sterile wire loop a colony of yeast was touched and emulsified in the serum. After incubation at 37 °C for 2-4 hours but no longer, then a drop of the serum was transferred into a slide for examination, cover slip was placed and examined microscopically using 40X objective.

Germ tubes are appendages half the width and 3 to 4 times the length of the yeast cell from which they arise. There is no constriction between the yeast cell and the germination tube.

Positive test: presence of short lateral filament (germ tube) for *C.albicans*,  
Negative test: yeast cell only for *C. non albicans* (Bhavan *et al.*, 2010).

#### **3.10.6.3. Chrom agar pigmentation test**

Chromogenic media prepared according to manufacture instruction and the organism inoculated in the media, then incubated at 37 °C for 48 hours. After that the growth of *Candida spp* was observed by the change in the colour of the colonies according to the pigment, as a result of reaction between chromogenic substrate and enzymes that secreted by different *Candida spp*, allowing organisms to be identified to the species level by their color and colony characteristics. The result was as the following : *Candida*, the product identifies *C.albicans* by growth as light to medium green colonies, *C.tropicalis* by growth as steel blue colonies accompanied by purple pigment diffusion into surrounding agar, and *C.krusei* by growth as large, fuzzy, ross-colored colonies with white edge, individual *non albicans* species was identified as : *C.glabrata* (dark pink and wet colonies), *C.krusei* (light pink and dry colonies) and *C.albicans* ( green and wet colonies) (Babic and Hukic, 2010) (Appendix 2).

#### **3.10.6.4. Corn meal agar sporulation technique (chlamydospore formation)**

Sterile inoculating loop was used, The appropriate yeast colony was touched and immediately “X” shape was scraped through prepared corn meal agar in the middle on one half of the agar plate, the arms of the x should be about 2 cm long. This procedure was repeated, a duplicate”X” was made in the middle on the other half of the agar plate. Sterile forceps was used, and center sterile cover slip over the cross of one of the “X”patterns was applied. Plate was inverted and incubated up to 4 days (96 hour) at 25 ± 2°C. Plates were examined daily for the development of chlamydospores with the aid of dissecting or stage microscope. The”X” without cover slip

serves as a growth control. Subculture where necessary, and perform appropriate biochemical test for identification. The result was seen by microscopic examination of the yeast under the cover slip revealed chlamydospores that appear as terminal double walled spheres on the pseudohyphae which indicated positive result (Bhavan *et al.*, 2010).

(Appendix 2).

#### **3.10.6.5. Zymogram (Carbohydrate fermentation test)**

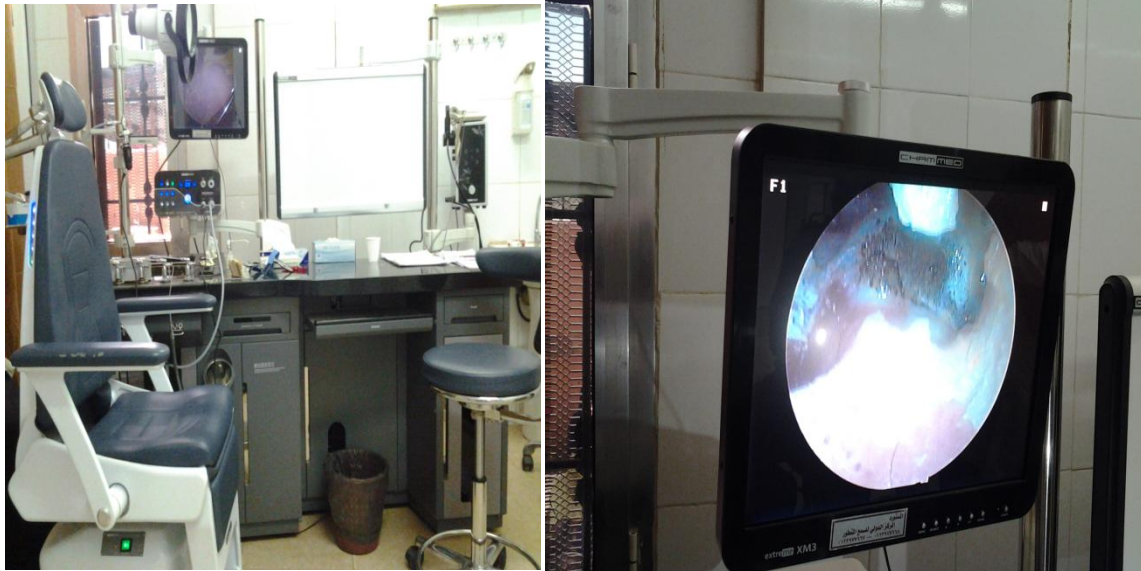
Fermentative yeasts recovered from clinical specimens produce carbon dioxide and alcohol. Production of gas rather than a pH shift is indicative of fermentation. Dextrose, maltose, sucrose, lactose, galactose and trehalose were used in the test.

The 5 ml of medium (pH, 7.4) containing 1 % peptone, 1 % sugar, 0.3 % beef extract and 0.5 % NaCl, 0.2 % Andrad's in distilled water, medium was dispensed in sterilized Durham tube and 0.2 ml of saline suspension of the test organism was added and incubated at 37° C for 10 days. The results were comparing with zymogram chart (Bhavan *et al.*, 2010).

#### **3.11. Data analysis**

Data was analyzed using SPSS (Statistical Package of Social Science) soft program version 11.5.

Graphs and chi- square were used in analysis of data.

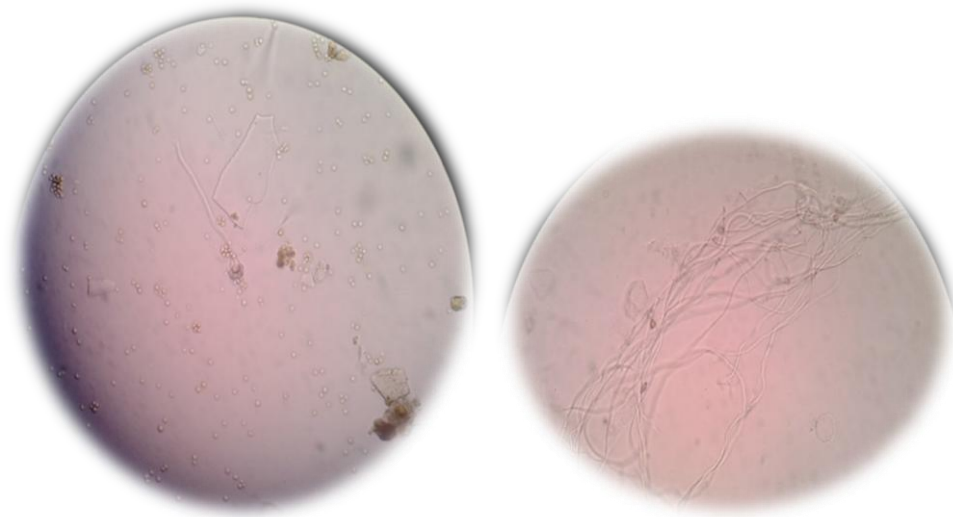


Otoscope for clinical diagnosis in aldoha hospital

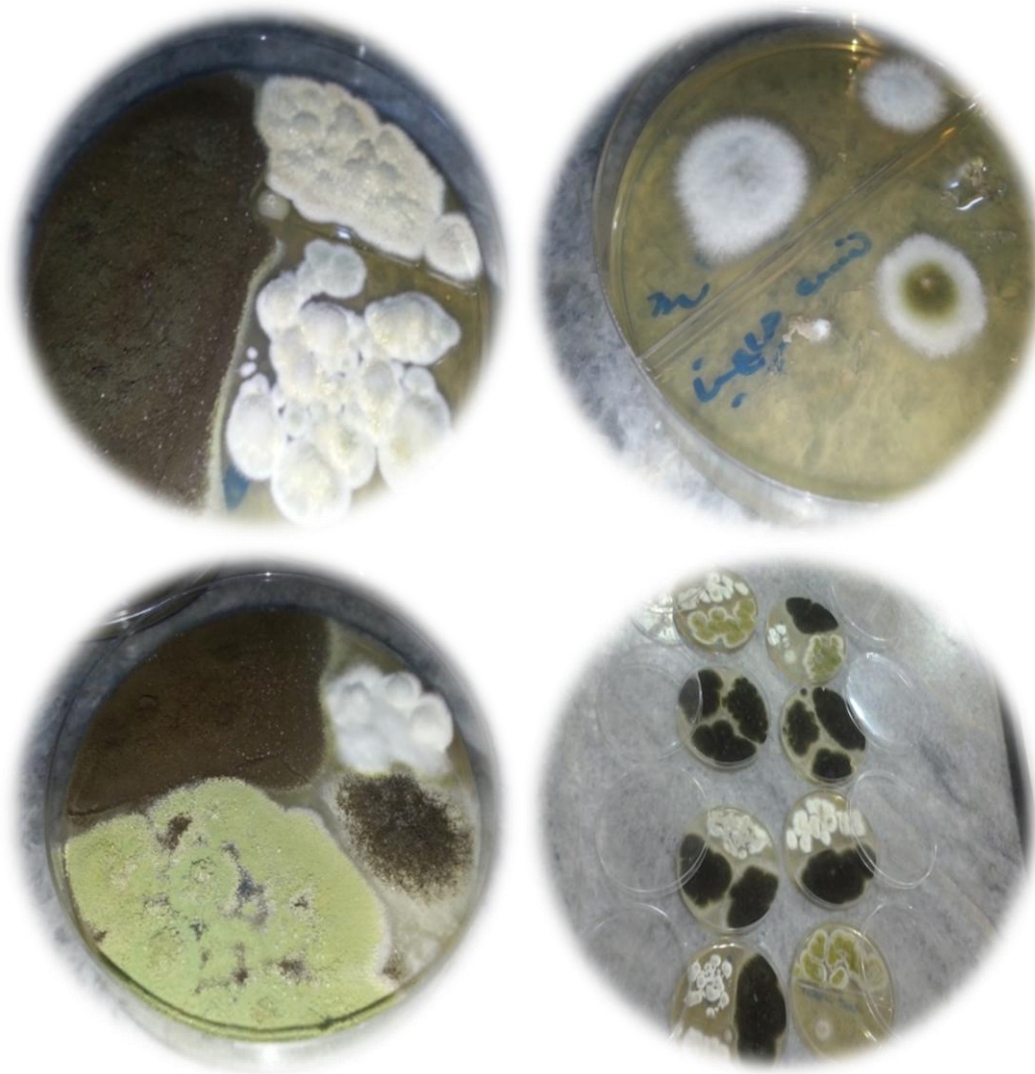


Black discharge and presence of hyphae inside ear by otoscope





Direct microscopy using KOH showed hyphae and head of Aspergillus



Aspergillus species on SDA showed

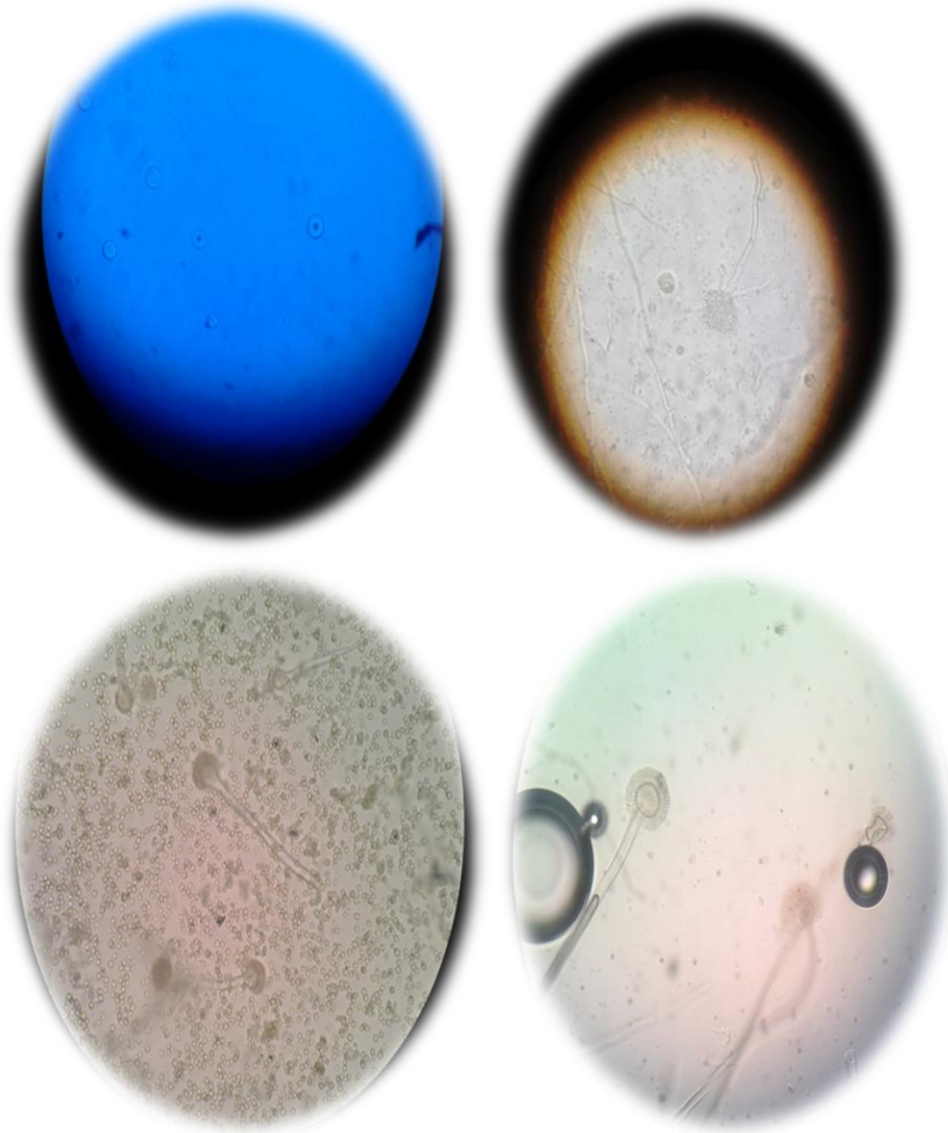
*A.niger* (Black color)

*A.flavus* (Green color)

*A.terrus* (Sinnsmon color)

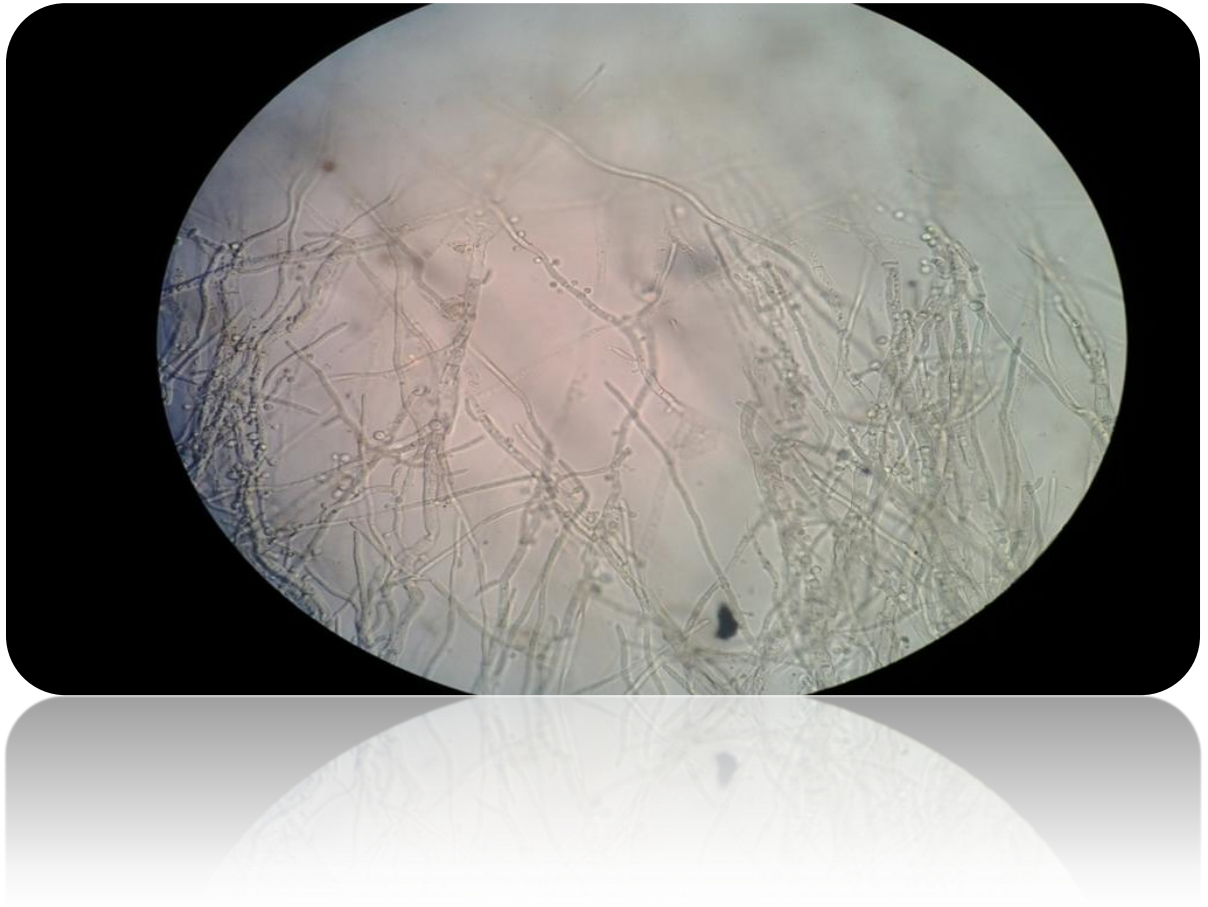
*A.nidulans* (grey to green color)

Alternaria (wooly with white color)

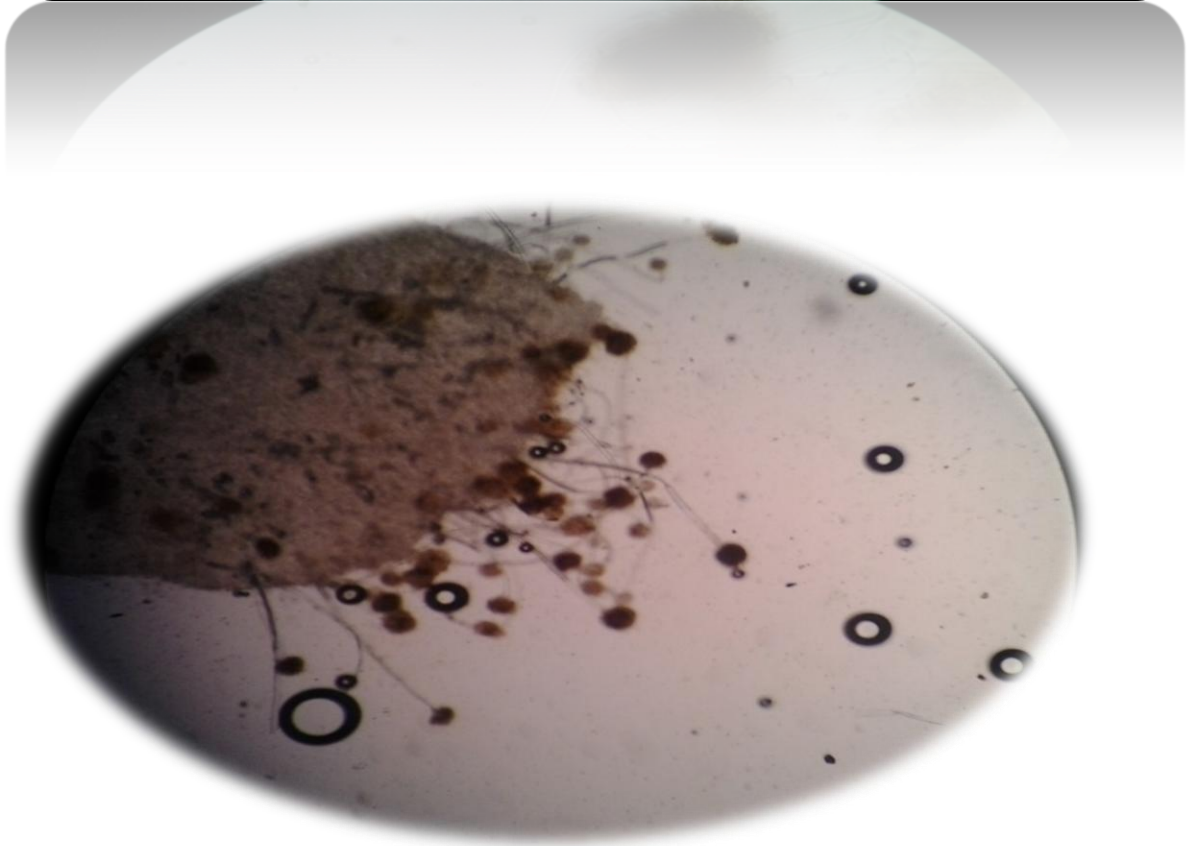
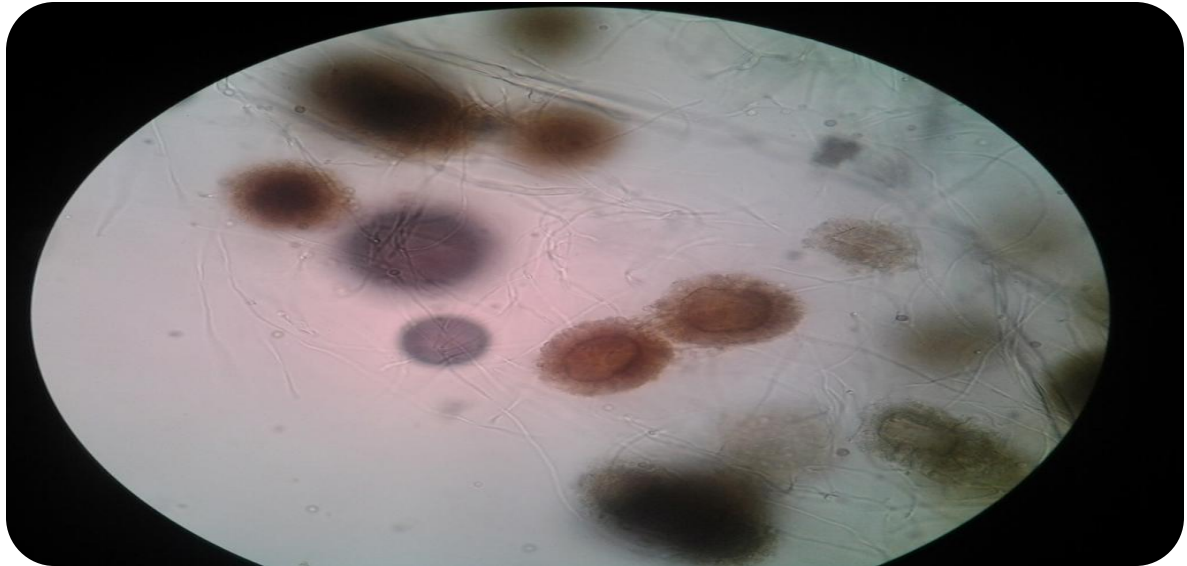


From the left to right

1. Needle mount by LPCB, showed Hull cells of *A.nidulans*
2. Needle mount by lactic acid showed *A.terrus*
3. Needle mount showed head and hyphae of *A. flavus*
4. Needle mount showed head and hyphae of *A.niger*

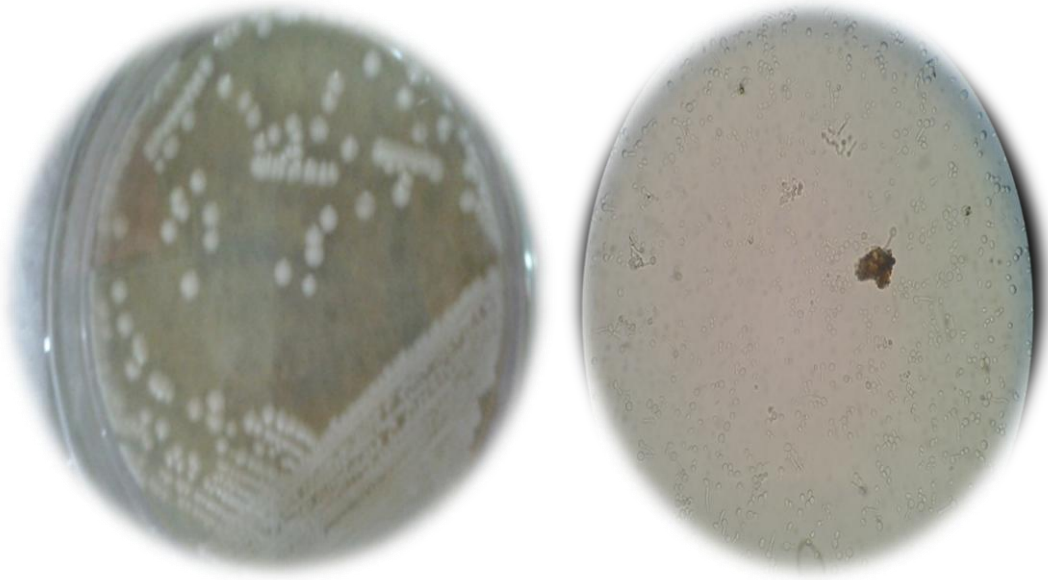


Needle mount by lactic acid showed septated hyphae



Needle mount showed head and hyphae of *A.niger*





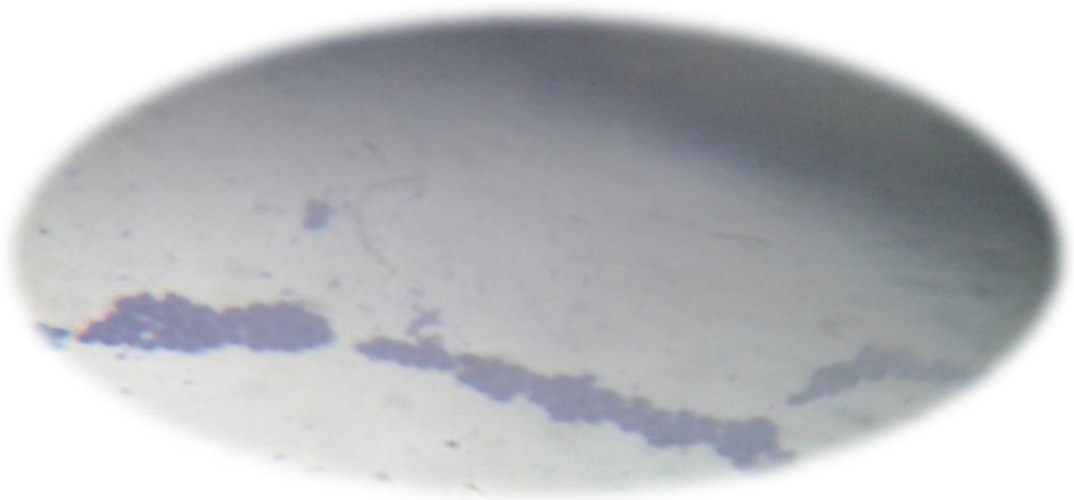
Left: *Candida* grow as white, pasty colonies on SDA

Right: GTT of *Candida albicans* showed positive result



Left: *Candida non albicans* on Chrom agar showed pink color

Right: *Candida albicans* on chrom agar showed green color

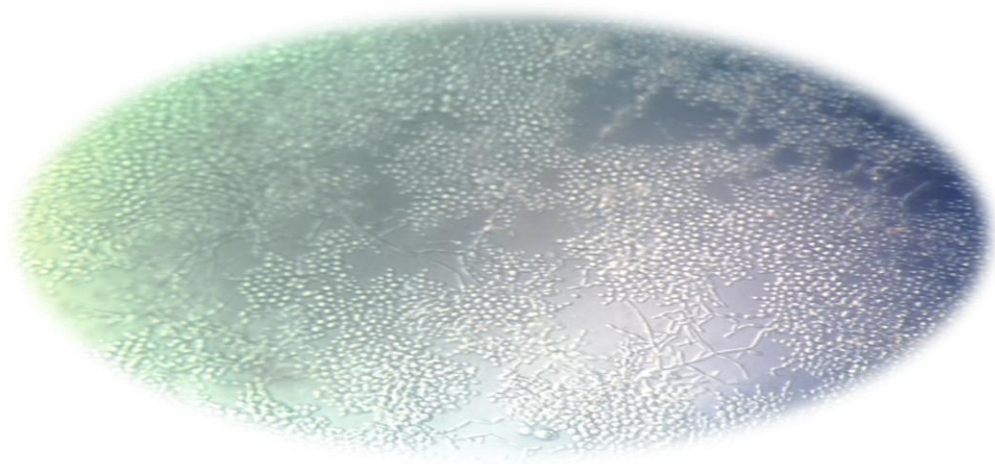
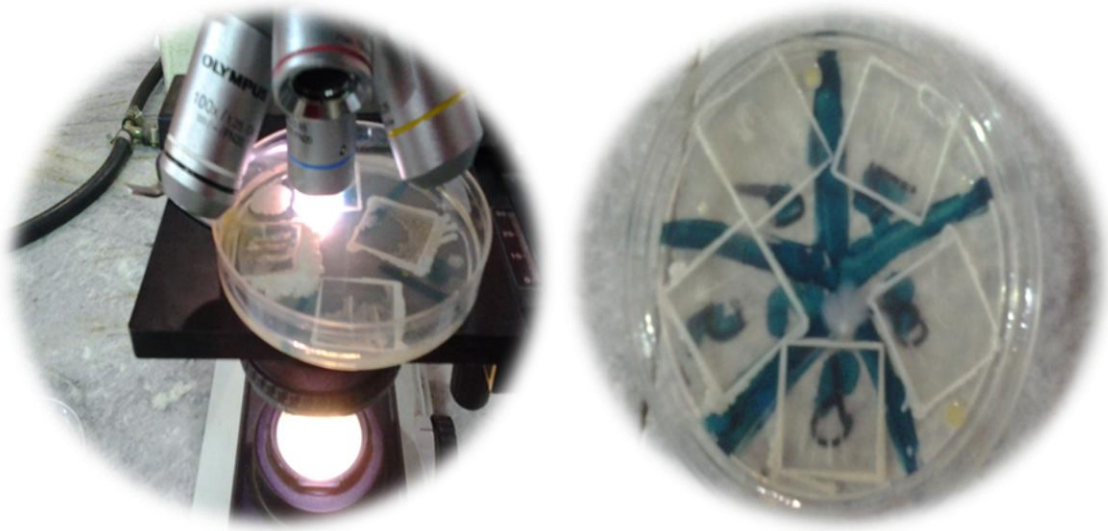


Gram's stain of *Candida* shown G+ve yeast



Candida species on Chrom agar





Corn meal agar test for chlamydospore

## CHAPTER FOUR

### 4. RESULT

A total number of 100 patients suffering of ear infection and attending Aldoha Specialized Hospital, Khartoum Ear, Nose and Throat Hospital and Africa Specialized Hospital during March to June 2014 were enrolled in this study in order to detect the frequency and etiology of otomycosis.

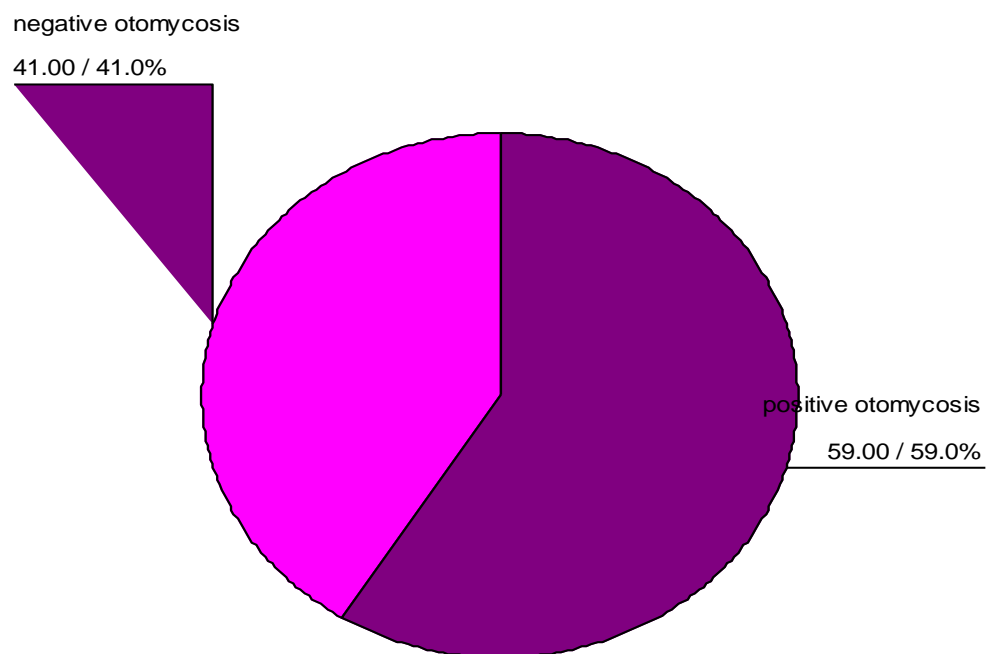
Out of the total, the overall frequency of otomycosis was 59 (59%), while the remain 41 (41%) were negative by microscopy and culture techniques (Figure1).

The study population age range was 3-80 years, with mean 38.50 year, among them the highest frequency of otomycosis 33 (33%) was observed among 31-60 year age range (Table 2).

Out of the total isolated fungi, the most frequently encountered species is *Aspergillus niger* 22 (37%), followed by *A.flavus* 21 (36%), *A.terrus* 6(10%), *C.glabrata* 5(8%) , *C.krusei* 2(3%) , *C. albicans* 1(2%) , *A.nidulans* 1(2%) and *Alternaria* 1(2%)(Figure 2).

**Table 1.** Show differences among species by different techniques

<i>Species</i>	<i>A.niger</i>	<i>A.flavus</i>	<i>A.terrus</i>	<i>C.glabrata</i>	<i>C.krusei</i>	<i>C.albicans</i>	<i>A.nidulans</i>	<i>Alternaria</i>
<b>Direct microscopy by KOH</b>	Hypahae	Hypahae	Hypahae	Budding yeast	Budding yeast	Budding yeast	Hypahae	Hypahae
<b>Direct Gram's stain</b>	/	/	/	G(+ ve) yeast	G(+ve) yeast	G(+ve) yeast	G(+ ve) yeast	/
<b>SDA</b>	Black	Green	White cinnamon	White & pasty	White & pasty	White & pasty	Grey	Black-olivaceous
<b>Chrome agar</b>	/	/	/	Dark pink & wet	Light pink & dry	Green & wet	/	/
<b>Corn meal agar(chlamydo sore formation)</b>	/	/	/	-ve	-ve	+ve	/	/
<b>Needle mount</b>	Septated hyphae, globose vesicle, biseriated & with radial conidia	Septated hyphae, Rough conidiophore, globose vesicle, biseriated & with radial conidia	Septated hyphae ,smooth, brownis, globose vwsicle, biseriated wih lon paralld conidia	/	/	/	Septated hyphae, globose vesicle, biseriated & with radial conidia with presence of huell cell	Branched acropetal chain of multicelled conidia wit short conidiophore
<b>Slide culture</b>	Same to needle mount			/	/	/	Same to needle mount	
<b>GTT</b>	/	/	/	-ve	-ve	+ve	/	/
<b>Zymogram</b>								
<b>Glucose</b>	/	/	/	+	+	+	/	/
<b>Lactose</b>	/	/	/	-	-	-	/	/
<b>Sucrose</b>	/	/	/	-	-	-	/	/
<b>Maltose</b>	/	/	/	-	-	+	/	/
<b>Galactose</b>	/	/	/	-	-	+	/	/



**Figure 1.** Frequency of otomycosis among study group (n = 100).

**Table 2.** Frequency of otomycosis according to the age range of study population.

AGE IN YEARS		PATIENT		Total
		Infected	non infected	
3-12	Count	19	18	37
	% of Total	32%	43%	37%
13-30	Count	33	18	51
	% of Total	56%	43%	51%
31-80	Count	7	5	12
	% of Total	12%	12%	12%
Total	Count	59	41	100
	% of Total	100%	41%	100%

**P value= 0.001**

**\*Sig value  $\leq 0.05$**

**Table 3.** Frequency of species among males and females

SPECIES	SEX		Total
	Male	female	
<i>A.niger</i>	6 6.0%	16 16.0%	22 37%
<i>A.flavus</i>	11 11.0%	10 10.0%	21 35.6%
<i>A.terrus</i>	3 3.0%	3 3.0%	6 10.2%
<i>C.albicans</i>	1 1.0%	0 .0%	1 1.7%
<i>C.glabrata</i>	4 4.0%	1 1.0%	5 8.5%
<i>C.krusi</i>	0 .0%	2 2.0%	2 3.4%
<i>Alternaria</i>	1 1.0%	0 .0%	1 1.7%
<i>A.nidulans</i>	0 .0%	1 1.0%	1 1.7%
Total	46 46%	54 54%	59 59%

**P value= 0.211****\*Sig ≤ 0.05**

## CHAPTER FIVE

### DISCUSSION

#### 5.1. Discussion

In recent years, incidence of otomycosis increased because number of immunocompromised patients has been raised (Nowrozi *et al.*, 2014).

Otomycosis is described as superficial mycotic infection of the External Auditory Channel (EAC) with infrequent complications involving the middle ear (Satish *et al.*, 2013).

An analysis of the age group revealed that otomycosis can affect any age from one year up to 80 years (Satish *et al.*, 2013).

In this study out of the total, the overall frequency of otomycosis was 59 (59%), while the remain 41 (41%) were negative by microscopy and culture techniques, and this result was slightly higher compared with another study achieved by Shadman Nemati *et al* in Iran, 2013, which was reported as 43%.

Overview of literature shows that among the fungus isolates, *Aspergillus niger* and *Candida* were the most common species causing otomycosis worldwide (Satish *et al.*, 2013). The present study recorded that the most frequently encountered species was *Aspergillus niger* 22 (37%), followed by *A. flavus* 21 (36%), *A. terreus* 6 (10%) , *C. glabrata* 5 (8%) , *C. krusei* 2 (3%) , *C. albicans* 1 (2%) , *A. nidulans* 1 (2%) and *Alternaria* 1 (2%) which agree with similar study conducted in India which showed *A. niger* (38%) was the most common followed by *A. fumigatus* (27%) and *A. flavus*(15%) and *Candida albicans* (4%) (Parsad *et al.*, 2014).

The present study results revealed that infection was high in the age group 31-60 years when compared with another study in Iran showed that the 20-39 year age group had the highest prevalence of otomycosis (Saki *et al.*, 2012).

Also according to gender the study slightly differ with the study of Satish and his college which showed 53% in males and 47% in females whereas in the present study, 46% of males were affected while 54% of females were affected (Satish *et al.*, 2013).

Also the present study agree with other study achieved by Kenan Değerli and his college in Turkey reveled that distribution of the molds out of 2279 patients were as follows; *Aspergillus niger* (180), *Aspergillus fumigatus*(95), *Aspergillus terreus* (32), *Aspergillus flavus* (23), *Aspergillus spp.* (14), *Penicillium spp.* (13), *Trichophyton spp.* (*T.rubrum* 1, *T.mentagrophytes* 1); while this distribution was as follows for the yeasts; *Candida tropicalis* (97), *Candida albicans* (39), *Candida parapsilosis* (21), *Candida glabrata* (19), *Candida kefir* (4), *C.guilliermondii* (2), *Candida krusei* (1), *Geotrichum candidum* (1) and *Trichosporon capitatum* (1) (Değerli *et al.*, 2012).

The present study is in agreement with other study conducted in sudan by Hashim and his colleges revealed that *Aspergillus niger* (59.6%) was the predominant species, followed by *A. flavus* (21.3%), *A. terreus* (12.8%), *Penicillium species* (2.1%), *Curvularia species* (2.1%) and *Scopulariopsis species* (2.1%). *Aspergillus species* constituted 93.6% of the total isolates and their isolation frequency from the total samples was 44%. Most of isolates (81%) were isolated from young cases, especially males (Hashim *et al.*, 2010).

## **5.2. Conclusion**

Otomycosis is seen across the world with a high incidence especially in tropical countries. This study analyzed the growth of fungi in 100 otomycotic ears.

The study concluded that the frequency of otomycosis was 59 % and the prevelant was more among female (54%) , above 30 years age group and were caused by *A.niger* 22 (37%).



### **5.3. Recommendations**

1. Diagnosis of infection should not be made on clinical criteria only due to misdiagnosis and cross reacting with bacterial infection (matching between clinical diagnosis and lab diagnosis) laboratory methods must be applied.
2. Specimens must be collected by experienced health workers by mean of otoscope.
3. In case of presence of severe itching and pain, otomycosis should be considered.

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**Appendix (1)**  
**Etiology of Otomycosis among Patients Attending Different Khartoum**  
**ENT Reference Hospitals**

**Questionnaire**

**Demographic data:**

1. Name: ..... 2. Age .....
3. Gender:
1. Male (.....) 2- Female (.....)
4. Current complains:
- .....
5. Remarks
- .....

**Laboratory investigation:**

- Macroscopic examination: .....
- Microscopic examination: Wet preparation by 10% KOH.....
- Gram's stain.....
- Culture on SDA: .....
- Identification: .....

## Appendix (2)

### Culture media

**Sabouraud dextrose agar (HiMedia Laboratories Pvt.Ltd. Mumbai, India)**

#### **Formula and preparation:**

Oxoid dehydrate medium formula (CN41)

Mycological peptone.....	10g
Dextrose .....	40g
Agar No.1.....	15 g
Distilled water 1 liter	

The medium is used at concentration of 6.5 gram in every 100 ml of DW. Prepared and sterilize the medium as structured by manufacture. Allow to cool to 50-55°C, add suitable volume of Chloramphenicol, mix well and dispense aseptically in 15-20 ml amount in sterile petri dishes (Cessbrough, 2000).

#### **Chromogenic agar medium**

For differentiation between *Candida spp.* This medium is based on sabouraud dextrose agar (Oxoid CM41) and contain (per liter) 40.0 g of glucose, 10.0g of mycological peptone, and 15.0g of agar along with a novel chromogenic glucosaminidase substrate, ammonium 4-{2-[4-(2-acetamido-2-deoxy-β-o-glucopyranosyloxy)-3-methoxyphenyl]-vinyl}-1-(propan-3-yl-oate)-quinolium bromide (0.32g/liter).

#### **Corn Meal Agar medium**

Corn Meal infusion provides nitrogen, vitamins, minerals and amino acids essential for growth. Bacteriological agar is the solidifying agent.

Formula / liter:

Corn Meal infusion .....	50g
--------------------------	-----

Agar .....15g

Final pH  $6.0 \pm 0.2$  at 25°C.

Suspend 17 grams of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete

dissolution. Sterilize in autoclave at 121°C for 15 minutes. Cool to 50°C, mix

well and dispense into Petri dishes.

### **Appendix (3)**

#### **Reagents and stains**

##### **Crystal violet Gram stain**

To make 1 litre:

Crystal violet..... 20 g

Ammonium oxalate..... 9 g

Ethanol or methanol, absolute.....95 ml

Distilled water.....1 liter

Weigh the crystal violet on a piece of clean paper (preweighed). Transfer to a brown bottle premarked to hold 1 liter. Add the absolute ethanol or methanol and mix until the dye is completely dissolved. Weigh the ammonium oxalate and dissolve in about 200 ml of distilled water. Add to the stain. Make up to the 1 litre mark with distilled water, and mix well. Store at room temperature (Chessbrough, 2000).

##### **Lugol's iodine solution**

To make 1 litre:

Potassium iodide..... 20 g

Iodine..... 10 g

Distilled water..... 1 liter



Weigh the potassium iodide, and transfer to a brown bottle premarked to hold 1 liter. Add about a quarter of the volume of water, and mix until the potassium iodide is completely dissolved. Weigh the iodine, and add to the potassium iodide solution. Mix until the iodine is dissolved. Make up to the 1 liter mark with distilled water, and mix well. Label the bottle, and store it in a dark place at room temperature. (Chessbrough., 2000).

### **Acetone-alcohol decolorizer**

To make 1 liter:

Acetone.....	500 ml
Ethanol or methanol, absolute.....	475 ml
Distilled water.....	25 ml

Mix the distilled water with the absolute ethanol (ethyl alcohol) or methanol (methyl alcohol). Transfer the solution to a screw-cap bottle of 1 liter capacity. Measure the acetone, and add immediately to the alcohol solution. Mix well. Store in a safe place at room temperature. The reagent is stable indefinitely.

### **Safranin**

For prepare safranin, 10g of safranin was weighed and transferred to a clean brown bottle. Then absolute ethanol (200ml) was added and mixed until the dye was dissolved, stored at room temperature.

To prepare the working solution, 100ml of safranin stock solution was taken to a clean bottle, then 400ml of distilled water was added and mixed (Collee, *et al.*, 1996).

### **Potassium hydroxide, 200 g/l (20% w/v)**

To make 50 ml:

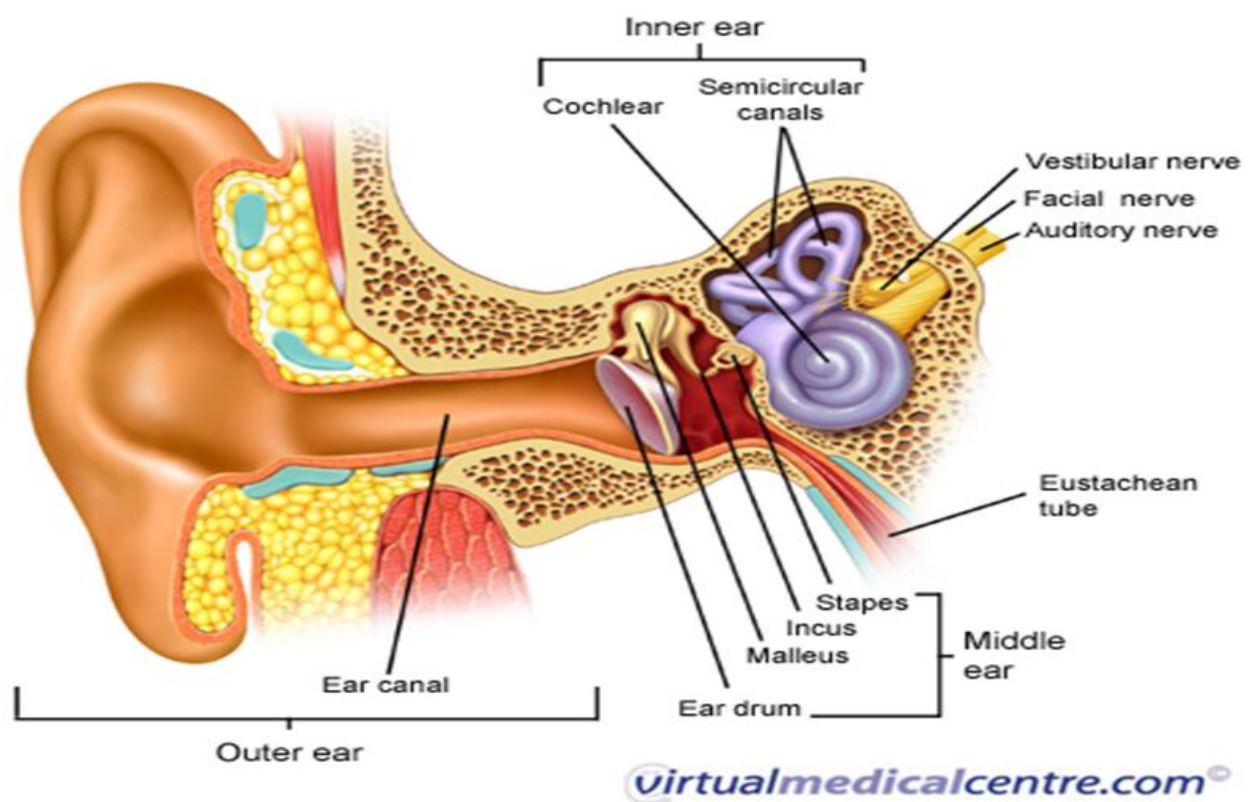
Potassium hydroxide (KOH).....	10 g
Distilled water.....	50 ml

Weigh the potassium hydroxide pellets. Transfer the chemical to a screw-cap bottle. Add the water, and mix until the chemical is completely dissolved. Store it at room temperature. The reagent is stable for up to 2 years.

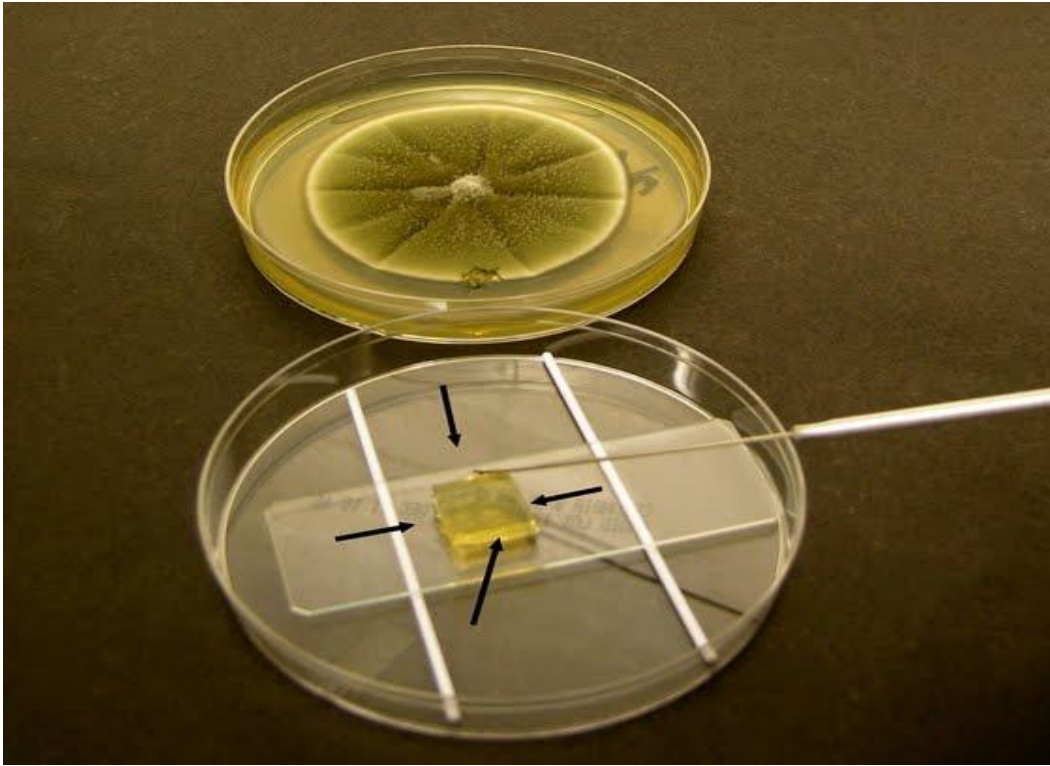
**Appendix (4): Table of zymogram test results**

<b>Candida spp.</b>	<b>Glucose</b>	<b>Lactose</b>	<b>Succrose</b>	<b>Trehalose</b>	<b>Maltose</b>	<b>Galactose</b>
<i>C. albicans</i>	+	-	-	+/-	+	+/-
<i>C. tropicalis</i>	+	-	+	+	+	+
<i>C. glabrata</i>	+	-	-	+	-	-
<i>C. kreusi</i>	+	-	-	-	-	-
<i>C. parapsilosis</i>	+	-	-	-	-	+/-
<i>C. guilliermondii</i>	+	-	+	+/-	-	+

(Bhavan *et al.*, 2010)



Anatomy of ear



Slide Culture Technique



Slide Culture technique show morphology of *Alternaria*