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Isolation and Identification of Fungi Associated with Toe and Finger Nails from School Aged Children

Research Project Submitted to the Department of Biology in Partial Fulfilment of the Requirements for the degree of B.Sc. in Biology

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

وَقُل رَّبِّ زِدْنِي عِلْمًا

Supervisor's Certification

I certify that this research project was prepared under my supervision at University of Salahaddin as a partial fulfillment of the requirements for the degree of B.Sc. in Biology.

Signature:

Supervisor: **Shna Ibrahim Ismail**

Dedicated to:

My parents

My beloved brothers and sisters

My friends and colleagues

Every one whose knowledge is his own path

Firdaws & Lana

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Firdaws & Lana

SUMMARY

Onychomycoses constitute pathologies frequently seen in dermatological practice worldwide. Usually, they are caused by two groups of pathogenic fungi: dermatophytes and yeasts of the *Candida* genus. However, in a small fraction of the cases, the etiologic agents comprise nondermatophyte molds, belonging to several genera and species.

This work was designed for isolation and identification of different saprophytic fungal genera as the etiologic agents of onychomycosis prevalent among school aged children in Erbil city. Sixty nail samples including toe and finger nails were collected from 30 students 16 (53.3%) males and 14 (46.7%) females ranged in age from 6-12 years.

For preliminary diagnosis, microscopic examination was carried out. A portion of the sample was covered with10% KOH solution, incubated for at least 3 hours and examined microscopically for detection of fungal elements. The remaining aliquot of nail specimen was cultured on Sabouraud Dextrose Agar (SDA) media supplemented with 0.5mg/ml chloramphenicol (to suppress bacterial growth). After 2 weeks of incubation, pure isolated fungi were identified according to the recommended references.

The results showed that a total of 6 different fungal genera were isolated. *Aspergillus niger* was the most common fungal species isolated accounting for 33.4%, followed by *Aspergillus flavus* 17.9%, *Cladosporium* sp. 12.9%, *Alternaria* sp & *Penicilium* sp. 10.2%, *Aspergillus fumigatus* 7.7%, *Trichophyton* sp. 5.1% and the least species is *Scytalidium* sp 2.6%.

Keywords: Onychomycosis, Saprophyte, *Aspergillus*, nail clipping, diagnosis.

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1. INTRODUCTION

Fungi are a diverse group of organisms belonging to the Kingdom Mycota (Arora, 2003). This kingdom is one of the six kingdoms of life, contains adversity of organisms, including macroscopic forms as well as filamentous or yeast-like microscopic structures (Richardson and Warnock, 2012). Characteristically, they are eukaryotes, with unicellular or multicellular structures bounded by a rigid cell wall containing chitin (Dóczi, 2006). They are non-photosynthetic organisms that propagate via spores (Arora, 2003). Some fungi, known as saprophytes, live on dead organic matter; these microorganisms do not damage any macroorganism. Other fungi are parasitic; they live at the expense of a host organism with or without injuring the latter (Nolting and Fegeler, 1987).

There are at least 100,000 named species of fungi. However, fewer than 500 have been associated with human disease, and no more than 100 are capable of causing infection in otherwise normal individuals. The remainder is only able to produce disease in hosts that are debilitated or immune-compromised in some way. Most human infections are caused by fungi that grow as saprophytes in the environment and are acquired through inhalation, ingestion or traumatic implantation (Richardson and Warnock, 2012).

Finger and toenails serve as visual advertisements of an individual's overall health and have unquestionable effects on patients' psychological, physical, social, and business activities (Halvaee *et al.*, 2021).

Onychomycoses are fungal infections of both the fingernails and toenails. Toenails are 4-10 times more frequently affected than fingernails, probably because of their slower growth and increased exposure to injury and infecting organisms. It is caused by dermatophytes, yeasts and non-dermatophytic (saprophytic) moulds (Baran and Kaoukhov, 2005). The dermatophytes *Trichophyton rubrum* and *Trichophyton mentagrophytes* are the main causative pathogens, responsible for 80-90% of cases (Foster *et al.*, 2004). Non-dermatophytic fungi such as *Acremonium* spp., *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Scytalidium* spp. and *Scopulariopsis* spp. have been found to be involved in 2-11% of the onychomycosis cases reported. Yeasts, including *Candida* spp., account for 2-10% of fungal nail infections. Dermatophytes are normally transmitted through infected moist floor areas and are less often transmitted via direct personal contact. Non-dermatophytic

fungi have been frequently associated with the infection of traumatized nails in aged patients (Thomas *et al.*, 2010).

Depending on its origin, onychomycosis can be divided into primary and secondary. In primary onychomycosis, fungal invasion affects an intact nail, whereas secondary onychomycosis occurs in an already abnormal nail affected by various diseases or traumas. It should be noted, however, that when strictly defined, primary onychomycosis is a rare occurrence (Tchernev *et al.*, 2012). While clinically, onychomycosis can be classified into three distinct categories based on the region of the nail unit that is affected. These categories include distal/lateral onychomycosis, proximal subungual onychomycosis, and superficial white onychomycosis. The infection is more common in toenails than fingernails. If left untreated, onychomycosis will often worsen in severity, leading to marked dystrophic changes in affected nails (Ghannoum *et al.*, 2018).

Onychomycosis represents 30% of superficial mycosis and 50% of all nail disorders, with increasing incidence as people age. The estimated prevalence is more than 10% in the general population and 40% in elderly individuals, probably because of suboptimal immune function, inactivity, and the inability to maintain good foot care (Moreno and Arenas, 2010). In children the disease is seen only occasionally. (Heikkilä, 1996). It is not a serious condition, but it affects patients comfort in various aspects: aesthetic, frequent medical consultation, long evolution, etc. and makes the patient feel stigmatized for its popular association with poor hygiene and contagious risk (Cuchí-Burgos *et al.*, 2021).

There are several risk factors which could facilitate the occurrence of onychomycosis, including old age, underlying conditions (peripheral vascular disease, diabetes, compromised immune system, psoriasis, obesity, smoking), and walking shoeless in moist environments like public swimming pools and bathing places, are associated with an increased risk of this infection (Jacobsen and Tosti, 2017). There is emerging evidence that suggests a genetic component in the susceptibility of an individual to fungal infection (defects in the innate and adaptive immune system) that, in addition to exposure to environmental risk factors, leads to chronic onychomycosis (Gupta *et al.*, 2014). Furthermore, hot and humid climate and specific environment, existing some jobs such as animal husbandry with non-

hygienic methods, which make direct contact human to animals are associated with a high risk for developing onychomycosis (Asadi *et al.*, 2009).

Onychomycosis is very common worldwide. It is more frequent in developing countries due to risk factors of crowding, low socio- economic status and improper personal hygiene. Risk factors lead to epidemic potential, most notably in overcrowded places like schools and refugee camps (Farag *et al.*, 2018). It represent a major public health problem in school age children especially in low- and middle-income countries (Oke *et al.*, 2014).

Onychomycosis considered as a common nail problem, accounting for up to half of all nail diseases. Several nail disorders may mimic the onychomycosis clinically. Therefore, a sensitive, quick, and inexpensive test is essential for screening nail specimens for the administration of the proper drug (Haghani *et al.*, 2013). The accurate treatment of onychomycosis is essential as this infection has an important impact on the quality of life and could lead to a more serious infection and complication if left untreated (Scher and Baran, 2003). Due to the composition of the human nail plates, it acts as a formidable barrier against permeation and diffusion of all drugs. In addition, the nail has a slow growth rate, requiring a long duration of therapy, usually 8-12 months or longer, until the nail has grown back (Thomas *et al.*, 2010).

Non-dermatophyte (NDMs) organisms are becoming increasingly prevalent in onychomycosis. This apparent emergence might be an artifact of improved diagnostic techniques and increased awareness that these fungi are potential etiologic agents. It is important to bear in mind that all isolated organisms should be evaluated as potential pathogens when diagnosing fungal infections, especially given the increasing use of immunosuppressive drugs and the increasing numbers of chronically immunocompromised individuals. While many patients with non-dermatophyte mold onychomycosis will respond to oral or topical antifungal therapy, poor or incomplete response might still be expected in some patients (Gupta *et al.*, 2003).

AIM OF THE STUDY

This study was undertaken for isolation and identification of different saprophytic fungal genera as the etiologic agents of onychomycosis prevalent among school aged children in Erbil city.

2. MATERIALS AND METHODS

2.1 Collection of Specimens:

This study was carried out on children without any clinical signs of onychomycosis. During October 2022, samples of healthy toe and finger nails from 30 students (16 males and 14 females) aged between 6-12 years were taken randomly from two basic schools (governmental and private) at Erbil city. Personal data and history taking including age, sex, residence, personal hygiene and history of pet contact.

After nail asepsis with 70% ethanol, the distal edge of the nail plate was clipped with a sterilized nail clipper. Nail-clipping specimens were placed in the clean sterilized disposable plastic cup, labeled and transferred to the mycological laboratory for fungal analysis. The samples were divided into two portions, one for microscopic examination and the remaining part for culture.

2.2 Media preparation:

Sabouraud dextrose agar (SDA) which used in this study, was prepared and sterilized according to the manufacturer's guideline. Bacterial contamination was inhibited by adding chloramphenicol into the agar solution after autoclaving and prior to pouring into petri dishes aseptically. Media was placed in to the autoclave to allow for homogenization and sterilization. Reliable sterilization with moist heat requires temperatures above that of boiling water. These high temperatures are most commonly achieved by steam under pressure in an autoclave. Steam at a pressure about 15 psi, attaining temperature 121°C will kill all organisms and their endospores in about 15 minutes. A basic principle of chemistry is that when the pressure of a gas increases, the temperature of the gas increase proportionally (Thliza *et al*. 2020).

2.3 Laboratory Diagnosis

The accurate diagnosis of onychomycosis is important for its successful treatment. The cost and long duration of the therapy, the risk of developing adverse drug reactions, and possible interactions with concomitant medications all underline the importance of accurate diagnosis of the condition before commencing therapy (Thomas *et al.*, 2010).

The diagnosis of onychomycosis is confirmed when fungal hyphae and fungal viability are demonstrated and the fungal species identified. A variety of laboratory and diagnostic techniques are currently used, all of which have benefits and limitations; thus accurate diagnosis is often achieved using a combination of these techniques (Gupta and Simpson, 2013).

2.3.1 Direct Microscope Examination

For preliminary diagnosis, direct microscopic examination was carried out. The potassium hydroxide (KOH) test is a popular method used to confirm onychomycosis, with accuracy dependent on proper specimen collection, preparation, and examiner experience. The test is inexpensive, and the results are quickly available (Weinberg *et al.*, 2003).

All nail samples were examined microscopically. A portion of the sample was covered with a solution of potassium hydroxide (KOH) 10% and incubated for at least 3 hours before examination (Cuchí-Burgos *et al.*, 2021). KOH was used to digest the keratin material. Some clinicians heat the slides to accelerate the process, or add color stains to make hyphae easier to identify (Tchernev *et al.*, 2012).

Each treated slide was then carefully examined under low 10X and high 40X power objective lens for the presence of hyphae, pseudohyphae, arthroconidia, spores, or budding cell (Umar *et al.*, 2016).

23.2 Culture Incubation

Fungal culture has been considered to be the ‘gold standard’ technique in the diagnosis of onychomycosis. Clinical samples are plated onto a properly selected general media such as sabouraud dextrose agar with added antibiotics to inhibit overgrowth by bacterial contaminants. A significant advantage to using fungal culture is that it is able to identify the causative agent, thus being more specific than KOH testing (Alberhasky, 2004).

The remaining aliquot of nail specimen was cultured on Sabouraud Dextrose Agar (SDA) media supplemented with 0.5 mg/ml chloramphenicol (to suppress bacterial growth). Cultures were incubated at 26- 28°C and examined three times weekly for the detection of fungal growth over 2 weeks after which it was considered negative if no growth had occurred (Farag *et al.*, 2018). Fungal growth was assessed daily. Any growth obtained was regularly observed for differentiated colonies. Growth rate, obverse, reverse pigmentation of the recovered colonies was reported (Diso *et al.*, 2020).

2.4 Purification of the Fungal Isolates:

Pure isolates were generated by sub-culturing on Saboraud Dextrose for both visual and microscopic examinations of cultural (color and growth pattern) and morphological characteristics respectively for further differentiation (Umar *et al.*, 2016). Occasionally identification of fungal species was done from the culture of the original petri dish (Abdel-Gawad and Zohri, 1993).

2.5 Identification of fungal isolates:

2.5.1 Macroscopic characteristics:

The macroscopic characteristics (color of the surface and reverse, topography, and texture) of fungal colonies observed after incubation were thoroughly studied and observations were recorded.

2.5.2 Microscopic characteristics:

Microscopic identification was done according to (Fawole and Oso, 2001). A drop of lactophenol cotton blue stain was placed on a clean slide and with the aid of a mounted needle, a small portion of the mycelium from the fungal cultures was removed and placed in the drop of the stain. The mycelium was spread very well on the slide with the aid of the two mounted needles and a cover slip was gently lowered on it. The slide was then examined under the microscope. The observation was done at high power objective 40X of the microscope. Morphological characteristics of the fungi such as type of hyphae and asexual reproductive structure were observed (Muhammad *et al*., 2018). Each filamentous fungal colony growing from the inoculum pieces was identified at least to genus level, whether or not it belonged to a species previously known as an agent of opportunistic onychomycosis.

3. RESULTS AND DISCUSSION

Onychomycoses constitute frequent fungal infections seen in dermatological practice worldwide. The clinical picture is very variable, but that in general is characterized by nail architecture alterations, such as changes in color, thickness, onycholysis and onycodistrophy. In most cases they are caused by species of filamentous fungi like the dermatophytes or yeasts of the genus *Candida*. However, in a small fraction of the cases, the etiologic agents comprise nondermatophyte filamentous fungi, belong to several genera and species. The prevalence of nondermatophyte onychomycosis varies widely, according to geographical location or the climate, but it is more frequent in hot and humid tropical areas. Often they are considered simple contaminants or secondary pathogens, invading nails previously damaged by trauma or disease, although in some cases they actually act as primary pathogens (Pontarelli *et al.*, 2005).

The study attempted to determine the prevalence rate and etiological agents of onychomycosis among healthy pupils from two different basic schools in Erbil city. A total of 16 (53.3%) males and 14 (46.7%) females student agreed to participitate in this study with ages ranging from 6-12 years (Table 3.1). Majority of the participants were 6 to 7 years age bracket. Most of them 17/30 (56.7%) had contact with household pets and birds especially cat, deer, squirrel, hen, cockatiel, peafowl…. etc. Animal contact represented as predisposing factor for many fungal diseases including onychomycosis.

Sixty (60) nail samples (30 finger and 30 toe nail) were included in the study. Of them, 24 had negative direct KOH and 36 had positive direct KOH. Direct microscopic examination showed irregular saprophytic septate hyphae associated with single or grouped conidia in a few samples. In the group of negative KOH samples, 12/24 had a positive culture. while, in the group of positive KOH samples, 27/36 had a positive culture based on laboratory finding.

The prevalence of isolated fungi is presented in Table 3.2. Out of 39 culture-positive specimens a total of 6 different fungal genera were isolated. *Aspergillus niger* was the most common fungal species isolated accounting for 33.4%, followed by *Aspergillus flavus* 17.9%, *Cladosporium* sp. 12.9%, *Alternaria* sp & *Penicilium*

sp. 10.2%, *Aspergillus fumigatus* 7.7%, *Trichophyton* sp. 5.1% and the least species is *Scytalidium* sp 2.6%.

The results of the present study are in compatible with many previous study done with onycomycosis. In Tehran-Iran, (Halvaee *et al.*, 2021) identified the etiologic agent of onycomycosis by molecular method, they confirmed that saprophytic fungi accounted for the vast majority of the nail isolates followed by dermatophytes and yeasts. In Izmir-turkey (Hilmioğlu-Polat *et al.*, 2005) reflected that majority of all mycologically confirmed cases of ungual mycosis were due to non-dermatophytic filamentous fungi, among them *Aspergillus niger* reported as the most prevalent nondermatophyte but not the leading cause of all cases. In India, *Aspergillus* spp. are found to be the predominating causative molds (RAMANI *et al.*, 1993). Also in Iran from Yazd a report presented that *Aspergillus niger* was the most common etiology of onychomycosis (Hossein Sadeghi *et al.*, 2017). *Aspergillus flavus* is found to be the major NDM isolated from onychomycosis in studies by (Nouripour-Sisakht *et al.*, 2015). Other fungi are reported as the most common NDMs etiology of onychomycosis, as well. For instance, (Gupta *et al.*, 2016) in Canada, indicated *Scytalidium dimidiatum* and *Acremonium* as the most common NDMs. Even though, according to the previous study, *Candida parapsilosis* was the predominantly isolated *Candida* species as the normal flora in different sites of the body skin particularly nails, in our study did not recovered as pathogenic agent of onychomycosis (Rafat *et al.*, 2019). The distribution pattern of NDMs varies based on geographical region. (Gupta *et al.*, 2012) in a systematic review described the *Scopulariopsis brevicaulis*, *Fusarium* species, *Aspergillus* species, *Scytalidium dimidiatum* and *Acremonium* species as the most frequently reported NDMs. Furthermore, according to their review, in South America *Fusarium* species and in European countries *Scopularipsis brevicaulis, Aspergillus* species, *Acremonium* species and Fusarium species are more likely to be reported as the most common NDMs. While, *Scytalidium dimidiatum* is reported from various counteries and frequently from Thailand. Thus, it is necessary to determine the accurate epidemiology of onychomycosis in different part of the world.

Our results on the distribution of onychomycosis etiological agents differ from other published reports. In the present study, saprophytic fungi accounted for the vast majority of the nail isolates, while (Cuchí-Burgos *et al.*, 2021) and (Vélez

et al., 1997) in Spain, (Ghannoum *et al.*, 2000) in America and (Asadi *et al.*, 2009) in Iran isolated dermatophyte as the major etiologic agents of onychomycosis. As in the present study nail samples were collected from healthy pupils without any signs of onychomycosis and other nail dystrophy.

Hot and humid weather participitates the spread of the diseases. The shoes, which cover completely the feet, make the feet more susceptible to the disease and cause some damages to the superficial veins of the feet. Temperature and humidity are two important predisposing factors for Onycomycosis (Asadi *et al.*, 2009). As a school going student, there is increase in transmission between them due to increased contact, overcrowding in classrooms, lack of awareness and apathy to personal hygiene, sharing of personal items, and exposure to soil and even animals on playgrounds (Diso *et al.*, 2020).

Table 3.1 Demographic Properties of the Subject

Parameters	**Frequency (n)**	**Prevalence(%)**
sex		
Male	16	53.3%
Female	14	46.7%
Age (years)		
6-7	12	40%
8-9	8	26.7%
10-12	10	33.3%

Table 3.2 Prevalence of fungal Isolates:

Fungi	Frequency (n)	Prevalence (%)
Aspergillus niger	13	33.4
Aspergillus flavus	7	17.9
***Cladosporium* sp.**	5	12.9
***Alternaria* sp.**	4	10.2
***Penicillium* sp.**	4	10.2
Aspergillus fumigatus	3	7.7
***Trichophyton* sp.**	2	5.1
***Scytalidium* sp.**	1	2.6
Total	39	100

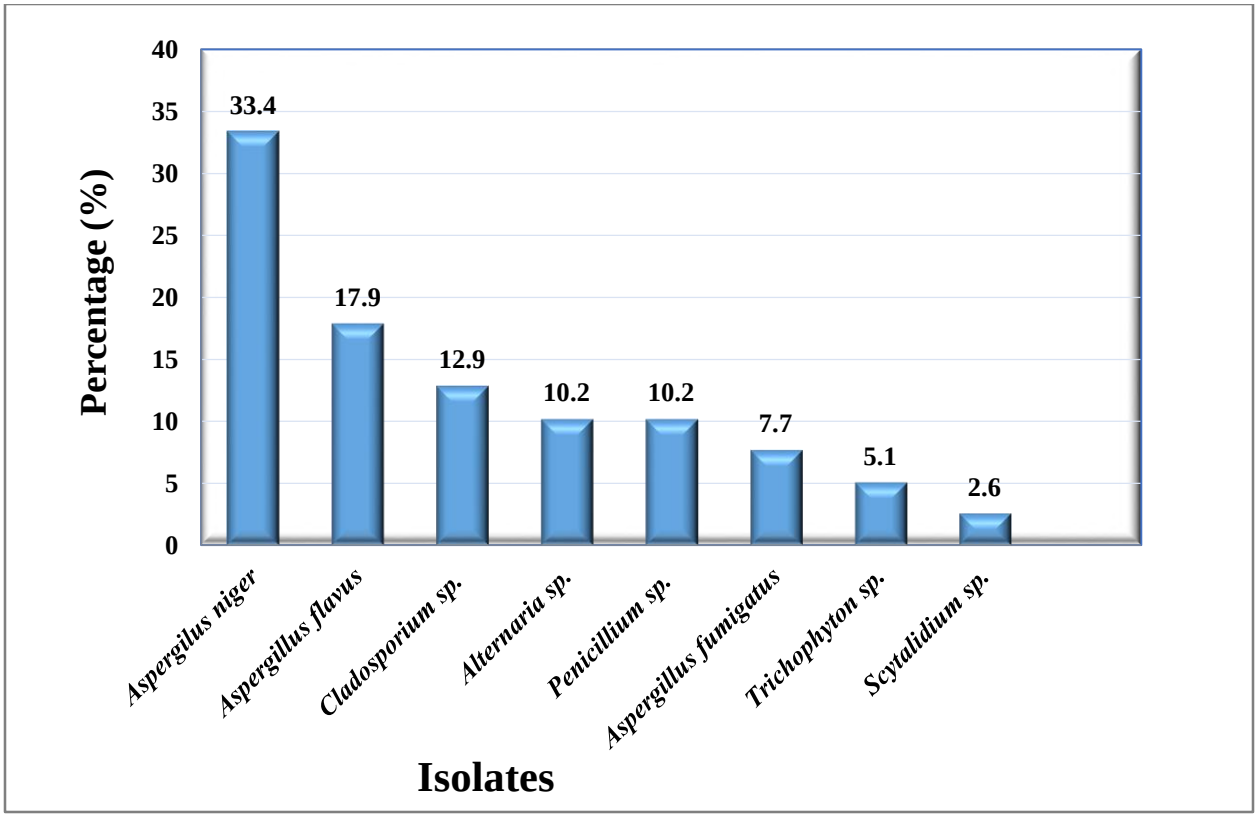
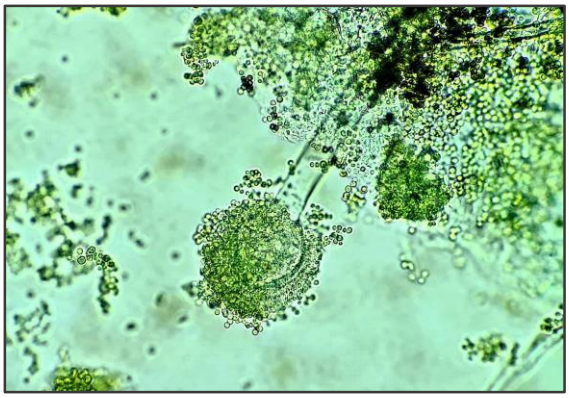
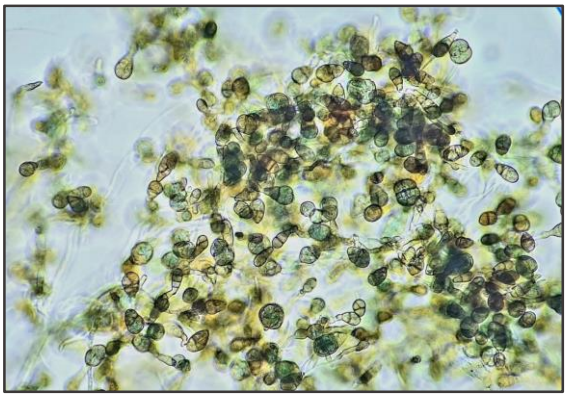


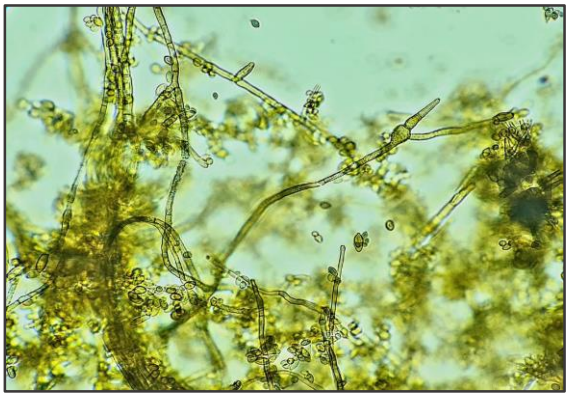
Figure 3.1: Percentage of etiologic agents from onychomycosis.



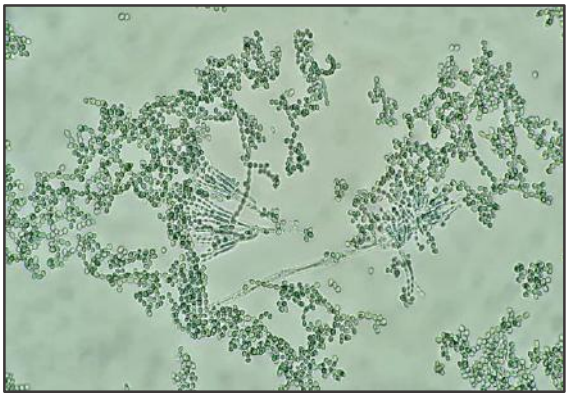
***Aspergillus* sp**



***Alternaria* sp.**

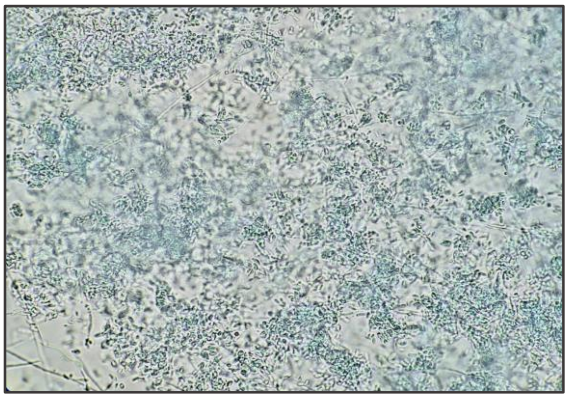


***Cladosporium* sp.**



***Penicillium* sp.**

***Scytalidium* sp.**



***Trichophyton* sp.**

Figure 3.2: Microscopic morphology of different fungal genera isolated from tested nail specimens.

4. CONCLUSIONS AND RECOMENDATIONS

CONCLUSIONS

- This study highlighted that the prevalence of onychomycosis among school puplis was relatively high.
- This study identified a markedly high occurrence *Aspergillus niger*, *Aspergillus flavus* and *Cladosporium* and they appear to be an emerging cause of saprophytic onychomycosis, while *Trichophyton* and *Scytalidium* were the least common isolated species.

RECOMMENDATIONS

- Studying topical antifungal drugs for the treatment of onychomycosis.
- Commercial real time PCR implementation for rapid diagnosis of onychomycosis.

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APPENDIX

Salahaddin University
College of Science
Biology department
Research Data Requirement

Data sheet of students

Case No.: Date: /10/2022

General History

Name:

Age: Gender:

Address:

School:

Animal contact: YES NO

Lab Diagnosis:

- Direct Microscopic Examination:

  Hand Foot

- Macroscopic morphology of culture:

  Hand Foot