بسم الله الرحمن الرحيم

Isolation and Identification of Mycelial Fungi Associated with Human Ear Infections

BY

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DEDICATION

To the soul of my father, whom I miss very much,
To my loving mother who brought me up and raised me kindly and graciously until now,
To my dear brothers Esam and Gamal and lovely sisters Huda, Nagat and Awatif, who stood beside me,
To my all friends.

With great love and gratitude
Shehab

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ABSTRACT

The aim of this study was to isolate and identify the mycelial fungi (mold) associated with human ear infections.

A total of 100 ear swabs were collected from patients attending Ear, Nose and Throat (ENT) Hospital, Khartoum, during the period from June to September, 2006. Samples were taken from patients irrespective to age or sex.

The samples were subjected to conventional mycological examination after being cultured on Sabouraud's Dextrose Agar and Corn Meal Agar. The mycological examination work was executed at the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum.

Out of the 100 samples collected, 47(47%) were positive for mycelial growth, while 53 (53%) were negative. After the purification process, the total number of isolates was 47 (no specimen gave more than one type of mycelial growth).

Based on their microscopic and cultural characteristics, the isolates were identified as: *Aspergillus niger* (n=28, 59.6%) as the most dominant suspected pathogen, followed by *A. flavus* (n=10, 21.3%), *A. terreus* (n=6, 12.8%), *Penicillium* species (n=1, 2.1%), *Curvularia* species (n=1, 2.1%) and *Scopulariopsis* species (n=1, 2.1%).

The *Aspergillus* species constituted 93.6% of the total isolates and their isolation frequency from the total samples as 44%.

It was observed that 84% of patients visiting the ENT Hospital were 30 years old or less; and the rate of fungal ear infection is inversely proportional with age of patients. Likely, 38 (80.8%) isolates were obtained from ages of 30 years or less; of which 34% were from ages of 10 years or less.

Isolation frequency of mycelial fungi was almost similar in both sexes; however, in young ages (up to 30 years), the rate of isolation was higher in males than in females and vise versa in older patients.

ملخص الأطروحة

هدف هذه الدراسة هو عزل و تعريف الفطريات المشيجية ذات الصلة بإصابات الأذن فى الإنسان. تمّ جمع 100 مسحة من الأذن من مختلف الأعمار ومن الجنسين من المرضي الذين يرتادون مستشفي الأذن والأنف والحنجرة بالخرطوم في الفترة من يونيو إلي سبتمبر 2006م. أخضعت العينات للفحص المعتاد للفطريات بعد أن تمّ زرعها في وسط غراء سابرود ووسط غراء وجبة القمح. تمّ فحص العينات بمعمل الأحياء الدقيقة، كلية الطب البيطري، جامعة الخرطوم.

سبعة و أربعون (47%) من العينات التي جمعت أعطت نتائج إيجابية لنمو الفطريات المشيجية، وقد تمّ الحصول علي عدد 47 عزلة نقية. اعتماداً علي الصفات المجهرية والمزرعية، فقد تمّ تعريف هذه العزلات الي الآتي: 28 (59.6%) عزلة من نوع الرشاشية السوداء، 10 (21.3%) عزلات من نوع الرشاشية الصفراء، 6 (12.8%) عزلات من نوع الرشاشية البنية،1 (2.1%) عزلة من جنس المكنسية،1 (2.1%) عزلة من جنس سكوبيولاريوبسيس و1 (2.1%) عزلة من جنس كورفولريا. مثلت أنواع الرشاشية نسبة 93.6% من المجموع الكلي للعزلات ونسبة 44% من العينات.

لوحظ أنّ 84% من المرضي الذين يرتادون مستشفي الأذن والأنف والحنجرة ينتمون الى الفئة العمرية 30 سنة أو أقل. حيث وجد أن معدل الإصابة يتناسب عكسياً مع عمر المرضي. كذلك تمت ملاحظة أنّ 38 (80.8%) عزلة تمّ الحصول عليها من المرضي في عمر 30 سنة أو أقلّ، 42.1% منها عزلت من مرضي في عمر عشر سنوات أو أقلّ.

لم يظهر في هذه الدراسة فرق في عدد العزلات بين الجنسين بصورة اجمالية، ولكن وجد انّ عدد العزلات في الأعمار الصغيرة (حتي عمر 30 سنة) أكثر في الذكورمن الاناث والعكس في حالة الأعمار الكبيرة.

INTRODUCTION

About 180 of the 250.000 known fungal species are recognized to cause disease (mycosis) in man and animals (Greenwood, Slack and Peutherer, 2002). Although formerly considered to be plants, they are now generally assigned to their own kingdom, Fungi (Strohl, Rouse and Fisher, 2001). Most are mycelial fungi (mold), but there are a number of pathogenic yeasts and many are dimorphic (form yeast in tissue and mycelial fungi in culture) (Greenwood, Slack and Peutherer, 2002). Mycelial fungi grow as branching filaments called hyphae; whereas the unicellular yeast has an oval or spherical appearance (Quinn *et al*., 2002).

Fungi are eukaryotic, non-photosynthetic heterotrophs which produce exoenzymes and obtain nutrients by absorption. They grow aerobically and many are strict aerobes, tolerate high osmotic pressures and low pH (5.0) and resistant to antibacterial drugs. Fungi reproduce both sexually and asexually with characteristic of spore formation. Fungal species may be saprophytic, parasitic or mutualistic, widely distributed in the environment and most of fungi grow at temperature 25-37°C (Quinn *et al*., 2002).

Human fungal diseases are classified by the location or body site attacked and virulence of the etiologic agent: cutaneous when limited to the epidermis, subcutaneous when the infection penetrates significantly beneath the skin, systemic when the infection is deep within the body or disseminated to internal organs and opportunistic

when the physiological status of the host is in some way altered (immune compromised host) (Strohl, Rouse and Fisher, 2001).

Andrall and Gavrret were the first to describe fungal infection of the ear (Joy *et al.*, 1980). Otomycosis, a fungal infection of the external auditory canal, is found throughout the world. It is a superficial chronic or subacute infection, usually unilateral, characterized by inflammation, scaling, pruritus, severe discomfort and pain (Emmons *et al.*, 1977). Its prevalence is greatest in hot, humid and dusty areas of the tropical and subtropical regions of the world (Kaur *et al.*, 2000). Furthermore, the pH level in the normal ear canal is on the acidic side and the common pathogenic *Aspergillus* species experience optimal growth at a pH range of 5 to 7 (Yehia, al-habib and Shehab, 1990). Although a wide spectrum of fungi is involved, members of the genus *Aspergillus* are the most common (Kaur *et al.*, 2000).

Otomycosis was more common in young men (Kaur *et al.*, 2000). Young men are more exposed to fungal spores because they generally spend more time outdoor than others do. It is well known that the outdoor air is an important vector for locally prevalent fungal flora (Yehia, al-habib and Shehab, 1990).

It has been estimated that cases of otitis externa were between 5 and 20 % of all otological consultations; the aetiology of the majority is bacterial, only 15 –20 % are attributed to fungi. Mixed infections are generally scarce as fungal flora tends to inhibit the bacterial kind (Hueso *et al.,* 2005).

The fungi that produce otomycosis are generally saprophytic species that abound in nature and that form a part of the commensal flora of healthy ear auditory canal (EAC) (Hueso *et al.,* 2005).

Otomycosis is sometimes difficult to manage due to different factors related to the micro-organism and to the local and general characteristics of the patient. In the last few years the participation of fungi in external otitis has increased, mainly because of the use of broad-spectrum antibiotics for the treatment of bacterial otitis and to factors related to changes in immunity (Ozcan *et al.*, 2003).

The literature on the role of mycelial fungi as aetiological agents of human ear infections is scarce, especially in this country.

Objectives of this study:

1. Isolation of mycelial fungi from samples collected from patients with ear infections attending to Ear, Nose and Throat (ENT) Hospital of Khartoum.
2. To identify the isolated mycelial fungi according to their microscopic and cultural characteristics.
3. To determine the prevalence of different types of mycelial fungi found associated with ear infection, in patients of different age and sex.

CHAPTER ONE

LITERATURE REVIEW

1.1. Anatomy of the ear:

1.1.1. The external ear:

The external ear includes the pinna, external auditory meatus and tympanic membrane. The outer part has a cartilage skeleton, and the deeper part is bony; both parts are covered by skin. The skin on the outer part contains hair follicles and wax glands, but these elements are absent in the deep meatus. Skin canal migrates outward from the deep meatus, but does not desquamate until it reaches the junction with the cartilaginous meatus. The normal mechanism may be disturbed by injudicious use of cotton buds. The eardrum is the window of the middle ear and is divided into the pars tensa and pars flaccida. The main landmark on the drum is the malleus handle (Snell, 2004; Dhillon and East, 1999) (Fig.1).

1.1.2. The middle ear:

The middle ear is an air-containing space connected to the nasopharynx via the eustachian tube; it acts as an impedance matching device to transfer sound energy efficiently from air to a fluid medium in the cochlea. The middle ear space, including the mastoid air cells, is closely related to the temporal lobe, cerebellum, and jugular bulb and labyrinth of the inner ear (Snell, 2004). The space contains three ossicles (the malleus, incus and stapes), which transmit sound vibrations from the eardrum to the cochlea. The middle ear also

contains two small muscles and is traversed by the facial nerve before it exists in the skull (Dhillon and East, 1999).

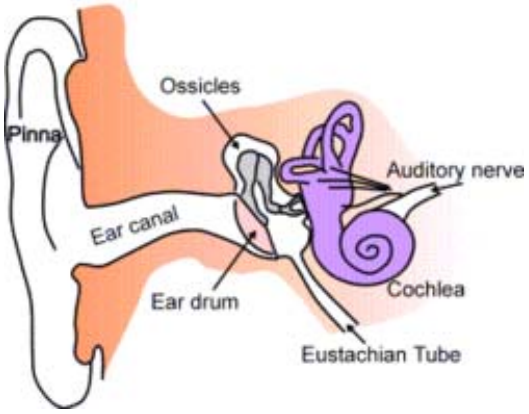


Figure 1: Anatomy of the human ear

1.1.3. The inner ear:

The inner ear comprises a dense bony capsule containing a membranous labyrinth, which forms the cochlea, vestibule and semicircular canals (Dhillon and East, 1999).

The membranous part is surrounded by fluid which communicates with the subarachnoid space and is sealed from the middle ear by the stapes footplate and round window membrane. The cochlea contains the organ of hearing which is connected by the auditory nerve to the brain stem (Snell, 2004).

Normal hearing depends on transmission of sound via a vibrating tympanic membrane through a mobile ossicular chain to the cochlea. Displacement of the basilar membrane and movement of the hair cell causes an organized volley of electrical discharge in the eighth nerve, perceived as sound in the cerebral cortex (Moore and Dalley, 1999).

The vestibule and semicircular canals form the peripheral balance organ. These have connections to the cerebellum and the eyes, and are important in the maintenance of posture and the ability to keep the eyes fixed when the head is moving (Dhillon and East, 1999).

1.2. Histology and physiology of ear:

The disease (otomycosis) is closely connected to the histology and the physiology of the ear auditory canal (EAC). This 2.5 cm long, 7-9 mm wide cylindrical canal is lined with a stratified keratinized squamous epithelium that continues along the external face of the tympanic membrane. Its interior tympanic recess, medial to the isthmus, tends to accumulate remains of keratin and cerumen; it is a difficult area to clean. The skin changes its morphology as it progresses from lateral to medial, being at its thickest in the cartilaginous canal (0.5 to 1 mm) and very thin (30 to 50 μm) in the bony canal (the internal third) (Heuso *et al.*, 2005). The external segment, besides being more adherent, is hairy and has sebaceous and ceruminous glands. The glandular secretions mix with flaked cellular epithelial elements to form an acidic ceruminous substance, impermeable to water, that protects the skin of the canal (Heuso *et al.*, 2005). Cerumen has antimycotic and bacteriostatic properties and is insect-repellent. It is composed of lipids (46 – 73%), proteins, free amino-acids and mineral ions; it also contains lysozyme, immunoglobulins and fatty polyunsaturated acids. The long chain fatty acids present in unbroken skin probably inhibit bacterial growth. Because of its hydrophobic composition, cerumen is capable of

repelling water, making the surface of the canal impermeable and thus avoiding maceration and epithelial damage (Gil-Carcedo, Vallejo and Gil-Carcedo, 2001).

1.3. Definition and synonyms of otomycosis:

Otomycosis is a superficial fungal infection of the external ear often associated with a bacterial infection or a co-existing lesion, including surgery that makes the area vulnerable to mycotic invasion. Commonly, infection involves *Candida* or *Aspergillus* spp. (Kurnatowski and Filipiak, 2001). Otomycosis is a name given to growth of *Aspergillus* spp. in the ear (Paulose *et al.*, 1989).

Mycotic otitis externa, myriangomycosis, fungal infection of the ear are synonyms of otomycosis (Emmons *et al.*, 1977).

1.4. Aetiology of otomycosis:

Otomycosis is caused by several fungi, but predominately, *Aspergillus niger, A. fumigatus, A. terreus* and *A. flavus* are the most important agents (Collee *et al.,* 1996). Species of *Scopulariopsis, Polypaecilum, Mucor, Rhizopus, Penicillium* and *Dermatophytes* also are occasional aetiologic agents, usually by extension of facial dermatophytosis (Emmons *et al.*, 1977; Kaur *et al.*, 2000). The studies by Lakshmipati and Murti (1960) and Pradhan, Tuladhar and Amayta (2003) revealed that all cases observed had been caused by either *Aspergillus* or *Candida* species.

1.5. History of otomycosis:

It was reported that 90% of the cases of fungal infection of the human ear are associated with aspergilli, of which *Aspergillus fumigatus* was one of earliest recorded and most frequently isolated species (Raper and Fennell, 1965). Their significance as causal organisms of otitis is not easily assessed, and Conant *et al*. (1954) suggested that only 15 to 20% of ear infections are true otomycosis and that the majority are of bacterial origin. The aspergilli most commonly concerned are frequently recorded from the air and from such sources as house dust, hay and straw (Raper and Fennell, 1965).

1.6. Predisposing factors of otomycosis:

Predisposing factors may play an important part in producing environments favorable for germination of the spores and subsequent hyphal growth. The accumulation of wax, epithelial debris and serious exudate provides suitable substrates and raises humidity by effectively blocking the ear canal. Infection may also occur following mastoidectomy and *Aspergillus* spp. sometimes form conidiophores within the ear canal (Raper and Fennell, 1965). The presence of excessive cerumen in patients with poor personal hygiene favors the germination of spores and conidia of the prevalent fungi (Lakshmpati and Murti, 1960).

Various factors influence the transformation of saprophytic fungi into pathogens. All of the following factors have been considered to encourage infection: changes in the epithelial covering (dermatological diseases, microtraumas), decrease in the pH level of the EAC, qualitative and quantitative alteration of the cerumen,

systemic factors (alterations in immunity, debilitating disease, corticoids, cytostatics, neoplasia), environmental factors (heat, excessive humidity), a history of bacterial infection, chronic secretory otitis media (CSOM), postsurgical mastoid cavities or instillation in the ear of oily substances or broad spectrum antibiotic therapy, etc (Hueso *et al.*, 2005).

1.7. Treatment of otomycosis:

In immunocompromised patients, systemic antifungal therapy appears necessary (Harley *et al.*, 1995). However, infections of lesser severity (without tissue invasion) or those that occur in immunocompetent patients may be managed with local measures, including cerumen removal.

A variety of such topical therapeutic options has been used, which includes cresyalte, alcohol, nystatin (ointment, powder), amphotericin B 3% topical solution, boric acid, thymol, gentian violet, iodochlorhydroxyquin (powder, lotion), 5-fluorocytosine ointment, nitrofungin, clotrimazole and ketoconazole. In these cases, prolonged therapy may be necessary (Georgiev, 1998).

1.8. Fungi implicated in otomycosis:

1.8.1. The genus *Aspergillus*:

1.8.1.1. General features:

Aspergillus is a very large genus containing over 185 species to which humans are constantly exposed. Only a small number of these species have, however, been associated with disease (Ajello and Hay, 1998).

Most *Aspergillus* species are grouped in the fungi imperfecti; some belong to the ascomycetes (Quinn *et al*., 2002).

When conditions in indoor situations are favorable for fungal growth, *Aspergillus* species are one of the primary colonizers of man-made substrata. Most species have relatively low moisture requirements and some are extremely xerophilic (dry tolerant) (Pitt and Hocking, 1999; Klich, 2002), allowing them to colonize areas that cannot support other fungi and where only minimal or intermittent moisture is available. Their rapid growth and production of large numbers of small, dry, easily aerosolized spores makes them a significant contaminant with regards to Indoor Air Quality and potential human exposure-related illnesses.

Many species are known to produce mycotoxins, including *A. flavus* (aflatoxin), *A. versicolor* (sterigmatocystin), *A. ochraceus* (ochratoxin), *A. ustus* (Austin), *A. terreus* (patulin) and *A. fumigatus* (fumitremorgin) (Frisvad and Thrane, 2000).

A few species are common opportunistic human pathogens, including *A. fumigatus,* the most common agent of aspergillosis and *A. niger*, a common agent of otomycosis (ear infection). Aspergillosis is considered the second most common type of fungal infection requiring hospitalization in the United States (Sigler and Kennedy, 1998). *Aspergillus* species are also well known allergens (type I or atopic allergy), and *A. fumigatus* is one of the most prevalent causes of type III allergy or hypersensitivity pneumonitis, and allergic sinusitis (De-Hoog *et al*., 2000).

The most important implicated species in human diseases are *A. fumigatus*, *A. niger*, *A. flavus*, *A. terreus* and *A. nidulans* (Greenwood, Slack and Peutherer, 2002).

All *Aspergillus* spp. grow in nature and in culture as mycelial fungi with septate hyphae and distinctive sporing structures; the spore-bearing hyphae (conidiophores) terminates in a swollen cell (vesicle) surrounded by one or two rows of cell (sterigmata) from which chains of asexual conidia are produced. *Aspergillus* spores are ubiquitous and in winter months counts may reach 600 spores/m^3 of air in the United Kingdom (Greenwood, Slack and Peutherer, 2002).

Aspergilli are saprophytic mycelial fungi with opportunistic pathogenic patterns depending on impaired overwhelmed or bypassed host defenses. Aspergilli grow on all common standard laboratory media (e.g. Sabouraud's Dextrose Agar, SDA) over a wide range of temperatures (up to 50°C) (Hirsh and Zee, 2002). *Aspergillus* spp. are thermotolerant such as *A. fumigatus* which can grow at temperature ranging from 20°C to 50°C (Quinn *et al*., 2002). Colonies of *Aspergillus* spp. are white at first, but later turn green to dark-green, flat and velvety. Colony color varies with different species (Carter and Wise, 2004). The color of the obverse side of colonies, which may be green, bluish-green, black, brown, yellow or reddish, varies with individual species and with cultural conditions. The color of the reverse side is pale yellow to lighten (Quinn *et al*., 2002).

1.8.1.2. Habitat (reservoir):

Aspergilli are widespread in the environment, they are commonly soil inhabitants and found in large numbers in dust and decomposing organic matter, spores are often found in the outside air (Goodley, Clayton and Hay, 1994). Also, they are found in soil, vegetation, feed, air, water and objects exposed to them (Hirsh and Zee, 2002).

1.8.1.3. Transmission:

Aspergillus is acquired from environmental sources, generally by inhalation, ingestion and direct contact (Hirsh and Zee, 2002).

1.8.1.4. Epidemiology and control:

For persons at risk for allergic disease or invasive aspergillosis, efforts are made to avoid exposure to the conidia of *Aspergillus* species. Most bone marrow transplant units employ filtered air-conditioning systems, monitor airborne contaminants in patient's rooms, reduce visiting, and institute other measures to isolate patients and minimize either risk of exposure to the conidia of *Aspergillus* and other mycelial fungi. Some patients at risk for invasive aspergillosis are given prophylactic low-dose amphotericin B or itraconazole (Brooks, Butel and Morse, 2004).

Otomycosis occurs around the world, more often in humid warm climates than arid or cold climates (Emmons *et al*., 1977).

1.8.1.5. Pathogenicity and Clinical Significance:

Aspergillus spp. are well-known to play a role in three different clinical settings in man: (i) opportunistic infections; (ii)

allergic states; and (iii) toxicoses. Immunosuppression is the major factor predisposing to development of opportunistic infections (Ho and Yuen, 2000). These infections may present in a wide spectrum, varying from local involvement to dissemination and as a whole called aspergillosis. Among all filamentous fungi, *Aspergillus* is in general the most commonly isolated one in invasive infections. It is the second most commonly recovered fungus in opportunistic mycoses following *Candida.*

Almost any organ or system in the human body may be involved. Onychomycosis, sinusitis, cerebral aspergillosis, meningitis, endocarditis, myocarditis, pulmonary aspergillosis, osteomyelitis, otomycosis, endophthalmitis, cutaneous aspergillosis, hepatosplenic aspergillosis, as well as *Aspergillus* fungemia, and disseminated aspergillosis may develop (Gupta and Summerbell, 1999; Gillespie and O'Malley, 2000). Nosocomial occurrence of aspergillosis due to catheters and other devices is also likely (Lucas, Tucker and Merz, 1999). Construction in hospital environments constitutes a major risk for development of Aspergillosis, particularly in neutropenic patients (Loo *et al*., 1996).

Aspergillus spp. may also be local colonizers in previously developed lung cavities due to tuberculosis, sarcoidosis, bronchiectasis, pneumoconiosis, ankylosing spondylitis or neoplasms, presenting as a distinct clinical entity, called aspergilloma (Glimp and Bayer, 1983). Aspergilloma may also occur in kidneys (Halpern *et al.*, 1992).

Some *Aspergillus* antigens are fungal allergens and may initiate allergic bronchopulmonary aspergillosis particularly in atopic host (Kurup and Banerjee, 2000). Some *Aspergillus* spp. produce various mycotoxins. These mycotoxins, by chronic ingestion, have proven to possess carcinogenic potential particularly in animals. Among these mycotoxins, aflatoxin is well-known and may induce hepatocellular carcinoma. It is mostly produced by *Aspergillus flavus* and contaminates foodstuff, such as peanuts (Quinn *et al*., 2002).

Aspergillus spp. can cause infections in animals as well as in man. In birds, respiratory infections may develop due to *Aspergillus*. It may induce mycotic abortion in cattle and sheep (St-Germain and Summerbell, 1996). Ingestion of high amounts of aflatoxin may induce lethal effects in poultry fed with grain contaminated with the toxin. Since *Aspergillus* spp. are found in nature, they are also common laboratory contaminants.

At present, the mechanisms of pathogenicity are not known. However, it is thought that toxic components may be, in part, associated with formation of hemorrhagic lesions. In addition, the activity of enzymes such as elastase may be associated with tissue damage. Primary and secondary infections occur in a wide variety of tissues and locations; lungs, skin, nasal sinuses, external ear, bronchi, bones and meninges. Infection occurs most frequently in immunocompromised patients (Carter and Wise, 2004).

1.8.1.6. Aspergillosis:

1.8.1.6.1. Definition of Aspergillosis:

Broadly defined, aspergillosis is a group of mycosis with diverse causes and pathogenesis. *Aspergillus* may colonize and then invade tissue in a traumatized cornea or in burns, wounds, or the external ear (otitis externa). *Aspergillus* species become opportunistic invaders in immunodeficient persons or individuals with anatomic abnormalities of the respiratory tract (Jawetz *et al*., 1987).

1.8.1.6.2. History of Aspergillosis:

One of the first identifiable cases of aspergillosis was cited by Virchow in 1856 (Emmons *et al*., 1977). More recent studies reveal that *Aspergillus* is becoming increasingly important in patients with malignant disease, especially with leukemia and lymphoma and in patient receiving immunosuppressive therapy for a wide range of illness (Emmons *et al*., 1977).

1.8.1.7. Otomycosis:

Otomycosis is a fungal infection of the ear that is seen in the tropical and subtropical regions of the world (Kaur *et al*., 2000).

It is the name given to growth of *Aspergillus* spp., usually *A. niger* or *A. fumigatus* within the external auditory canal (Paulose *et al*., 1989). Patients present with decreased hearing, itching, pain or discharge from the canal. Otoscopy reveals greenish or black fuzzy growth on the cerumen or debris in the auditory canal (Ajello and Hay, 1998). *Aspergillus* spp. invade the external auditory canal of

immunocompromised patients, extending into contiguous bone or even brain (Ajello and Hay, 1998).

Kerato-mycosis can be caused by several *Aspergillus* species and mostly follows trauma. *A. niger* is the species predominantly isolated from otitis externa, although it is less common in air than *A. fumigatus* and so a degree of specialization or adaptation to the microenvironment of the ear and the respiratory tract is evident (Collee *et al.*, 1996).

A. fumigatus and other spp. sometimes form conidiophore within the ear canal (Raper and Fennell, 1965). Although otomycosis may be caused by any one of several fungi (*A. niger* grows on cerumen, epithelial scales and detritus deep in the external canal). The resulting plug of mycelium and debris causes irritation, pruritus and impairment of hearing. The dermatophytes may invade the ear canal usually be extension of facial dermatophytosis (the lesion is pruritic, dry or eczematous and usually without plug formation). In rare cases of otomycosis *Mucor*, *Absidia*, *Rhizopus* and species of *Aspergillus* other than *A. niger* may grow in the ear canal (Emmons *et al.*, 1977).

1.8.1.7.1. Clinical types and differential diagnosis:

Simple otomycosis without complicating bacterial infection is characterized by inflammation, pruritus, exfoliation of epithelium and often by partial deafness when the ear canal is occluded by a plug of hyphae, cerumen and epithelial debris. Inflammation and scaling may extend to the inner end of the ear canal. The tympanic membrane rarely is perforated. Otomycosis must be differentiated from bacterial

otitis, which usually is exudative with a foul odor, and from which species of *Corynebacterium*, *E. coli*, *Pseudomonas*, *Proteus*, *Micrococcus* or *Streptococcus* can be isolated. It is also must be differentiated from seborrheic dermatitis (which usually occurs below and back of the ear canal), from impetigo, furunculosis and contact dermatitis (Emmons *et al.*, 1977).

Prior to 1999, the diagnosis of otomycosis as a cause of persistent otorrhea was rare. An increase incidence has been seen in outpatient pediatric otolaryngology practice (Jackman *et al.*, 2005).

Acute diffuse otitis externa (swimmer's ear), otomycosis, exostoses, traumatic eardrum perforation, middle ear infection, and barotraumas of the inner ear are common problems in swimmers and people engaged in aqua activities (Wang *et al.*, 2005), which are needed to be considered in differential diagnosis.

The high incidence can be attributed to the high degree of humidity and heat, the dusty environment, and the fact that a large proportion of the population is made up of outdoor laborers and reasons of low socioeconomic status. Other contributing factors might be such habits as cleaning the ear with a matchstick and applying oil or fatty acids to the area (Kaur *et al.*, 2000).

Other manifestations of aspergillosis include Allergic aspergillosis, Infection of the paranasal sinuses, Aspergilloma (fungus ball), Chronic necrotizing aspergillosis, Infection of the central nervous system, invasive aspergillosis, ocular infections, endocarditis and myocarditis, osteomyelitis, skin infections, infection of the

gastrointestinal tract and hepatic and splenic infection (Ajello and Hay, 1998).

1.8.1.8. Treatment of *Aspergillus* infections:

Treatment of invasive aspergillosis is still troublesome with high rate of mortality (Denninge, 1996). While amphotericin B (including its lipid formulations) and itraconazole are the currently available therapeutic options, the clinical success rate is still unsatisfactory due both to the low efficacy and/or high toxicity of the drugs and existence of unfavorable immune status of the host, such as lack of recovery from neutropenic state (Fisher *et al*., 1999; Elgamal and Murshid, 2000).

The novel azoles (e.g., voriconazole, posaconazole, or ravuconazole), glucan synthesis inhibitors (e.g., caspofungin, V-echinocandin, FK463) and liposomal nystatin are active in vitro against *Aspergillus* and remain promising for future therapy of aspergillosis (Arikan and Rex, 2000; Moore, Walls and Denning, 2000).

1.8.1.9. Pathological and morphological differentiation of *Aspergillus* species:

1.8.1.9.1. *Aspergillus niger:*

Aspergillus niger is less common cause of aspergillosis and it has a musty odor. It is commonly found in the environment on textiles, in soils, grains, fruits and vegetables (Al-Doory and Domson, 1984).

Aspergillus niger has been reported to cause skin and pulmonary infections. It is a common cause of otomycosis (Al-Doory and Damson, 1984; Crissy and Lang, 1995).

In tropical areas, otomycosis does occur due to *A. niger* invasion of the outer ear canal, but this may be caused by mechanical damage of the skin barrier. *A. niger* strains produce a series of secondary metabolites, but it is only ochratoxin A that can be regarded as a mycotoxin in the strict sense of the word (Schuster *et al.*, 2002). The organism is a common secondary invader following bacterial otitis (Steinbach and Stevens, 2003). It may also cause pulmonary disease in immunocompromised patients and the production of oxalate crystals in clinical specimens (Nakagawa *et al.*, 1999).

1.8.1.9.1.1. Cultural characteristics:

Colonies on potato dextrose agar at 25°C are initially white, quickly becoming black with conidial production. Reverse is pale yellow and growth may produce radial fissures in the agar.

1.8.1.9.1.2. Microscopic features:

Hyphae are septate and hyaline. Conidial heads are radiate initially, splitting into columns at maturity. The species is biseriate (vesicles produces sterile cells known as sterigmata that support the conidiogenous phialides). Conidiophores are long (400-3000 μm), smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle (30-75 μm in diameter). Sterigmata and phialides cover the entire vesicle. Conidia are brown to black, very rough,

globose, and measure 4-5 µm in diameter (Sutton, Fothergill and Rinaldi, 1998; De-Hoog *et al.*, 2000).

Aspergillus niger at first look may resemble *Syncephalastrum*. The absence of phialides and the presence of tubular sporangia in *Syncephalastrum* isolates are noticed by careful examination. In contrast to *Aspergillus*, the hyphae of *Syncephalastrum* are nonseptate (Larone, 1995).

1.8.1.9.2. *Aspergillus flavus*:

Aspergillus flavus grows on moldy corn and peanuts, it can be found in warm soil, foods and diary products (Al-Doory and Domson, 1984). Some strains are capable of producing a group of mycotoxins in the aflatoxin group. Aflatoxins are known animal carcinogen. There is limited evidence to suggest that this toxin is a human carcinogen. The toxin is poisonous to humans by ingestion. It may also result in occupational disease via inhalation (Roberts, Hay and Mackenzie, 1994). It is toxic to the liver (Al-Doory and Domson, 1984) also it is reported to be allergic, its presence is associated with reports of asthma; also it can be found in water damaged carpets.

The production of the fungal toxin is dependent on the growth condition and on the substrate used as a food source. This fungus is associated with aspergillosis of the lungs and or disseminated aspergillosis. Aspergillus flavus is occasionally identified as a cause of corneal, otomycotic and naso-orbital infections (Al-Doory and Domson, 1984; Crissy, Lang and Parish, 1995).

This species is the aetiologic agent in a wide range of infections including mycotoxicoses owing to aflatoxins, hypersensitivity pneumonitis (De-Hoog *et al.*, 2000), otitis (Harley *et al.*, 1995), sinusitis and invasive disease. Some reports suggest the disease process may be potentiated by aflatoxins (Mori *et al.,* 1998), particularly in the immunocompromised host. Organism is extremely angioinvasive with resultant necrosis and infarction.

1.8.1.9.2.1. Cultural characteristics:

Effuse, lime green colonies with rough conidiophores and smooth to very finely roughed conidia distinguish this species from the similar *Aspergillus parasiticus* that produces very rough conidia.

Colonies on potato dextrose agar at 25°C are olive to lime green with a cream reverse. Rapid growth, texture is woolly to cottony to somewhat granular. Sclerotia, when present, are dark brown. A clear to pale brown exudate may be present in some isolates (Raper and Fennell, 1965; Sutton, Fothergill and Rinaldi, 1998; De-Hoog *et al.*, 2000).

1.8.1.9.2.2. Microscopic features:

Hyphae are septate and hyaline. Conidial heads are radiate to loosely columnar with age. Conidiophores are coarsely roughened, uncolored, up to 800 µm long x 15 – 20 µm wide, vesicles globose to sub-globose (20 – 45 µm), metulae (8 – 10 x 5 – 7 µm) covering nearly the entire vesicle in biseriate species. Some isolates may remain uniseriate, producing only phialides (8 - 12 x 3 – 4 µm) covering the

vesicle. Conidia are smooth to very finely roughened, globose to subglobose, 3 - 6 μm in diameter (Raper and Fennell, 1965; Sutton, Fothergill and Rinaldi, 1998; De-Hoog *et al.*, 2000).

1.8.1.9.3. *Aspergillus terreus:*

This species is noteworthy for its refractoriness to amphotericin B therapy (Steinbach *et al.*, 2004).

1.8.1.9.3.1. Cultural characteristics:

Colonies on potato dextrose agar at 25°C are beige to buff to cinnamon. Reverse of the colony is yellow and yellow soluble pigments are frequently present, moderate to rapid growth rate. Colonies become finely granular with conidial production. Isolates that are initially white and producing only accessory conidia may be mistaken for *Histoplasma capsulatum* (Sutton, Fothergill and Rinaldi, 1998).

1.8.1.9.3.2. Microscopic features:

Hyphae are septate and hyaline. Conidial heads are biseriate and columnar. Conidiophores are smooth-walled and hyaline, 70 to 300μm long, terminating in mostly globose vesicles. Conidia are small (2-2.5 μm), globose, and smooth. Globose, sessile, hyaline accessory conidia (2-6 μm) frequently produced on submerged hyphae (Sutton, Fothergill and Rinaldi, 1998; De-Hoog *et al.*, 2000).

1.8.2. *Penicillium* species:

1.8.2.1. General features:

The genus *Penicillium* comprises a diverse group of species of tremendous importance in the human environment. It contains over 200 species, among which, *Penicillium marneffei* is the species known to be a primary pathogen of humans and animals (Ajello and Hay, 1998).

Some species are known agents of food decay, while others are prevalent on various organic substrata. A wide variety of species are known to grow on indoor surfaces including drywall, wood, carpet, painted surfaces, wallpaper, and various types of household contents (Abbot, 2002).

The *Penicillium* spp. are common and as cosmopolitan as the aspergilli; they are the so-called green and blue mycelial fungi, which are also frequently found on citrus, fruits and other feed stuffs that has become contaminated with their spores. The conidia of *Penicillium*, like those of *Aspergillus*, are found in everywhere (Alexopulos, 1962).

Some of *Penicillium* spp. have been found to be associated with animal and human diseases; and some *Penicillium* spp. grow and produce toxins in stored barley, maize and wheat grains (Quinn *et al.*, 2002).

Penicillium spp. produce septate mycelium and conidia in chains, but their conidiophores, unlike those of aspergilli, are branched, bearing several phialides creating a broom-like structure (brush-like) (Hirsh and Zee, 2002). The colonies grow at rates

comparable to *Aspergillus* and develop a blue-green pigmentation (Hirsh and Zee, 2002).

1.8.2.2. Description and natural habitats:

With only one exception (*Penicillium marneffei*, which is thermally dimorphic), the members of the genus *Penicillium* are filamentous fungi. *Penicillium* spp. are widespread (ubiquitous) in our environment and are found in soil, decaying vegetation, and the air. Showing again how it is distinct from other species in this genus, *Penicillium marneffei* is endemic specifically in South East Asia where it infects bamboo rats which serve as epidemiological markers and reservoirs for human infections.

Penicillium spp., other than *Penicillium marneffei* are commonly considered as contaminants but may cause infections, particularly in immunocompromised hosts. *Penicillium marneffei* is pathogenic particularly, in patients with AIDS and its isolation from blood is considered as an HIV marker in endemic areas. In addition to their infectious potential, *Penicillium* spp. are known to produce mycotoxins (Pitt *et al*., 2000). Some *Penicillium* spp. have teleomorphs included in genera *Eupenicillium*, *Talaromyces*, *Harnigera*, and *Trichocoma*.

1.8.2.3. Common species of *Penicillium*:

The genus *Penicillium* has several species. The most common ones include *Penicillium chrysogenum*, *Penicillium citrinum*, *Penicillium janthinellum*, *Penicillium marneffei* and *Penicillium purpurogenum*.

Identification to species level is based on macroscopic morphology and microscopic features (De-Hoog *et al*., 2000).

1.8.2.4. *Penicillium* toxins:

Many species are known to produce mycotoxins, including *P. aurantiogriseum* (penicillic acid), *P. viridicatum* (viridicatin), *P. verrucosum* (ochratoxin), *P. islandicum* (luteoskyrion), *P. variabile* (rugulosin), *P. crustosum* (penitrem A), *P. griseofulvum* (patulin, griseofulvin), *P. citrinum* (citrinin), and *P. crateriforme* (rubratoxin), as well as many others (Pitt and Hocking, 1999; Abbott, 2002). Potential mycotoxicoses from ingestion of moldy food and feed are significant hazards. Inhalation of *Penicillium* spores containing mycotoxins is implicated as a contributing factor for organic dust toxic syndrome (ODTS) and non-infectious fungal indoor environment syndrome (NIFIES). *Penicillium* is also a proven causal agent of allergy (atopic allergy and hypersensitivity pneumonitis) and one species (*P. marneffei*) is a well known opportunistic pathogen (Sigler and Kennedy, 1998).

1.8.2.5. *Penicillium* infections:

Penicillium species are occasional causes of infection in human and the resulting disease is known generically as penicilliosis. *Penicillium* has been isolated from patients with keratitis (Deshpande and Koppikar, 1999), endophthalmitis, otomycosis, necrotizing, esophagitis, pneumonia, endocarditis, peritonitis and urinary tract infection. Most *Penicillium* infections are encountered in immunosuppressed hosts. Corneal infections are usually

posttraumatic (Deshpande and Koppikar, 1999). In addition to its infectious potential, *Penicillium verrucosum* produces a mycotoxin (ochratoxin A) which is nephrotoxic and carcinogenic. The production of the toxin usually occurs in cereal grains at cold climates (Pitt, 2000).

Penicillium marneffei is a pathogenic fungus and specifically infects patients with AIDS, especially in South East Asia (Thailand and Adjacent countries, Taiwan and India), where the fungus is endemic (Ajello and Hay, 1998). *Penicillium marneffei* infections have also been reported in patients with haematological malignancies and receiving immunosuppressing therapy.

1.8.2.6. Cultural characteristics:

The colonies of *Penicillium* other than *Penicillium marneffei* are rapid growing and mature within four days, flat, filamentous, velvety and woolly or cottony in texture. The colonies are initially white and become blue green, gray-green, olive gray, yellow or pinkish in colour. The plate reverse is usually pale to yellowish (De-Hoog *et al.*, 2000).

Penicillium marneffei is thermally dimorphic and produces filamentous, flat colonies at 25°C. These colonies are bluish-gray-green at center and white at the periphery. The red, rapidly diffusing, soluble pigment observed from the reverse is very typical. At 37°C, *Penicillium marneffei* colonies are cream to slightly pink in colour and glabrous to convolute in texture (De-Hoog *et al.*, 2000).

1.8.2.7. Microscopic features:

For species other than *Penicillium marneffei*, septate hyaline hyphae (1.5 to 5 μm in diameter), simple or branched conidiophores, metulae, phialides, and conidia are observed. Metulae are secondary branches that form on conidiophore. The metulae carry the flask-shaped phialides. The organization of the phialides at the tips of the conidiophores is very typical. They form brush-like clusters which are also referred to as "penicilli". The conidia (2.5-5 μm in diameter) are rounding, unicellular, and visualized as unbranching chains at the tips of the phialides (Larone, 1995).

In its filamentous phase, *Penicillium marneffei* is microscopically similar to the other *Penicillium* species. In its yeast phase, on the other hand, *Penicillium marneffei* is visualized as globose to elongated sausage-shaped cells (3 to 5 μm) that multiply by fission.

Penicillium marneffei is easily induced to produce the arthroconidial yeast-like state by subculturing the organism on an enriched medium like brain heart infusion (BHI) and incubating it at 35°C, in which after a week, yeast-like structures dividing by fission and hyphae with arthroconidia are formed (St-Germain and Summerbell, 1996; Sutton, Fothergill and Rinaldi, 1998).

Compared to other fungi, *Penicillium* differs from Paecilomyces by having flask-shaped phialides and globose to sub-globose conidia; from *Gliocladium* by having chains of conidia; and from *Scopulariopsis* by forming phialides. *Penicillium marneffei* differs as well by its thermally dimorphic nature.

1.8.2.8. Treatment of *Penicillium* infections:

Amphotericin B, itraconazole, and fluconazole have so far been used in treatment of penicilliosis (Lortholary, Denning and Dupont, 1999). Itraconazole was found to be efficient when used prophylactically against *Penicillium marneffei* in patients with HIV infection (Chariyalertsak *et al.*, 2001).

1.8.3. *Curvularia* species:

1.8.3.1. General features:

Curvularia is a prevalent member of the dematiaceae family of the hyphomycetes class of fungi. These fungi contain melanin in their hyphae. Recently, reports have surfaced that saprobic fungi, as well as fungi pathogenic for plants have evolved as human pathogens: reported cases have involved skin infection, endocarditis, brain abscess, onychomycosis, keratitis, mycetoma, bronchopulmonary disease and rhinosinusitis. As most of these infections occurred in immunocompromised patients, other patients appeared to be immunocompetent (Schell, 2000).

1.8.3.2. Description and natural habitats:

Curvularia is a dematiaceous filamentous fungus. Most species of *Curvularia* are facultative pathogens of plants, and cereals in tropical or subtropical areas, while the remaining few are found in temperate zones. As well as being a contaminant, *Curvularia* may cause infections in both humans and animals (Larone, 1995).

1.8.3.3. Common species of *Curvularia*:

The genus *Curvularia* contains several species, including *Curvularia brachyspora*, *Curvularia clavata*, *Curvularia geniculata*, *Curvularia lunata*, *Curvularia pallescens*, *Curvularia senegalensis*, and *Curvularia verruculosa*. *Curvularia lunata* is the most prevalent cause of disease in humans and animals.

1.8.3.4. Pathogenicity and clinical significance:

Curvularia species are among the causative agents of phaeohyphomycosis, wound infections, mycetoma, onychomycosis, keratitis, allergic sinusitis, cerebral abscess, cerebritis, pneumonia, allergic bronchopulmonary disease, endocarditis, dialysis-associated peritonitis, and disseminated infections may develop due to *Curvularia* spp. (Anaissie *et al*., 1989). Importantly, the infections may develop in patients with intact immune system. However, similar to several other fungal genera, *Curvularia* has recently emerged also as an opportunistic pathogen that infects immunocompromised hosts (Ebright *et al*., 1999; Guarro *et al*., 1999).

1.8.3.5. Cultural characteristics:

Curvularia produces rapidly growing, woolly colonies on potato dextrose agar at 25°C. From the front, the color of the colony is white to pinkish gray initially and turns to olive brown or black as the colony matures. From the reverse, it is dark brown to black (St-Germain and Summerbell, 1996; Sutton, Fothergill and Rinaldi, 1998).

1.8.3.6. Microscopic features:

Septate brown hyphae, brown conidiophores, and conidia are visualized. Conidiophores are simple or branched and are bent at the points where the conidia originate (De-Hoog *et al*., 2000; Larone, 1995). This bending pattern is called sympodial geniculate growth. The conidia (8-14 x 21-35 μm), which are also called the poroconidia, are straight or pyriform, brown, multiseptate, and have dark basal protuberant hila. The septa are transverse and divide each conidium into multiple cells. The central cell is typically darker and enlarged compared to the end cells in the conidium. The central septum may also appear darker than the others. The swelling of the central cell usually gives the conidium a curved appearance (St-Germain and Summerbell, 1996; Sutton, Fothergill and Rinaldi, 1998).

Compared to other fungi, *Curvularia* is distinguished from *Bipolaris* and *Drechslera* by its conidial septa lying from one side wall to the other (not distoseptate). Also, unlike that of *Bipolaris*, conidium of *Curvularia* is usually curved, has an enlarged, darker central cell, thinner cell wall, and narrower septations between the cells (Larone, 1995; St-Germain and Summerbell, 1996)

1.8.3.7. Treatment of *Curvularia* infections:

Treatment modalities for *Curvularia* infections have not been standardized yet. Amphotericin B, itraconazole, and terbinafine have so far been used to treat *Curvularia* infections. However, the prognosis

is usually poor, particularly for immunocompromised patients (Ajello and Hay, 1998).

1.8.4. *Scopulariopsis* species:

1.8.4.1. Description and natural habitats:

Scopulariopsis is a filamentous fungus that inhabits soil, plant materials, feathers, ears, dust and insects. It is distributed worldwide. Several species of *Scopulariopsis* have teleomorphs which are classified in the genus *Microascus*. While *Scopulariopsis* is commonly considered as a contaminant, it may cause infections in humans, particularly in immunocompromised patients (Larone, 1995; De-Hoog *et al*., 2000).

1.8.4.2. Common species of *Scopulariopsis*:

The genus *Scopulariopsis* is unique in that it contains both moniliaceous (hyaline) and dematiaceous species, with several being clinically significant. The most common species is *S. brevicaulis*, a hyaline mould. Other non-pigmented species include *S. candida*, which remains white at maturity rather than becoming buff-colored, *S. koningii*, *S. acremonium*, and *S. flava*. Dematiaceous or phaeoid members include *S. cinerea*, the anamorph of *Microascus cinereus*, *S. trigonospora*, the anamorph of *M. trigonosporus*, the *Scopulariopsis* anamorph of *M. cirrosus*, *S. brumptii*, *S. chartarum*, *S. fusca*, and *S. asperula*.

1.8.4.3. Pathogenicity and clinical significance:

Scopulariopsis spp. may cause various infections in humans (De-Hoog *et al.*, 2000). It is among the fungi that cause onychomycosis

especially of the toe nails. Skin lesions, mycetoma, invasive sinusitis (Kriesel *et al.*, 1994), keratitis, endophthalmitis, pulmonary infections, endocarditis (Migrino, Hall and Longworth, 1995), brain abscess (Baddley *et al.*, 2000) and disseminated infections due to *Scopulariopsis* spp. have been reported. Invasive *Scopulariopsis* infections are seen mainly in immunocompromised hosts, such as bone marrow transplant recipients. These infections are highly mortal (Neglia *et al.*, 1987).

1.8.4.4. Cultural characteristics:

Scopulariopsis colonies grow moderately rapidly and mature within 5 days. They are granular to powdery in texture. From the front, the color is white initially and becomes light brown or buff tan. Reverse color is usually tan with brownish center. Some species may form dark colored colonies (Sutton, Fothergill and Rinaldi, 1998; de-Hoog *et al.*, 2000).

1.8.4.5. Microscopic features:

Septate hyphae, conidiophores, annelids, and conidia are seen. Chlamydospores may occasionally be present. Conidiophores are hyphae-like and simple or branched. Annelids are solitary, in clusters, or form a penicillus; they are cylindrical and slightly swollen. Conidia are one-celled, globose to pyriform, smooth, but more commonly rough-walled, spiny, truncate, and forming basipetal chains (Larone, 1995; Sutton, Fothergill and Rinaldi, 1998).

Compared to other fungi, *Scopulariopsis* differs from *Penicillium* by forming annelids. *Scedosporium inflatum* differs from *Scopulariopsis* by forming annelloconidia in wet clumps at the apices of annelids with swollen bases.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Collection of samples:

Using sterile cotton swabs, a total of 100 ear samples were collected from patients attending ENT Hospital, Khartoum State, Sudan. Samples were collected randomly from patients suffering from ear infections during the months of June to September, 2006. Samples were taken from different ages and both sexes (Table I).

Table I: Distribution of samples according to age and sex

Age group	No. of patients		Total	Accumulative %
	Male	female		
≤ 10	20	17	37	37
11-20	15	12	27	64
21-30	8	12	20	84
31-40	1	3	4	88
41-50	2	4	6	94
>50	2	4	6	100
Total	**48**	**52**	**100**	

Prior to collection of samples, ears were first cleaned by sucking the discharge, if present, using sucking pump apparatus by aid of a physician and then rubbed by cotton soaked with 70% alcohol.

The sterile cotton swabs which were used to take the samples were first moistened by sterile normal saline to prevent the drying of specimens. Each sample was labeled with date, sex, age and number of the patient.

After collection, the specimens were kept inside a clean thermos flask containing ice and transported within 3 - 4 hours to the Microbiology Laboratory, Faculty of Veterinary Medicine, University of Khartoum for processing.

In the Laboratory, samples were processed as soon as possible to avoid over growth of the commensal saprophytic flora and the deterioration of the pathogenic flora.

2.2. Asepsis (disinfection) and sterilization:

Sterilization is the complete destruction or elimination of all viable organisms in a material being sterilized (Carter, 1986). While, disinfection is to reduce the count of pathogenic organism in a potential source of infection to below that required to cause infection, rather than to eliminate completely the organism from the source (Shanson, 1989).

2.2.1. Autoclaving (moist heat):

The majority of culture media, solutions (e.g. normal saline, distilled water), screw-capped bottles with rubber stoppers and plastic ware were sterilized by autoclaving at 121°C for 15 minutes. Also it was used for decontamination of discarded cultures and contaminated glass and plastic ware.

2.2.2. Hot air oven (dry heat):

It was used for objects that do not melt such as scissors, scalpels, glass-ware such as Petri dishes, pipettes, flasks, glass rods, set of slide culture technique, and metal containers and tools. It was done at 160°C for one hour and it was allowed to cool until the temperature inside the oven has dropped to 50°C to avoid the cracking of the glass ware (Shanson, 1989).

*Notice***:** The set of slide culture technique was sterilized by hot air oven as a complete unit.

2.2.3. Flaming (dry heat):

Flaming was used for cotton-plugged flasks, tubes, glass slides and bottle necks. Inoculation of media, transfer and addition of supplements and pouring media into Petri-dishes were done under flaming.

It was done by exposing the object to the direct flame for few seconds.

2.2.4. Red heat (dry heat):

Mycological loops, scalpels with blade, forceps and scissors were sterilized by holding the object inside the flame until it became reddish (Shanson, 1989). It is used in the laboratory for objects not destroyed by heat (Carter, 1989).

2.2.5. Irradiation and disinfectants:-

Phenolic disinfectants were used to disinfect floors, while 70% alcohol was used to disinfect benches and stage of work. In addition,

in media pouring room, ultraviolet (UV) irradiation was used for 20 minutes before work.

UV light is used to reduce air-borne organisms. It acts by causing errors in the replication of DNA (Carter, 1986).

Formalin (formaldehyde) as 1% aqueous solution was used for 18 – 24 hours used to disinfect incubator after being rubbed by 70% alcohol (Shanson, 1989).

2.3. Stain and reagents:

2.3.1. Lacto phenol cotton blue (LPCB) stain:

LPCB stain is a mounting medium used to stain fungal preparations on microscope slides (Hendrix, 2002).

Contents:

Phenol crystals... ……..20.0 g

Lactic acid…….….20.0 ml

Glycerol……..40.0 ml

Cotton blue…….0.05 g

Distilled water ……..20.0 ml

Preparation:

Twenty grams of phenol crystals were added to 20 ml distilled water and 20 ml lactic acid and then heated by holding the mixture in hot water bath. After dissolution, 0.05 g of cotton blue was added till it dissolved, and then 40ml of glycerol were added and mixed thoroughly. The solution was kept in stock bottles at room temperature and labeled with date of preparation. It was refiltered if the dye had precipitated (Hendrix, 2002).

2.3.2. Alcohol (70%):

Contents:

Absolute alcohol…….70 ml

Distilled water……30 ml

Preparation:

Seventy milliliters of absolute alcohol were dissolved in 30ml of distilled water, mixed well and kept until being used.

2.3.3. Normal saline:

Contents:

Sodium chloride……...8.5g

Distilled water……..1000ml

Preparation:

An amount of 8.5g of the Sodium chloride was suspended in one litre of distilled water and mixed well until the salt was fully dissolved then dispensed in bottles, autoclaved, labeled and stored at room temperature.

2.4. Culture media:

2.4.1. Sabouraud's Dextrose Agar (SDA) (Oxoid):

Contents:

Mycological peptone……10.0g

Dextrose (D-glucose)……40.0g

Agar…….... 15.0g

Chloramphenicol…….. 0.05g

Distilled water…….……1000ml

Preparation:

An amount of 65.0g of the dehydrated medium was suspended in one litre of distilled water in suitable flask, dissolved completely by heating in boiling water bath. After cooling down to about 60 °C, the pH was adjusted to 5.6 by addition of hydrochloric acid solution. Then it was sterilized by autoclaving at 15 lbs/sq. inch (121°C) for 15 minutes. After sterilization, the medium was allowed to cool to approximately 50-55°C and then aseptically chloramphenicol (0.05 g/L) was added into the medium and mixed well. The medium was then dispensed into sterile Petri dishes, 20ml each, and 10ml volume in bottles to make slants for culture and maintenance of mycelial fungi and allowed to solidify at room temperature. The prepared medium was kept at 4°C until being used.

2.4.2. Corn Meal Agar (CMA):

*Contents***:**

Corn meal extracts……... 2.0g

Agar……....15.0g

Distilled water……...1000ml

Preparation:

An amount of 17g was suspended in one litre of distilled water, dissolved by boiling and sterilized by autoclaving at 15 lbs/sq. inch

(121°C) for 15 minutes. The medium was allowed to cool to about 60°C and then the pH was adjusted to 6.0. The medium was then poured into sterile Petri dishes or bottles, and allowed to solidify. It was stored at 4°C until being used.

2.5. Culture methods:

Specimens were cultured on two sets of media: Corn Meal Agar (CMA) and Sabouraud's Dextrose Agar (SDA) supplemented with chloramphenicol (0.05 g/l) to inhibit the growth of bacteria. Each medium was inoculated by the swabs in the dried up medium and incubated at 30°C and examined daily for mycelial fungi for as long as four weeks before they were discarded as negative.

Growth of the mycelial fungi was observed for production of aerial mycelia, development of pigments, the appearance of colonial morphology, the color of the culture and growth rate.

2.6. Purification of isolates:

For purification, pieces of mycelia from suspected colonies were picked up carefully by mycological needle tip and cultured separately on SDA. Each time a part of colony was removed and put on a glass slide, containing a drop of LPCB, teased and covered with coverslip and examined under the microscope.

Material for subculture was taken between the center and edge of the colony, because the old mycelia at the center of the colony may be not viable (Hendrix, 2002).

2.7. Staining method and microscopic examination:

To study the microscopic features of the mycelial growth, three techniques were used:

a. Slide culture technique:

Procedure: A thin plate of agar (about 2mm thick) was cut into pieces (about 6 mm square) with a sterile scalpel blade. A square of agar was transferred to a sterile slide and shielded with Petri dish lid. The four sides (edges) of the agar square were inoculated with the fungal culture under study and a sterile coverslip was applied on the top. Then, the slide was placed on a bent glass rod to raise it above a filter paper, which was moistened with sterile distilled water. The culture was incubated at room temperature or in an incubator at 25 – 28°C for 3 – 5 days.

Every other day the slide was removed and examined visually and under the microscope, for the presence of characteristic growth of the fungus. When sufficient growth is attained, both slide and coverslip had growth on them.

To make a permanent mount preparation, the agar square was remove and discarded; a drop of alcohol was put on the preparation on the slide; and when nearly dry, a drop of lacto phenol cotton blue (LPCB) was added. A coverslip was placed on the slide and warmed gently to drive out air bubbles. Excess stain was cleaned with blotting paper and the coverslip was sealed with nail varnish (Koneman *et al.*, 1997).

The microscopic features of the mycelial growth were observed under the light microscope using the 10x and 40x lenses.

b. Adhesive tape mount technique:

Procedure: A small clear adhesive tape was attached on one edge to a needle or wooden applicator, the sticky side of the tape was then pressed down gently, but firmly onto the surface of aerial growth of the colony using second stick to ensure good contact. The tape is then removed and mounted face-down in a drop of LPCB and stretched over the stain, gently so that mycelium becomes permeated with stain.

The tape was then examined under the microscope for presence of the hyphae and the mode of spore formation.

Precautions:

Care was taken that the exposed fingers do not come in contact with the mycelial fungi surface. For maximum safety, gloves and masks were worn.

c. Needle mount technique:

With a mycological needle, a small portion of fungal growth approximately mid-way between the center and the edge of the colony was picked and transferred to a drop of LPCB. The fungal mat is gently teased a part with two needles, a coverslip applied, and the preparation examined microscopically using lower power of 10x and 40x objectives (Koneman *et al.*, 1997).

2.8. Preservation method:

Agar slants of SDA were used for preservation of pure cultures. The slant was inoculated by the organism and incubated at 28-30°C, when sufficient growth is attained; slants were maintained at refrigerator for 3 months. Slants were inoculated in duplicate

preparations to afford a desirable margin of safety. Purity was always checked under microscope before used. Bottle caps were flamed before and after inoculation. Transfer was done after 3 months (Raper and Fennell, 1965).

2.9. Identification of isolates:

Identification was performed for pure cultures of the isolates according to their microscopic and cultural properties. Preparations were stained by lacto phenol cotton blue.

The identification was performed using the slide culture technique to observe the following characters: the mycelium (septate or aseptate), type of conidiophores, the arrangement of conidia, color of the culture, pigmentation and the colonial morphology.

CHAPTER THREE

RESULTS

3.1. Mycological growth findings:

Mycological examination of the 100 samples collected from ENT Hospital revealed the presence of mycelial fungi in 47 (47%) samples, while 53 (53%) showed no growth (Fig.2). The mycelial growth in positive samples was found composed of only one fungus. According to their microscopic and cultural characteristics, out of the 47 mycelial fungi isolated, 44 (93.6%) isolates were identified as *Aspergillus* species, 1 (2.1%) as *Penicillium* species, 1 (2.1%) as *Curvularia* species and 1 (2.1%) as *Scopulariopsis* species (Table II). The *Aspergillus* isolates were identified as: *A. niger* (n=28, 59.6%), *A. flavus* (n=10, 21.3%) and *A. terreus* (n=6, 12.8%) (Table III).

It was observed that the growth of mycelial fungi on SDA was rapid and helped in isolation and identification, but the growth on CMA was slow and weak.

Distribution of mycelial fungi isolates according to age of patients is shown in table IV and figure 3. From tables I and IV, it was observed that 84% of patients visiting the ENT Hospital were 30 years

old or less; and the rate of ear infection is inversely proportional with age of patients. Similarly, 38 (80.8%) isolates were obtained from patients 30 years old or less.

When the sex of patients was considered, it was noticed that almost no difference in isolation frequency of mycelial fungi. Twenty three (48.9%) isolates were from males and 24 (51.1%) were from females. However, it was observed that young male patients were more affected by ear infection than female ones (Table V and Fig. 4).

3.2. Cultural and microscopic features of isolated mycelial fungi species:

3.2.1. *Aspergillus* species:

Cultural characteristics:

Aspergillus spp. were slow to rapid growers, the colonies were white at first and turned to blue, green, yellowish-green, black or cinnamon-brown. The surface was velvety, cottony, granular or fluffy.

Microscopic features:S

Microscopically the *Aspergillus* spp. mycelium was septate, conidiophores were long with vesicle-like tip, the surface contained many flask-shaped sterigmata (phialides) and chains of conidia (phialospores) which were spherical to elliptical, smooth or rough-walled.

The species have been described based on differences in colony pigmentation, the size and length of the conidiophores, the shape of the vesicle, the presence of sterigmata (one row or two rows), the

position of the phialides, the size and length of the chain of spores, and other criteria.

3.2.1.1. *Aspergillus niger*:

Cultural characteristics:

The colonies were white at first and turned to yellow, but soon developed a black colour on the surface as conidia were produced, which become so dense by time to produce a black matt. The reverse (underside) of the colony was buff or yellow-gray and this gives the characteristic of *Aspergillus niger* (Fig.7).

Microscopic features:

The hyphae were septate and the conidiophores were long and smooth. The vesicles were spherical (globose) and gave rise to large sterigmata and smaller phialides from which dense clusters (aggregates) of jet black conidia were produced, the conidia were black and roughened (Fig.13).

3.2.1.2. *Aspergillus flavus:*

Cultural characteristics:

The colonies were granular to wooly and yellowish-green in colour (Fig.6).

Microscopic features:

The conidiophores were long and tend to be roughened just beneath a globose vesicle, phialides arised from the entire circumference of the vesicle surface and had one or two rows of phialides. Conidia were spherical, smooth and form relatively long chains (Fig.12).

3.2.1.3. *Aspergillus terreus:*

Cultural characteristics:

The colonies were cinnamon buff, brown, or orange-brown. Radial folds emanating from the center of the colony were often observed (Fig.5).

Microscopic features:

The vesicles were relatively small and dome-shaped. The phialides were biseriate (tow rows), and the primary phialides were shorter than the secondary. Smooth, elliptical conidia were formed in a long chain, which is a unique character to *Aspergillus terreus*. The conidiophores were short and borne laterally from the hyphal cells (Fig.11).

3.2.2. *Penicillium* species*:*

Cultural characteristics:

The colonies of *Penicillium* species were fast growing (4 days), at first, white, then becoming green, blue-green-brown, yellow and brown colonies. The margin or edge was white. The surface was velvety to powdery due to abundances of conidia. The reverse colonies were reddish (Fig.10).

Microscopic features:

Brush-like conidiophores developed from septate hyphae were seen. Chains of conidia were one-celled, globose to elliptical, smooth or rough, cut-off from flask-shaped phialides (metulae). Species were separated on the basis of variations in branches (metulae) of the conidiophores, conidia and colonial characteristics.

For confirmation of identification of the mycelial fungi isolated in this study, the Mycology Unit, National Health Laboratory, Ministry of Health, was consulted (Fig.16).

3.2.3. *Scopulariopsis* species*:*

Cultural characteristics:

Scopulariopsis colonies grew rapidly and matured within 5 days; they were hairy or fluffy in texture. From the front, the colonies were white at first then became light brown or buff tan and yellow pigmented.

Reverse color of the colony was buff or yellow, orange-yellow with brownish center (Fig.9).

Microscopic features:

The species characterized by septate hyphae and simple or branched conidiophores. Conidia were one celled, globose, rough-walled, formed in chains, and lemon-shaped (Fig.14).

3.2.4. *Curvularia* species*:*

Cultural characteristics:

Curvularia species colonies grew rapidly and matured within 4 days, they were wooly colonies on SDA. For the front, the color of the colony was white to gray at first and turned to gray-brown or dark gray as the colony matures. The reverse of the colony was dark brown to black (Fig.8).

Microscopic features:

The mycelium was hyaline or brown, septate; conidiophores were simple or branched and were bent at the points where the conidia originate.

The macroconidia were brown, multiseptate and dark in color in central cells than the other, curved and divided into 3 -5 cells (Fig.15).

Table II: Mycelial fungi isolated from patients with ear infection

Fungal species	**No. of isolates**	**% from total number of isolates**
Aspergillus species	44	93.6%
Penicillium species	1	2.1%
Scopulariopsis species	1	2.1%
Curvularia species	1	2.1%
Total	**47**	**99.9 %**

Table III: Isolation frequency of *Aspergillus* species isolated from patients with ear infection

Aspergillus species	No. of isolates	% from total number of isolates
A. niger	28	59.6%
A. flavus	10	21.3%
A. terreus	6	12.8%
Total	**44**	**93.7%**

Table IV: Distribution of mycelial fungi isolates according to age

Age group (yrs)	No. of patients (%)	Accumulative number of patients	No. of isolates (%)	Accumulative number of isolates (%)
≤ 10	37 (37)	37	16 (34)	16 (34)
11-20	27 (27)	64	13 (27.7)	29 (61.7)
21-30	20 (20)	84	9 (19.1)	38 (80.8)
31-40	4 (4)	88	3 (6.4)	41 (87.2)
41-50	6 (6)	94	4 (8.5)	45 (95.7)
>51	6 (6)	100	2 (4.3)	47 (100)
Total	**100 (100)**		**47 (100)**	

Table V: Distribution of isolates according to sex of patients

Age group (yrs)	No. of patients		No. of isolates	
	Male	**Female**	**Male**	**Female**
≤ 10	20	17	10	6
11-20	15	12	7	6
21-30	8	12	5	4
31-40	1	3	0	3
41-50	2	4	1	3
> 50	2	4	0	2
Total	**48**	**52**	**23**	**24**

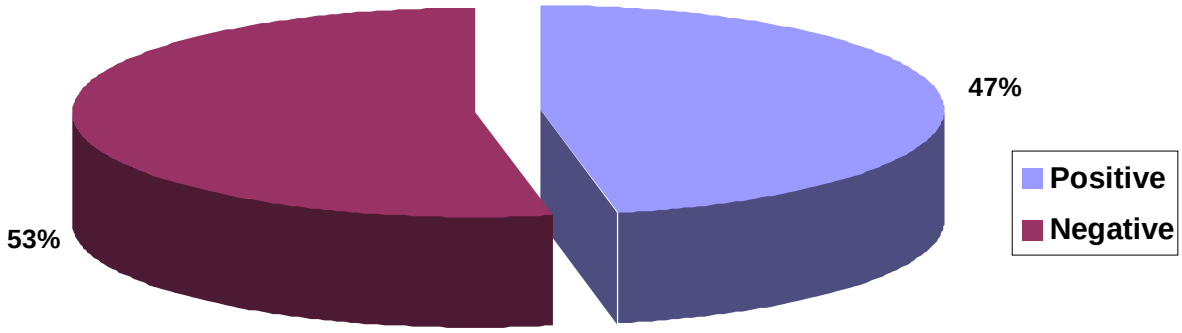
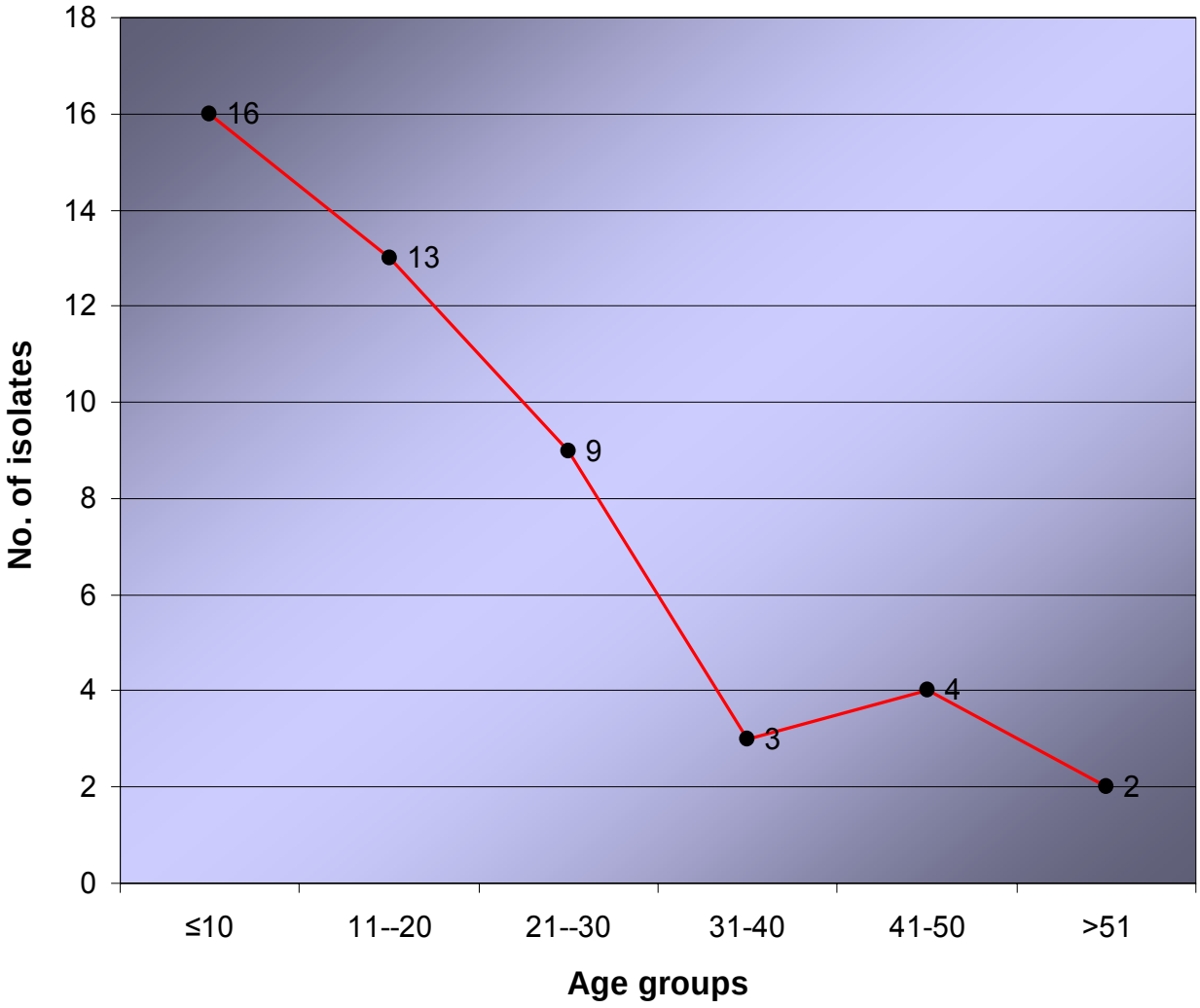


Figure 2: Precentage of positive and negative samples for growth of molds



Figture 3. Correlation between isolation frequency of molds and age of patients

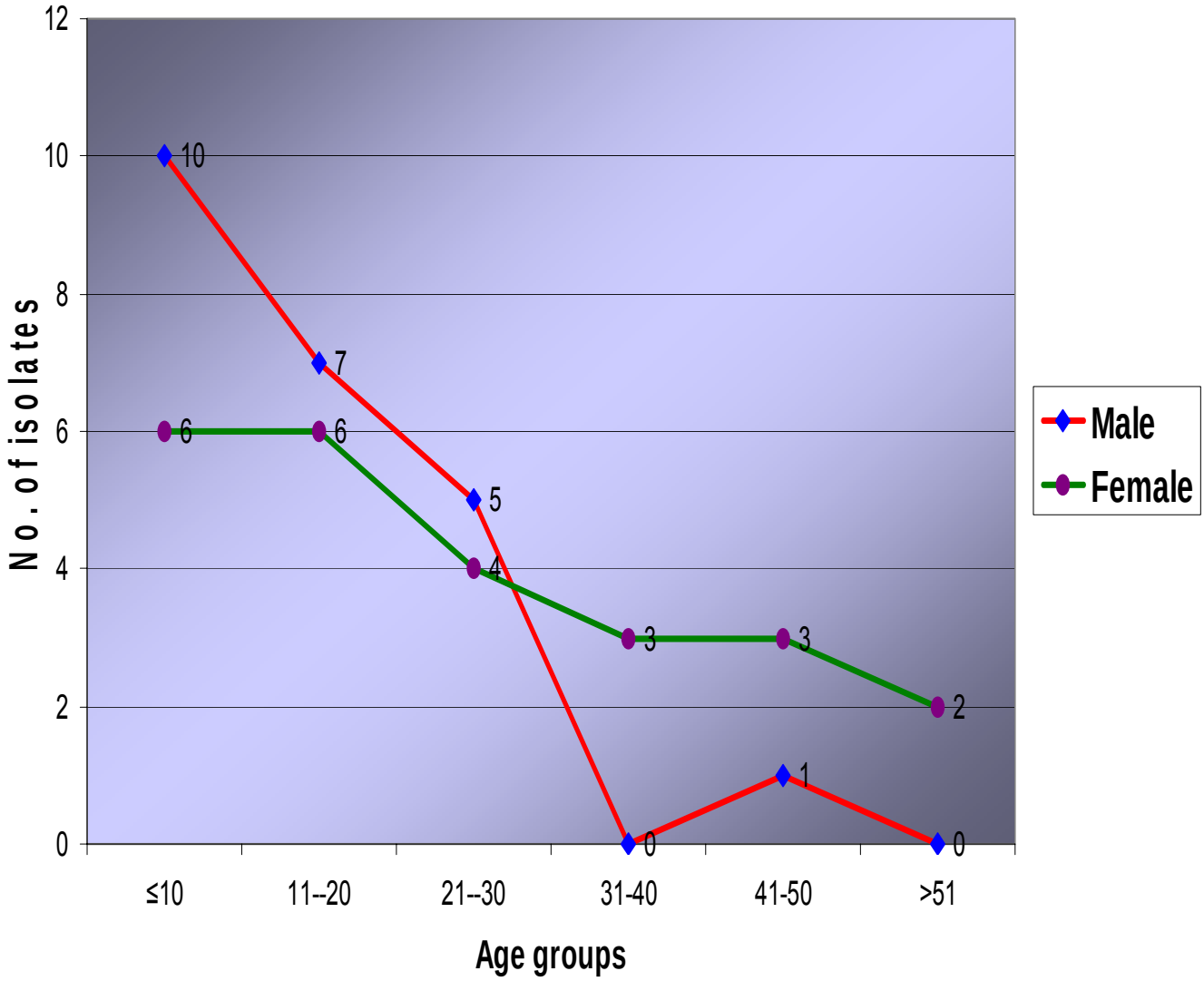


Figure 4: Distribution of mold isolates according to age and sex

Figure 5: Morphology of *Aspergillus terreus* culture on Sabouraud's Dextrose Agar medium.

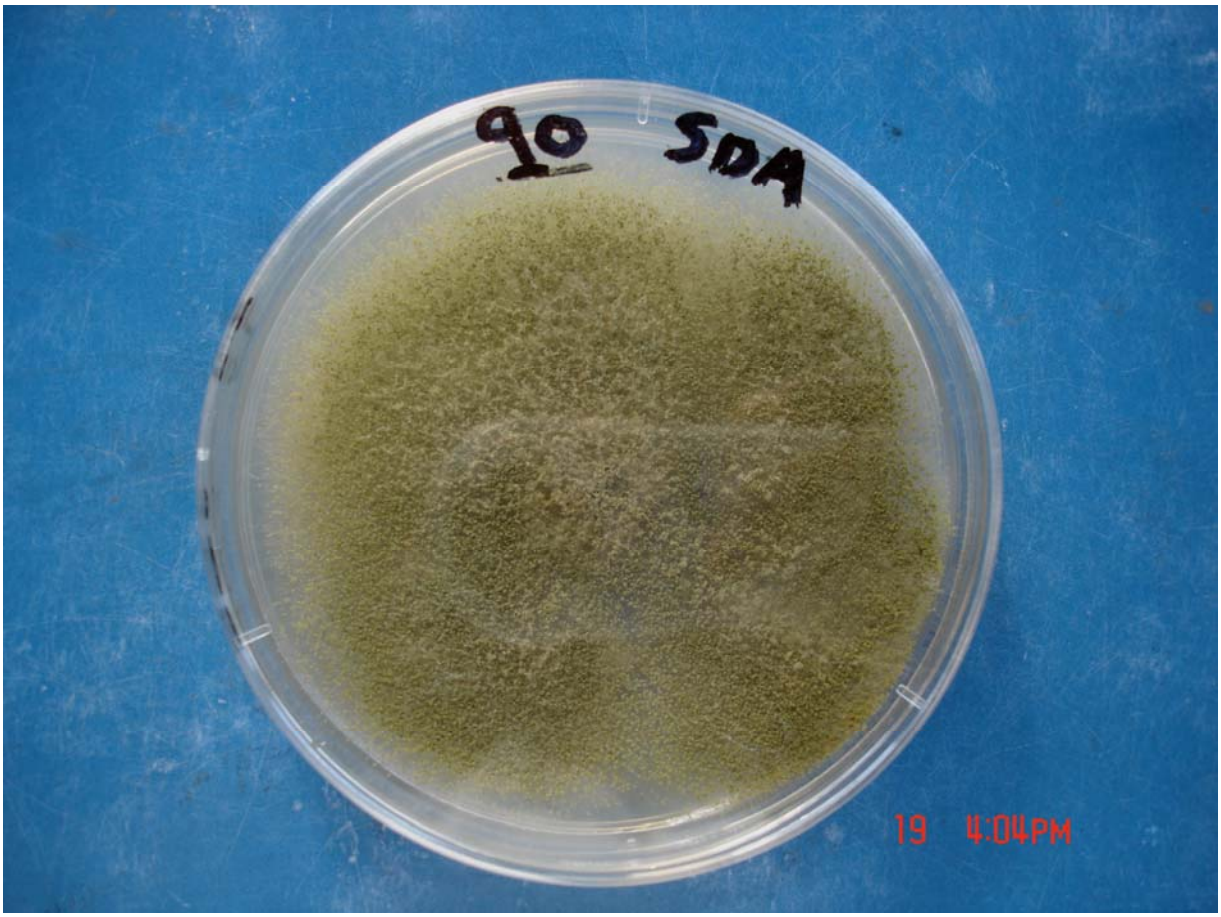


Figure 6: Morphology of *Aspergillus flavus* culture on Sabouraud's Dextrose Agar medium.

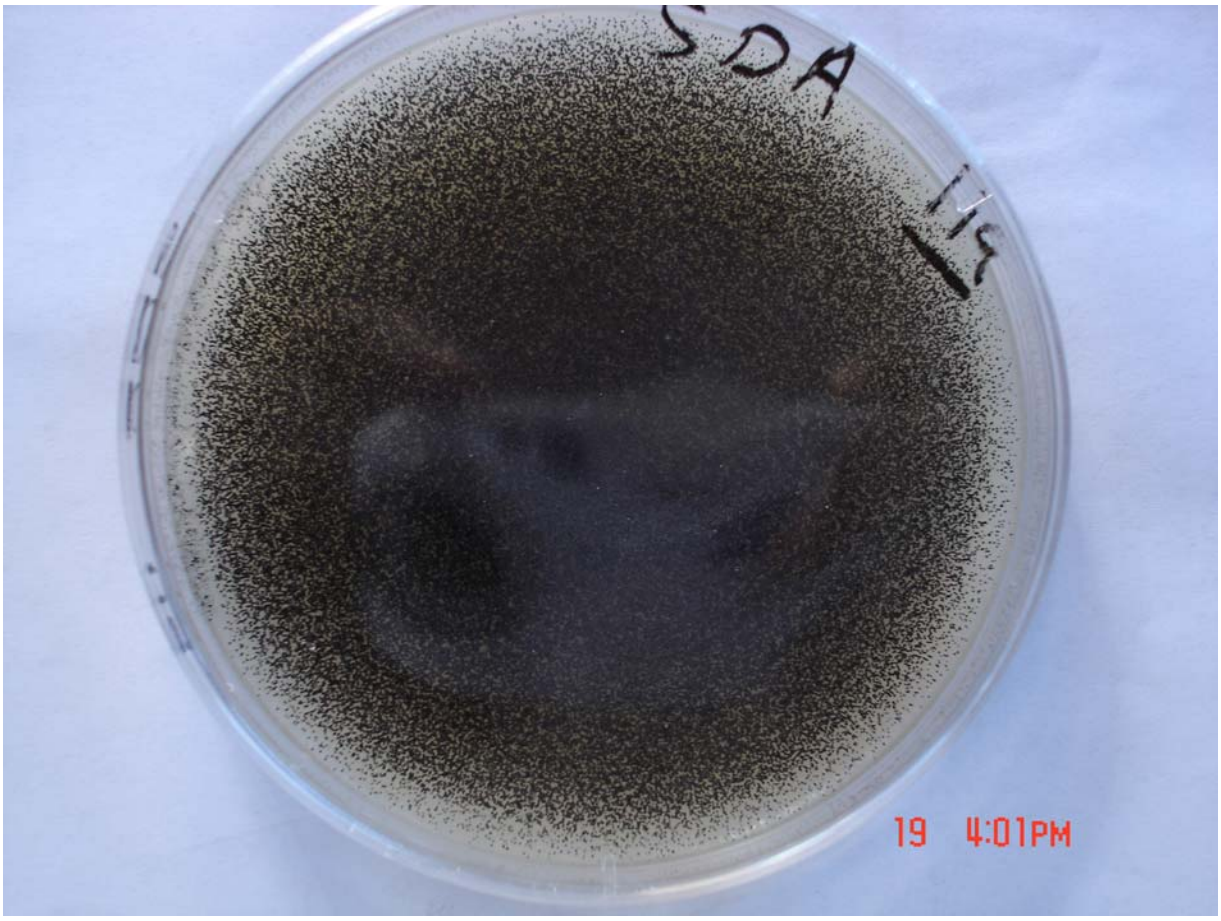


Figure 7: Morphology of *Aspergillus niger* culture on Sabouraud's Dextrose Agar medium.

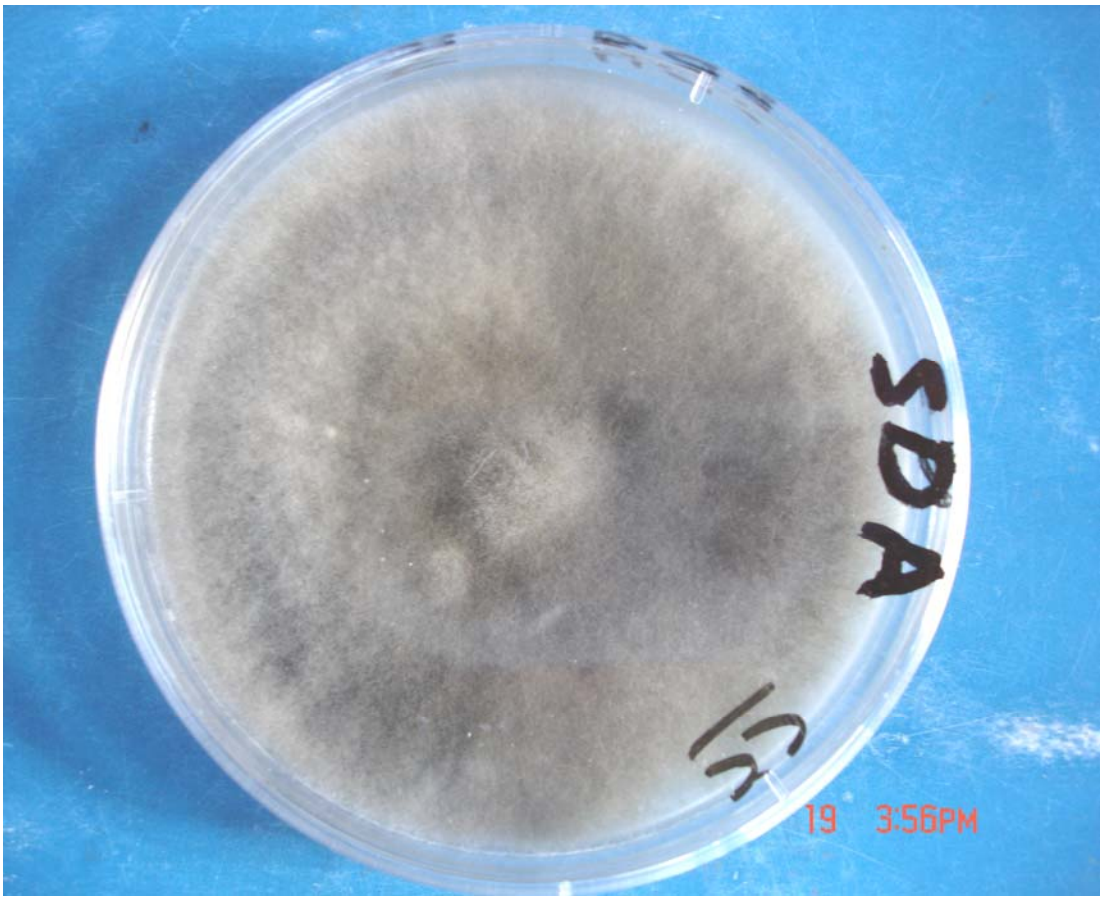


Figure 8: Morphology of *Curvularia* culture on Sabouraud's Dextrose Agar medium.

Figure 9: Morphology of *Scopulariopsis* culture on Sabouraud's Dextrose Agar medium.

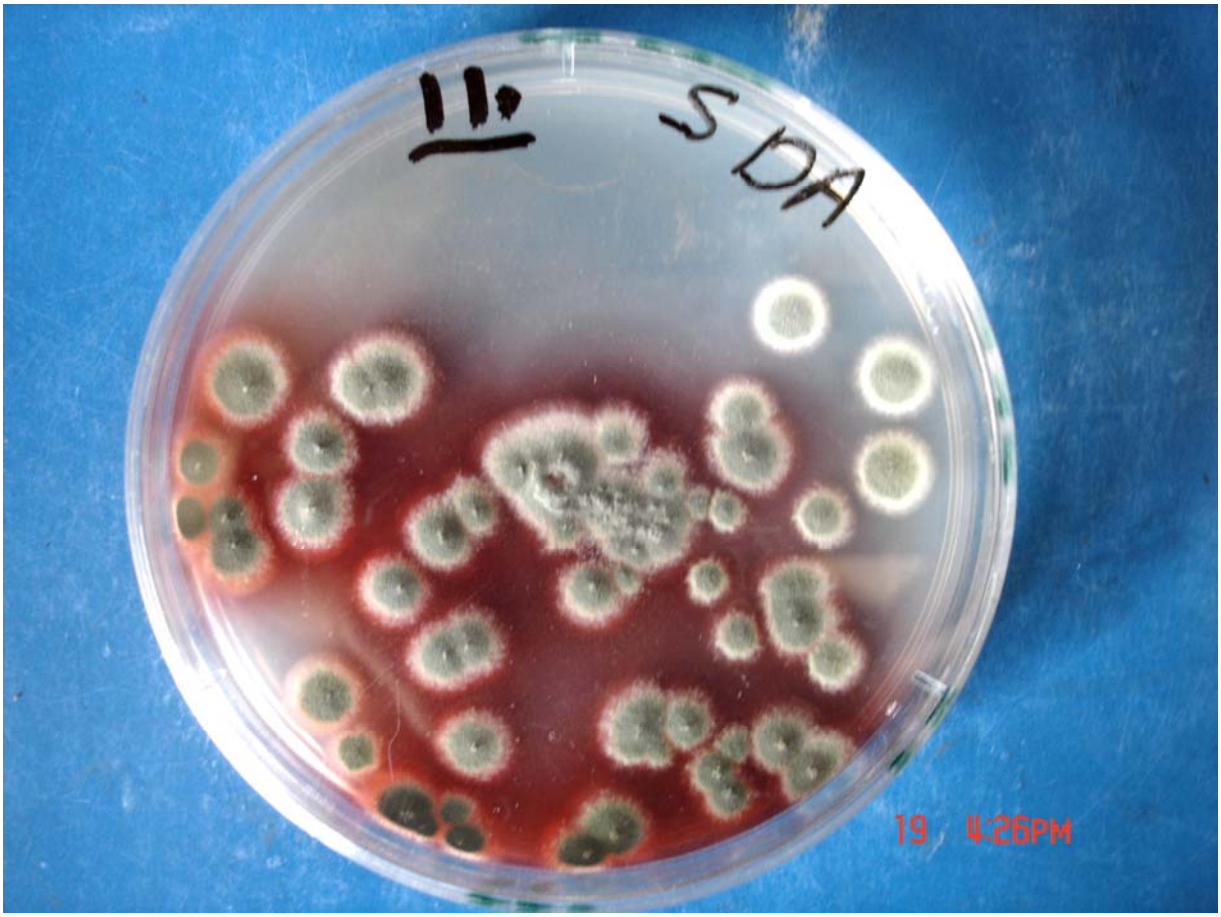


Figure 10: Morphology of *Penicillium* culture on Sabouraud's Dextrose Agar medium.

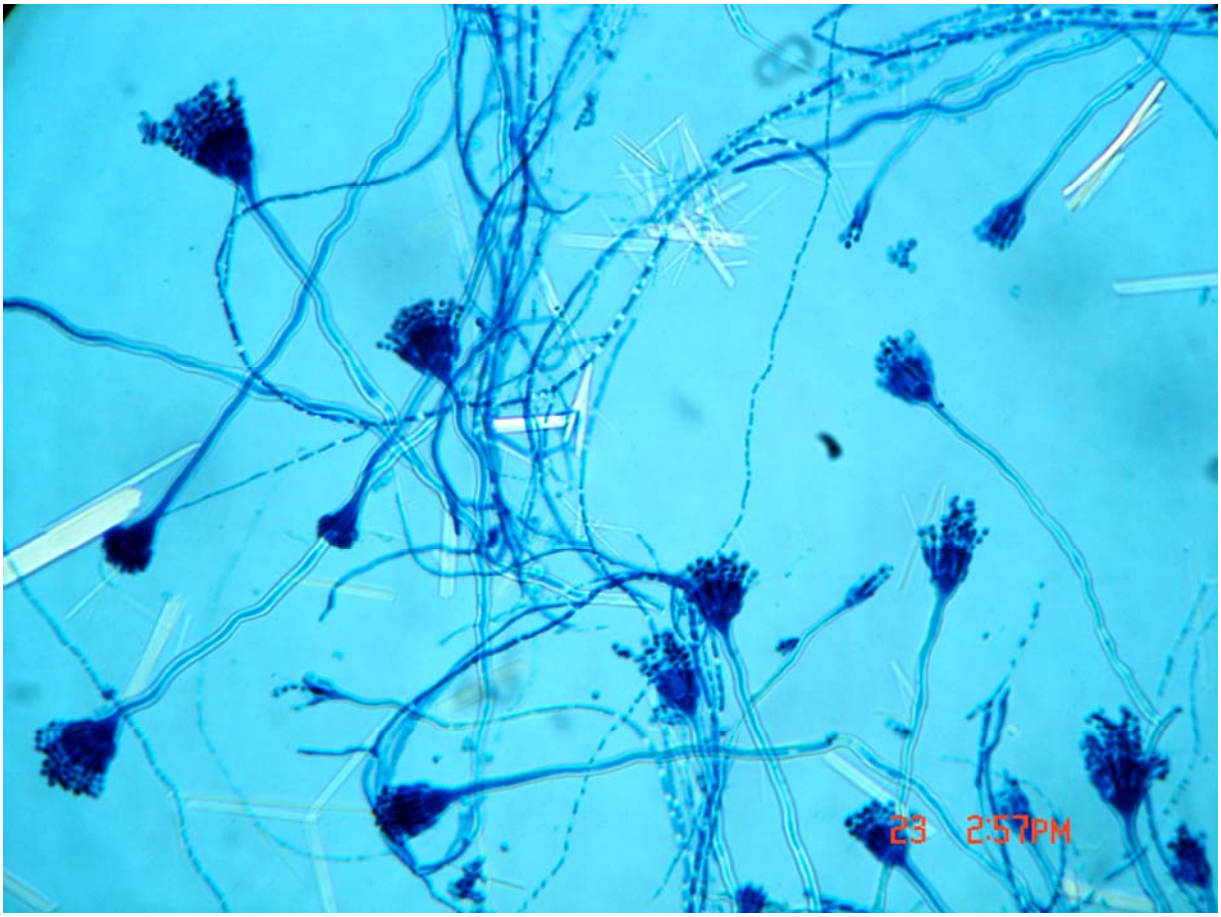


Figure 11: