CHARACTERIZATION AND ANTIFUNGAL DRUG PROFILE OF DERMATOPHYTES FROM CLINICAL SOURCES IN NAIROBI, KENYA.

BY

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DECLARATION

This Research Thesis is my original work and has not been presented for the award of any degree at any other University or Institute.

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DEDICATION

To my Aunt Mary Amalemba, my late Uncle David Amalemba, my Dad, Jackson Mashedi, my Mum Felistas Mashedi, my siblings and my friends; Betty Nkatha and Mary Njenga. It's been a tough journey, but with your constant re-assurance, I have come this far.

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ABBREVIATIONS AND SYMBOLS

MIC: Minimum Inhibition Concentration.

ITS: Internal spacer transcriber

mt DNA: Mitochondrial deoxy ribonucleic acid

rDNA: Ribosomal deoxy ribonucleic acid

RFLP: Restriction fragment length polymorphism

PCR: Polymerase chain reaction

RAPDS: Randomly amplified polymorphic DNAs

NTS: Non- transcribed spacers

SSU: Small sub units

RNA: Ribonucleic acid

KTZ: Ketaconazole,

FLU: Fluconazole

ITR: Itraconazole.

VOR: Voriconazole

KOH: Potassium hydroxide

LPCB: Lacto-phenol cotton

SDA: Sabouraud dextrose agar (SDA)

MEA: Malt extract agar

CTAB: Cetyltrimethylammonium Bromide

$Mgcl_2$: Magnesium chloride

DMSO : Dimethyl sulphoxide

MOPS: Morpholine propane sulfonic acid

DMF: Dimethylformamide

ATCC: The American Type Culture Collection

CLIS: Clinical and Laboratory Standard Institute

CFU: Colony forming unit

PPV: Positive predictive value

NPV: Negative predictive value

ABSTRACT

Dermatophytes are fungi that invade the keratinized tissue (skin, hair and nail) to produce infections referred to as dermatophysis. The severity of dermatophysis may be as a result of the host reactions to the metabolic products of the fungus, the anatomic location of the infection, the virulence of the infecting species, the immune status of the patient and local environmental factors. The HIV/AIDS and other immunocompromised infections and diseases have led to the steady rise in opportunistic fungal infections including dermatophytes. When incorrect diagnosis, treatment and management of dermatophysis are encountered, it leads to predisposition to secondary bacterial and other microbial infections, scarring and drug resistant strains are created. Data have shown that there has been a steady rise of these infections from patients attending a clinical laboratory. Increasing concerns are evidenced on the genotypic and phenotypic characteristics of these dermatophytes and why the relapse infections. These observations are explained by incorrect diagnosis, excessive use over the counter drugs or mutated and drug resistant dermatophytes. This study aimed at characterizing dermatophytes isolated from clinical sources by conventional and molecular typing methods. The study also determined the antifungal drug patterns of the isolated organisms. The dermatophytes were identified by conventional culture techniques and molecular methods. *In vitro* activity against triazoles and azoles antifungals drug profile was determined in accordance to Clinical and Laboratory Standards Institute (CLSI) M 38-A document. Results of the study revealed that dermatophytic and non dermatophytic infections were isolated from the study population, with the genera of *Trichophyton* being the most commonly isolated dermatophyte. Tinea corporis (23%) showed the highest manifestation of dermatomycoses followed by Tinea capitis (14%) respectively. The commonly isolated dermatophytes were *Trichophyton rubrum* (16%), *Trichophyton*

mentagrophyte (14%), *Trichophyton terrestre* (12%) and *Trichophyton violaceum* (10%) respectively. There was a significant variation in the prevalence of the different dermatophytes was at (P = 0.011).The molecular characterizations of the isolates were more sensitive compared to the conventional methods because it correctly identified *Chrysosporium keratinophilum* species that had been wrongly identified by the conventional methods. The antifungal assay showed that among the Azoles Fluconazole was resistant to all the isolated tested against. Posiconazole and Isaconazole were the most potent drugs against dermatophysis.

CHAPTER ONE

1.0: INTRODUCTION

Dermatophytes are a group of morphologically and physiologically related fungi some of which cause well defined infection: dermatophytoses (ringworm or tinea) that are the infections of the skin, hair and nails. They are parasitic fungus that invades the skin due to their ability to digest keratin *in vitro* in their saprophytic state and utilize it as a substrate thus making them as keritonophilic and keritionolytic. Dermatophytes thrive by growing on nail, skin and hair where they have adapted to these environments by using a variety of host proteins especially the keratin as nutrients. They secrete proteases that degrade skin and hair protein, which provide mechanism for fungal adherence and invasion of the skin (Summerbell, 2003). Table 1 shows the different forms of dermatophysis (Kwon-Chung and Bennet, 1992).

Table 1: Different kinds of dermatomycoses associated with the human body.

Diseases	Site of infection
Tinea manuum	interdigitale spaces and palms of the hands
Tinea capitis	scalp, eyebrows and eyelashes
Tinea cruris	groin, perineum and perianal
Tinea corporis	glabourous skin of the trunk
Tinea pedis	infections of the toe webs and plantar of the feet
Tinea barbae	bearded areas of the face and neck
Tinea faciei	glabourous skin of the face, the chin and upper lip
Tinea unguium	nail plates

The dermatophyte fungi manifests asymptomatically causing chronic infections that are most common or it may lead to an acute phase associated with inflammation. Dermatophysis results in scaling, fissuring, maceration and erythema which are accompanied by itching or burning, (Kwon-Chung *et al.*, 1992).

Dermatophytes have vegetative structures, which exhibit morphological arrangement of the hyphae in form of, chlamydospores, chandeliers, spirals, pectinate, nodular and racquet hyphaes. Dermatophytes are communicable; they cause infections in healthy immunocompetent and in immunocompromised individuals. They are transmitted by shedding of infected skin cells and by direct body contact especially in school going children (Kwon-Chung *et al.,* 1992). It has been estimated that 30% to 70% of adults are asymptomatic carriers of these fungi (Woodfolk *et al.*, 1998).The etiological agents are commonly spread in public facilities like swimming pools, gyms and sharing of combs, fomites garments (Tietz *et al.,* 2003).Tinea pedis is more common in men due to warm and moist condition associated with wearing of socks with shoes. Dermatophytes are associated with specific age groups (teenagers as they approach puberty, young adults and the elderly). Dermatophytes are divided into three different groups based on their location and mode of transmission. These groups are zoophilic, geophilic and anthropophilic species. The geophilic species are commonly found in soil and rarely cause and infect humans; Zoophilic species are associated in both human and animals, rarely they do not infect human and when encountered they are characterized with acute inflammation upon infection (Summerbell, 2003). Chronic infections are standard with anthropophilic species suggesting that the fungi have adapted to their human host

(Summerbell, 2003). Within the Anthropophiles, polymorphous morphological variations are common, with numerous atypical and variant types being recognized, suggesting an indication of further genetic drift (Weitzman *et al.,* 1995). The need for species identification of dermatophyte in clinical settings is often related to epidemiological concerns by providing a preview on the scope of infection which may have animals as carriers. They may also be linked to recurrent institutional or family outbreaks such as *Trichophyton tonsurans* and *T .violaceum* (Arnow, 1991), causing rapidly progressing epidemics. These are geographically endemic, reflecting exposure during travel or residence in the area of endemicity or contact with persons with infection.

The dermatological conditions in Kenya have not been highlighted due to the fact that is usually considered as cosmetic problem or associated with poverty and poor sanitation. Very few studies to show the problem of superficial mycoses have been conducted in Kenya and thus limited data is available to portray the real impact, (Ayaya, 2001).

The studies which have been conducted basically have targeted school going children and the results have indicated that Tinea capitis with *Trichophyton violaceum* as etiology agent of the dermatomycoses. This study was conducted to try and bridge the gap in lack of data and also to characterize dermatophytes from the clinical sources in Nairobi, Kenya.

1.1: JUSTIFICATION

The HIV/AIDS and other immunocompromised infections and diseases have led to the steady rise in opportunistic fungal infections; with significant increase in the incidence of mycoses. Incidences of widespread of superficial fungal infection have been reported in Kenya. Inaccurate diagnosis has led to improper prescription leading to resistance of the fungus to antifungal and prolonged suffering in patients especially whose immune system is depressed. Accurate identification of the causative agent is therefore significant for quick accurate prescription. This study was carried out among the patients attending the Mbagathi District Hospital clinic to determine the prevalence of *Epidermophyton, Trichophyton* and *Microsporum*; and non dermatophytes isolated in the hospital. The hospital is located only a few hundred meters away from the Kibera slum, which is an informal settlement where health and environmental are poor predisposing the population to a variety of fungal infections. Therefore leads to a good coverage of low class patients who are most susceptible. Information about the variability of dermatophytes species and the evolutionary relationship between them is important for the understanding of the mechanisms of pathogenicity. While morphological appearance is essential for fungal taxonomy in general; it is inadequate for species identification or differentiation of dermatophytes. In addition to sharing a series of morphological characteristics, some species present atypical variations resulting from sensitivity to culture conditions. Thus, a combination of morphology, genetic and pathogenicity testing is the least option for identification of these fungi. Despite their medical importance, few studies are available on the taxonomy of *Trichophyton, Microsporium* and *Epidermophyton;* their intraspecific and interspecific variability. This study determined the phylogeny of dermatophytes using their molecular profiles. The antifungal drug pattern was also established to provide information on the extent of resistance to available commercial antifungal drug therapy.

1.2: OBJECTIVES

1.2:1 Overall objective

To determine the molecular and antifungal drug profile of dermatophytes isolated from clinical sources from Mbagathi District Hospital in Nairobi, Kenya.

1.2.2: Specific objectives

- To determine the prevalence of *Epidermophyton, Trichophyton* and *Microsporum* species isolated from Mbagathi District Hospital.
- To determine the molecular profile of *Epidermophyton, Trichophyton* and *Microsporum* species.
- To determine the antifungal drugs patterns of the isolated dermatophytes using the Minimum Inhibition Concentration (MIC) methods.

1.2.3: HYPOTHESIS

1. Dermatophytes species from clinical sources exhibit phenotypic and genotypic polymorphism.

2. Different strains of dermatophytes vary in their susceptibility to antifungal drugs.

CHAPTER TWO

2.0:LITERATURE REVIEW

2.1: Classification of dermatophytes

The main group of keritonophilic *Onygenales* is the family *Athrodermataceae;* that includes the dermatophytes and other soil fungi. Dermatophytes are defined as those *Athrodermataceae* that are agents of human and animal disorders (Sybren *et al.,* 2005, Atlas of clinical fungi). They usually have two types of conidia: microconidia and macroconidias which are multi-celled. The telemorph of dermatophytes have spherical evanescent asci containing eight ascopores; the ascoma wall has a loose network of hyphae with complicated branching and ornamentation. The ascomata are produced in a special media after mating.

Dermatophytes are classified into three anamorphic genera *Epidermophyton, Trichophyton* and *Microsporum* of anamorphic class Hyphomycetes of the Deuteromycota (Summerbell, 2003).The *Epidermophyton* genus has one species; the *Epidermophyton floccosum*. In this genus microconidia are not produced, it exhibits large macroconidia that are club shaped, thin walled multicellular and clustered .The macroconidia are broadly clavate with smooth, thin to moderate thick walls measuring 20 µm by 60 µm by 4 µm to 13µm in size and has one to nine septa, which are abundant in singles or in clusters.

Trichophyton genus exhibits both micro and macro conidia which are smoothed walled. The walls of the macroconidia are thin and cigar shaped, while those of the microconidia are pear shaped or are irregular in form. Sixteen species of *Trichophyton* are found in this genus.

Microsporum genus contains twelve species that exhibit both micro and macroconidias. The macroconidia have either a thick or thin echinulated cell wall. The main distinguishing feature in this genus is the presence of small spines projecting from cell walls hence making the cell wall to appear echinulated. The macroconidia are spindle shaped, having few or numerous septation.

The spectrum of dermatophysis in Kenya showed Tinea capitis as the most clinically manifested condition encountered especially in school going children; *Trichophyton* species was the most isolated fungi (Ayaya, *et al.*, 2001 and Schmeller *et al.*, 1994). Studies were done in Addis Ababa, Ethiopia, whereby 539 dermatological patients with signs of dermatophysis were investigated. Tinea capitis was diagnosed in 69% of the 374 children, Onchomycosis was 91 %(132/145) in the infected fingernails. *Trichophyton violaceum* was the most isolated dermatophyte with 71% (362/490), (Woldeamanuel *et al.*, 2005).

In Nigeria, a prevalence study was conducted among miners and office workers (Ozumba *et al.*, 2004) and *Trichophyton Spp* was the most isolated. In another survey of 13 schools in Western Kenya, showed the prevalence of 74 % which was clinical manifestation of Tinea capitis (Schmeller *et al.*, 1997) 18% was *Trichophyton Spp* and *Microsporum canis* was 12% respectively. Studies done in Malawi from 1987 to 1989, showed that 1.2%-1.5% of the population in Karonga District in Northern Malawi, were diagnosed with having Tinea facieci, Tinea corporis and Tinea inguinalis. With regard to the relative frequency of isolation of the dermatophytes the main findings were the rarity of *Trichophyton Spp* (1%) and the predominance of *Microsporum audouinii* (57%) in this part of Africa.

The identification of dermatophytes was based on the conventional and the molecular systems. The conventional system had the following tools that were based on purely morphologically features: growth on different kinds of Medias -Sabouraud peptone glucose agar with chloramphenicol, gentamicin and cycloheximide and vitamin test agars. *In vitro* hair perforation test was used for the detection of perforating organs formed by some species. Autoclaved polished rice was used to distinguish *Microsporum canis* from *Microsporum audouinii*; and for the induction of macroconidia in *Microsporium equinum* species. Christensen agar was used for the evaluation of urease production. Lacto phenol cotton blue staining technique and slide culture methods were used in microscopy identification.

The molecular system included the sequencing of the internal spacer transcriber (ITS) 1 and 2 (Gråsier *et al.,* 1998), amplification of ribosomal DNA, polymerase chain reaction finger printing and Restriction fragment length polymorphism. The difficulties associated with the conventional method were a tedious method and a lot of prior expertise is required; the molecular system of identification was quite expensive and applicable in advanced research setting.

2.1.2: Molecular identification of dermatophytes

Identification of dermatophytes was complicated and laborious due to the morphological similarity, variability and polymorphism exhibited: it required a significant level of knowledge and technological expertise. The use of mating test as a tool of identification is not practical because most of the anamorphic species lack a telemorph. When the G (guanine) +C (cytosine) content of the chromosomal DNA of the three dermatophytes genera *Trichophyton*, *Microsporum* and *Epidermophyton* were analyzed by (Davidson *et. al.*,1980) the result showed that there was a genetic homogeneity among the genera in contrast to their phenotypic and ecological variation.

This paved way for further investigation that focused on mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) of the dermatophytes. DNA sequence data can also be extremely useful for differentiating between fungal species. Molecular analyses of ribosomal DNA sequences have permitted inferences to be made on the relationship between different levels of divergence and are being increasingly explored as a taxonomic tool.

Analysis of ribosomal nucleotide sequences is well suited for use as taxonomic tool because they are universally present and their function in protein synthesis is highly conserved both in prokaryotes and eukaryotes.

Data from these investigations gradually lead to the development of phylogenetic relationship and epidemiology among the dermatophytes species and molecular technology used in identification. The use of molecular biology based techniques in identification has made it possible to identify the dermatophyte to species level and to distinguish the isolates to strain level. (Kanbe 2008)Methods that are used in molecular identification are:

(i) Restriction fragment length polymorphism (RFLP).

(ii) Polymerase chain reaction (PCR).

(iii) The Polymerase Chain Reaction with ribosomal DNA.

(iv) Randomly amplified polymorphic DNAs (RAPDs).

(i) Restriction fragment length polymorphism (RFLP).

Restriction fragment length polymorphism: the term polymorphism refers to the occurrence of many allelic variants of a particular gene or DNA sequence. Therefore RFLP are variation in length of restriction fragments generated by a particular enzyme cutting within a given locus. The basic method of RLFP is as follows;

DNA isolation

↓

Restriction digestion

↓

Agarose Gel Electrophoresis

↓

Southern Transfer

↓

DNA labeling

↓

DNA Hybridization

↓

Stringent washing

↓

X-ray film Exposure and Visualization

The small size and the availability of numerous copies of mtDNA have frequently been used in phylogenetic studies.

(ii) Polymerase chain reaction (PCR).

This is an in vitro method for synthesizing defined DNA sequences thus amplifying DNA. The reaction uses two oligonucleotide known as primers, which hybridize to opposite strands and flank the target DNA sequence that is to be amplified. The elongation of the primers is catalyzed by Taq DNA polymerase. A complementary sequence is found in the genome of the microbe DNA of interest to which the primers bind. The Taq DNA polymerase then adds nucleotides to the primer and extends it using the microbe genome as a template so that a double strand is formed. This follows the synthesis of the second strand that occurs in the 5'→3' direction.

The principal of PCR is the repetitive cycle of denaturation, polymerization and renaturation, (Gräser, 1999).

(iii) The Polymerase Chain Reaction with ribosomal DNA.

The ribosomal DNA contains repeats, that consists of a small subunit (SSU, 18S) an internal transcribed spacer (ITS1) 5.8S, an internal transcribed spacer (ITS2) and the large subunit (LSU, 26S). These repeats are separated by NTS (non- transcribed spacers).With the use of 2 primers, mostly a 17 to 20 mer sequence, part of the ribosomal DNA is selected and elongated, by means of an enzyme, DNA polymerase.

The product of RNA polymerase I is processed in the nucleolus, where the ITS1 and ITS2 are cut and three types of rRNAs are produced. For this purpose the DNA should be single stranded, at high temperature (denaturation).

The ITS regions form specific secondary specific structures which are needed for the correct recognition of cleavage site and the binding site for nucleolar proteins and RNAs during ribosome maturation.Fig1, shows the rDNA region with primers ITS1 and ITS4 localization indicated by the arrows and the formation of three mature rRNAs,(Gräser, 1999 and Kanbe,2008).

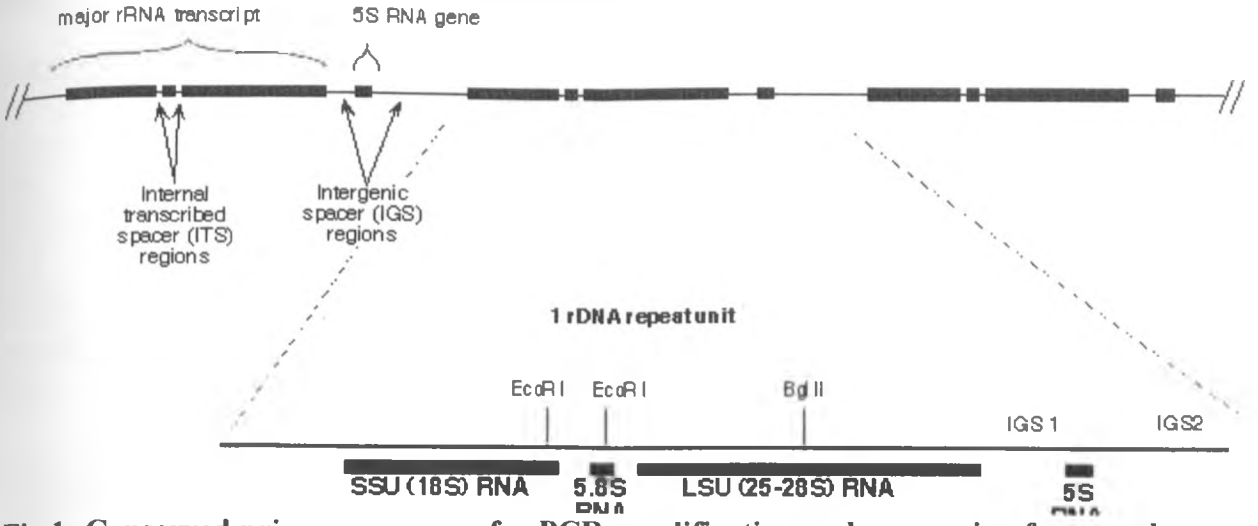


Fig 1: Conserved primer sequences for PCR amplification and sequencing from nuclear ribosomal RNA adapted from (White *et al.*, 1990).

The diagram below shows the primer location in the ribosomal cassette consisting of SSU, ITS1, 5.8S, ITS2 and LSU shown in figure 2 below. The primers are positioned above (forward primers) and below (reverse) their sequences positions.

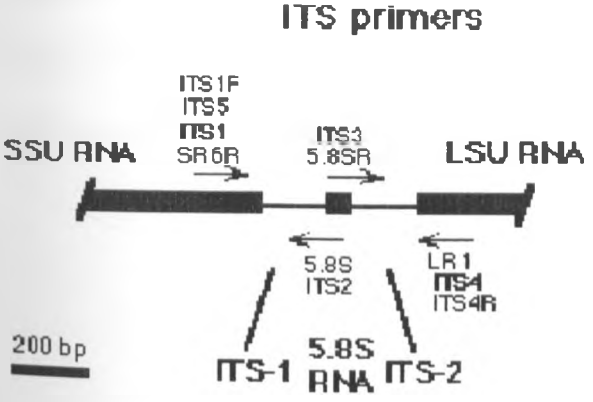


Fig2: Internal transcribed spacer (ITS) region primers.

Table 2: Different types of primers used in identification of filamentous fungi.

Primer name	Sequence (5'->3')	Reference
ITS1	TCCGTAGGTGAACCTGCGG	White *et al,* 1990
ITS2	GCTGCGTTCTTCATCGATGC	White *et al*, 1990
ITS3	GCATCGATGAAGAACGCAGC	White *et al,* 1990
ITS4	TCCTCCGCTTATTGATATGC	White *et al*, 1990
ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al,* 1990
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns, 1993
ITS4-B	CAGGAGACTTGTACACGGTCCAG	Gardes & Bruns, 1993
5.8S	CGCTGCGTTCTTCATCG	Vilgalys lab

The Polymerase chain reaction (PCR) and the analysis of the amplified rDNA using the endonucleases have been used to achieve efficient interspecies discrimination in medical mycology (Iwen *et al.,* 2002). Studies done by Hsin *et al.,* 1999, where they identified dermatophytes by sequencing and analyzing the rRNA gene internal transcribed spacer regions. They evaluated 17 dermatophytes species who's ITS 1 and ITS 2 regions were amplified and sequenced. From the result it was found out that some clinical isolates were misidentified by conventional methods. The clinical isolate *Microsporium gypseum* was misidentified as *Microsporium fulvum* as revealed by the ITS 1 and ITS 2 sequences. It has been reported by De Hoog *et al.,* 2002, that it was not easy to differentiate *Microsporium gypseum* from *Microsporium fulvum* on the basis of their morphological characteristics.

(iv) Randomly amplified polymorphic DNAs (RAPDs).

In the RAPD technique, a single short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from complex DNA template,(Gräser, 1999).

The basic method for RAPD is:

DNA isolation

↓

DNA amplification

↓

Agarose gel electrophoresis

↓

Ethidium Bromide staining

↓

Illumination with UV light and Visualization of band patterns

RAPD technique is a useful tool in identification and sub-typing of dermatophytes. It is however acknowledged that the amplification profiles of the amplified products from random primers are frequently complicated due to low affinity of the primers. Also the amplified genes are unknown. This suggests that the RAPD method has low specifity and reproducibility when compared to PCR using gene specific primers. It is an expensive and wasteful procedure when used to identify one unknown single fungi isolate because numerous reference DNAs must be prepared for precise identification. However, the RAPD method could be used useful since it is possible to adapt many random primers for many fungal species. It is better to use RAPD technique in combination with gene specific PCR- RLFP for fungal identification to the species level.

A study done by Liu *et al.*, 1997; by assessing 90 individual primers, it was possible to selects several potential primers which could be used in the identification and differentiation of dermatophytes. The OPAA11 (5'-ACCGACCTG-3') and OPD18 (5'-GAGAGCCAAC-3'), primers were used with RAPD technology to identify 90 suspected dermatophytes species. The result showed that, out of the 25 dermatophytes species and subspecies examined 16 of them displayed distinct DNA patterns after amplification with random primer OPAA11(Liu *et al.*, 1996). The random primer OPD18 was used to identify 19 out of the 25 dermatophytes.

The other aspect of this study revealed that DNA from non dermatophyte (*Fusarium, Aspergillus* and *Scytalidium Spp*), generated DNA products with these primers. The band patterns formed by these fungi were quite distinct from those formed by the dermatophytes. Hence they could be differentiated from dermatophytes species and subspecies. When comparing this method of identification with that of convectional, data has shown that RAPD measures the genotypic characteristics as compared to phenotypic which is usually in the case of convectional method. Some of the dermatophytes species like *Trichophyton rubrum, Trichophyton violaceum* at times posses a non pigmented appearance and they may be identified in correctly based on this and in such circumstance correct identification is done via RAPD.

2.1.3: Antifungal agents and Management of fungal infections

Dermatophytes are located in the stratum corneum within the keratinoncytes.Symptoms and signs that appear in infected individuals are due to acute and inflammatory changes in the dermis. For these reasons, antifungal agents should have the ability to penetrate the stratum corneum cells to be efficient when applied topically. Majority of antifungal drugs are fungistatic and when applied topically, the concentration is achieved in the skin. The growth of the dermatophyte is delayed and these are shed with the skin renewal and healing is achieved .The choice of treatment is determined by the site, the extent of the infection, the species involved, the efficacy, safety profile and the kinetics of the drugs being administered.

The Management of dermatophytes begins with topical agents which are morpholine derivatives, tolfnaftate and imidazole; while allylamines are generally used on localized non-extensive lesions. Systemic antifungal drug therapies are used for Tinea unguium, scalp ringworm and extensive dermatophytoses. Skin lesions located on the face, limbs and trunks usually require two to three weeks of treatment. Inflammatory dermatophyte infections of the feet should be treated for four to six weeks. The application of the medication should be done by rubbing it in gently in to the affected skin area and should surpass one centimeter.

Oral treatment is used in treatment of skin lesion with folliculitis, Tinea capitis, Tinea barbae, Tinea unguium, hyperkeratotic lesions of the palms and the soles and when there is no response to topical treatment or tolerance is not adequate. Once clinical cure is achieved, it is advisable to prolong treatment for two weeks and the patient is emphasized to follow the application of treatment as the doctor has prescribed.

(i) Griseofulvin antifungal

This oral antifungal agent is still the gold standard for treatment of dermatophytoses excluding Tinea unguium. Following the observation of Gentles, 1958; this drug was produced from *Penicillium* species and was introduced in the sixties. Griseofulvin inhibits fungal cell mitosis by disrupting mitotic spindle formation which is a critical step in cellular division.

Once the Griseofulvin reaches the stratum corneum, reversible protein binding and lipid solubility results in its concentration in the horny layer .When administration is discontinued the drug is rapidly cleared, hence its excellent safety profile. Absorption of Griseofulvin is poor in the gastrointestinal tract; it is influenced by particle size, dietary fat intake and the dissolution rate of the various preparations. Micronized and ultramicronized formulations are absorbed better, (Arujo, 1990).Griseofulvin is eliminated as different metabolic waste in the urine and faeces and a daily dose is required (Gupta *et al.*, 1994)

(ii)Azoles antifungals

The azoles inhibit the fungal cytochrome P-450-dependent enzymes resulting in the impairment of ergosterol synthesis and depletion of ergosterol in the fungal cell membrane and accumulation of toxic intermediate sterols, causing increased membrane permeability and inhibition of fungal growth. This class is made up of Ketaconazole, Miconazole, Fluconazole and Itraconazole.

Itraconazole is a triazole agent, poor water soluble and its bioavailability improves when the drug is taken with fatty meal (Haria *et al.*, 1994).

The oral absorption is dose dependant, extremely lipophilic and achieves high concentration in fat, skin and nails and in vaginal/cervical tissues (Haria *et al.*, 1994). Approximately 65% of the compound is eliminated in the faeces and 35% in the urine in the form of diverse metabolites.

It has been suggested to be a safe drug (Haria *et al.*, 1994), with the frequency of the side effects appearing to depend on the duration of therapy. Nausea, vomiting and headache are the most frequent side effect, with liver function abnormalities occurring in less than 1% of patients. Itraconazole is available in liquid and capsule form.

Fluconazole is a triazole that is water soluble and extremely well absorbed, it has a high bioavailability which is unaffected by food intake, pH, or antiacids (Pierard *et al.*, 1996). Fluconazole is widely distributed in the body tissues including the cerebrospinal fluid (Gupta *et al.*, 1994) Most of the drug is excreted unchanged in the urine since it undergoes no hepatic metabolism. It is eliminated more slowly from the skin, and therefore clinical cures may be achieved after the withdrawal of the treatment. It requires dosage adjustment in patients with impaired renal function. Nausea, vomiting and liver test abnormalities are most common side effects and occur in approximately 16 % of the patients.

(iii)Allylamines antifungals

Terbinafine

Allylamines work in a conceptually similar fashion to azole antifungals by inhibiting the synthesis of ergosterol. However, allylamines acts at an earlier step in the ergosterol synthesis pathway by inhibiting the enzyme squalene epoxidase. Like the azoles, Terbinafine has the potential for drug interactions with other medications metabolized through the mammalian cytochrome P-450 pathway (Darkes *et al.*, 2003).

Terbinafine is a fungicidal allylamine that is absorbed from the gastrointestinal tract, with a bioavilibity of 70-80%. It binds to strongly to plasma proteins and about 8% binds to blood cells (Gupta *et al.,* 1994), achieving high concentrations in skin and skin structures. Almost 80% of the administered drug is eliminated as metabolite in urine. Dosage adjustment is required in patient with severe hepatic or renal dysfunction or both. When treatment with Terbinafine ceases the concentration in stratum corneum remains high (0, 10μg/ml) for eight weeks and enables the use of short courses or treatment. The side effect appears in the first week of treatment and tends to disappear with continued treatment.

Treatment failures are summed up as; presence of concomitant bacterial infection and the likely hood of re-infection or relapses. When incorrect diagnosis of dermatophytes are encountered which are rectified by performing KOH (potassium hydroxide) test and culture identification. Other factors such as presence of underlying skin diseases such as lichen, psoriasis and patients adherence to the drug treatment as described by the doctor (Amalia *et al.,* 2000).

CHAPTER THREE

3. 0: MATERIALS AND METHODS

3.1: Study site

The specimens were collected from Mbagathi District Hospital in Nairobi; it serves as a district hospital for Nairobi area which is situated near the Kibera slum. The dermatology unit of the hospital serves a population from different social classes within Nairobi. The specimens were made up of scrapings from nail, hair and skin from patients suspected with dermatophyses.

Primary identification and the antifungal drug profile of the dermatophytes were done at Center for Microbiology Research located in the Kenya Medical Research Institute. The fungal strains were transferred to Centraalbureau voor Schimmelcultures Fungal Biodiversity Center, Institute of Royal Netherlands Academy of Arts and Sciences, located in Netherlands for molecular characterization.

3.2.1: Specimen Collection

The sample size was based on the average prevalence of fungi isolation from clinical sources of between 10%-30% as described by (Woodfolk *et al.*, 1998). Assuming a prevalence of 21% a sample size of 101 patients was used. This figure was derived from (Woodfolk *et al.*, 1998) formula below:

$$n \geq [Z^2_{(1-\alpha/2)} \sqrt{P (1 - P)/n}]/\delta$$

Where: $Z_{(1-\alpha/2)} = 1.96$ - The value corresponding to the 95% Confidence interval

$\delta = 0.079$ - allowable error

$P = 0.21$ - assumed probability of fungi isolation

n = Minimum sample size

$n \geq (1.96)^2 * (0.21 * 0.79)/ (0.079)^2 = 0.6373/(0.079)^2 = 102$

The sample size estimate of 102 non replica samples is at the 95% confidence interval and 5% level of significance with an error margin of 5%.

Patients who reported at the hospital with suspected dermatophytes had their data taken which included age and sex, after which the area for sampling was cleaned with 70% alcohol. Suspected Tinea capitis, hairs were plucked from the scalp; while skin scraping samples was collected using a blunt scalpel.

When patients presented with minimal scaling, adhesive tape was used to collect the samples. Infection of the nails were sampled by scrapings and clipping. The collected samples were put into envelopes.

3.2.2: Sample culturing and identification

The samples were seeded on to Sabouraud's dextrose agar (SDA) medium with chloramphenicol (0.05mg/ml) (Oxoid, U.K.) and incubated at 30°C for two weeks. Colony and microscopy characteristics were observed and recorded during the growth period. These were the colour, texture and topography of the colony; mode of hyphal growth (aerial and submerged). Microscopic morphology was based on the appearance and arrangement of macro and microconidias depicted by slide culture. Sporulation was enhanced by re- seeding the cultured plates on to malt extract agar (MEA) and incubated for one week at 25°C.This nutrient rich media was used for slide culture. Using a flamed spatula, a block of fresh solidified MEA of approximately 5X5 mm, was placed on to the sub-cultured dermatophyte plates. Sterilized cover slips measuring 22X22 mm was placed on the cut block medium and pressed gently using a flamed forceps. Culture was then incubated at 30°C for 14 days till sporulation was observed (Harris, 1986).

a) Microscopic identification

Lacto-phenol cotton blue (LPCB) staining technique was to distinguish the three anamorphic genera by their characteristic microconidia and macroconidia. Standard microscope slides (75X25 mm) were labeled and a drop of mounting medium was placed at the center of each slide. Using a flamed forceps, the top cover slip was removed from block of culture that had been incubated for 14 days after sporulation and dried by applying two to three drops of 95% of ethanol to the growth on the glass. After drying, two drops of LPCB was placed on to the glass slide and the cover slip with the growth was placed on top of the glass microscope.

The slide was then observed at 40X and 100X for the arrangement of hyphae, micro and macro conidias, (Sigler 1992).

3.2.3: Molecular characterizations

a) DNA extraction

Approximately 1 cm^2 of the fungal material from the pure culture was transferred in to the 500µl TEs buffer solution and five glass beads (size 4mm, fisher Scientific) were added. The samples were then homogenized for 6 minutes using the tissue lyser and spun for 2 minutes at 14,000 rpm. 10 µl of proteinase K was added to the grinded material and vortexed to homogenize the material and incubated for 30 minutes in a water bath at 55^0C. 120 µl of 5M sodium chloride and 62 µl of CTAB were added, vortexed for 2 seconds and incubated at 65^0C for 60 minutes. The samples were homogenized for 3 minutes at (30 Hz) using the tissue lyser after which 700 µl of chloroform was added and spun for 5 minutes at 4^0C at 14.000 rpm.600 µl of the top layer (water phase) was then transferred into a clean sterile eppendorf cup and 330 µl of isopropanol was added to the supernatant.

The solution was carefully mixed by hand and incubated at -20°C overnight to allow the DNA to precipitate. This mixture was spun at 4^0C at 14.000 rpm for 10 minutes. The pellet was washed twice with 700 µl 70% ethanol and spun for 5 minutes at 14,000 rpm; the ethanol was removed with care not to lose the (sometime clear) pellet. The pellet was then air dried, and re-suspended in 50 µl TE buffer.

b) The Polymerase Chain Reaction with ribosomal DNA

The thermocycle conditions were programmed as follows; Cycle 1X was at 95° C for 2 minutes with initial denaturation for 35 seconds at 95° C. This was followed by DNA amplification and annealing for 30 seconds at 52° C. Elongation was done 60 seconds, at 72° C for 35 cycles. The last cycle of the final extension was for a period of 7 minutes at 72 ° C The amplified PCR products were visualized on a 2% agarose gel electrophoresis containing ethidium bromide. Whereby 10 µl was loaded (2 µl of the loading buffer and 8µl of PCR product) into each well, with the marker ladder being loaded on to the first gel well. The electrophoresis was ran at 75V, until DNA/bromophenol blue had migrated into the gel, then at 150V, for 1.5-2 hours while the voltage remained constant. This was followed by observing the gel under ultraviolet light. The pictures were taken and the fragment sizes recorded (http://www.protocol-online.net/Protocol.htm).

Table 3. Recipe for PCR preparation.

ITS 1 AND 4; PRIMERS LS266 and V9G.						
MASTER MIX			PCR PROGRAME			
	1 isolate	36 isolates	Denature	5(2) mins	95°C	1X
Biotaq buffer	2.5	90	Annealing	35 sec	95°C	35X
$MgCl_2$ 50mM	0.75	27	Elongation	30 sec	52°C	35X
dNTPs 1mM	1.85	66.6		60 sec	72°C	35X
LS 266	1	36	final extension	7 mins	72°C	1X
V9G	1	36	∞		4°C	
BioTaq 5U/ul	1	36				
deminerilized H2O	14.6	525.6				
DMSO	1.3	46.8				
DNA	1	36				
TOTAL	25ul	900ul				

c) Construction of the phylogenetic tree

The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.,* 1987). Neighbor-Joining method worked by clustering taxa to minimize the total distance in the tree. First, a star phylogeny showed each taxon as equally distant from every other taxon. Then the initial distance in the star was calculated. Then the Neighbor-Joining algorithm temporarily removed a pair of taxa from the star, clustering the pair at a new node. The total distance in the tree was recalculated for the tree that had two new nodes. This was followed by returning the first pair of taxa to the star and the next pair of taxa was clustered at a new node. This process continued until the total distance in the tree had been recalculated with every possible pair of taxa forming a new node. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

3.2.4 : Antifungal susceptibility testing of Dermatophytes isolates

The susceptibility testing of dermatophytes was performed as suggested by the Clinical Laboratory Standard Institutes M38-A2, which was the reference method for Broth dilution of antifungal susceptibility testing of filamentous Fungi. The *in vitro* activities of the micro-dilutions of all the antifungals tested the MIC minimum inhibitory concentrations (MIC), the MIC range, and the MIC at which 50% (MIC_{50}) and 90% (MIC_{90}) of the dermatophytes isolates tested were inhibited.

a) Preparation of the micro dilution plates

Antifungal drug testing.

The modified protocols described by CLSI, 2008 referred to as (M38-A2) standard method for conidium forming filamentous fungi to test for antifungal drug susceptibility dermatophytes. Fluconazole, Isaconazole, Posiconazole, Itraconazole and Voriconazole (Janssen- Cilag Beerse, Belgium) were used in this study.

The drug was prepared by dissolving 20 mg of fluconazole antifungal in 1ml of dimethylformamide (DMF) (Sigma chemicals Co., St. Louis, Mo., U.S.A.).One milliliter of fluconazole was diluted in 14.63ml of RPMI to make an initial concentration of 1280 µg/ml. For Itraconazole, 10mg of ITR was dissolved in 1ml of polyethylene glycol. This solution was diluted in 30.25ml of RPMI to make an initial concentration of 320µg/ml.

These stock solutions were kept frozen in 1ml aliquots at -70°C. A working solution of each antibiotic was prepared by diluting 100 µl of the stock solution in 900 µl of RPMI-1640 medium containing L-glutamine and 0.165M morpholine propane sulfonic acid (MOPS) without bicarbonate (GIBCO BRL, Life Technologies, Paisley, Scotland) to get a concentration of 32µg/ml for Isaconazole, Posiconazole, Itraconazole and Voriconazole and 64 µg/ml for Fluconazole. *Candida parapsilosis* ATCC 22019 (The American Type Culture Collection (ATCC) was used as quality control strain to test for the antifungal drugs that were used. The reference strain was grown in 10ml brain heart infusion broth (Difco) at 35°C overnight.

The suspension was diluted to two folds with brain heart infusion broth containing 20% glycerol (Sigma), and dispensed in screw-capped tubes, sealed and stored at -70°C. The reference strain was tested with every batch of antifungal susceptibility of the isolated dermatophytes species. Sterility control (negative control) and growth control (positive control) was included in each plate. With each batch of antifungal susceptibility, antifungal control using *C. parapsilosis* ATCC 22019 reference strain was inoculated to test for the validity of the five antifungal agents according to the (CLIS), M38-A2 ,standard method.

b) Preparation of dermatophyte inocula.

Dermatophyte isolates were re-seeded on oatmeal cereal agar and potatoes dextrose agar slants for 7 days at 28°C; which supported the conidial growth (Jessup *et al.,* 2000). Sterile normal saline (0.85%) was added to the slant culture and was gently swabbed with a cotton tip applicator to dislodge the conidia from the hyphal mat. This suspension was adjusted to 5 ml with sterile normal saline. The cell density was adjusted to give final inoculum concentration of 104 (colony forming unit) CFU/ml.

The suspension was counted on a hemocytometer and diluted in RPMI 1640 to the desired concentration. 100µl of the organism suspension was transferred into all wells of the micro-dilution plates except for the negative control wells. The plates were incubated aerobically 7-10 days at 28°C, except for *E. floccosum* and *M. canis* at 35°C.

c) Reading and interpretation of the panel.

The minimal inhibitory concentration (MIC) endpoints was determined according to CLSI M38-A2 standards as the point at which no visual turbidity where the organism was inhibited 80% when compared to the growth control. For the quality control *Candida parapsilosis* MIC endpoint was determined as ≥80% inhibition of the positive growth control for Itraconazole (CLSI M38-A).

3.2.5: Data analysis.

The raw data were coded and analysed using Statistical Package for Social Sciences (SPSS) version 11 to determine chi square distributions for qualitative data such as the distribution of both dermatophytes as well as non-dermatophytes. The charts and tables were used to represent these data. The mean and the range were calculated for quantitative data such as minimum inhibition concentration (MIC).

The test sensitivity was calculated using the following formula;

$$\text{Sensitivity} = \frac{\text{number of true positive (TP)}}{\text{Sum of the number of TP and number of false negative (FN).}}$$

$$\text{Specificity} = \frac{\text{Number of true negatives (TN)}}{\text{Sum of TN and the number of false positives (FP).}}$$

The positive predictive value (PPV) which is the proportion of patients with positive test results who were correctly diagnosed was determined as follows; PPV = TP/sum TP+FP. While the negative predictive value (NPV) defined as the proportion of patients with negative test results who were correctly diagnosed was determined as follows; NPV = TN/TN+FN. The 95% confidence interval for the sensitivity and specificity were calculated using an online exact confidence interval for proportion method.
(http://www.causascientia.org/math_stat/ProportionCI.html).

CHAPTER FOUR

4.0: RESULTS

A total of one hundred and one samples were obtained from different parts of the body with their diversity shown in table 4 below. Tinea corporis (23%) showed the highest manifestation of dermatomycoses followed by Tinea capitis (14%) respectively.

Table 4: Diversity of clinical samples collected from the patients

Suspected site of manifestation	Total number of samples Collected.	Positive for fungal elements	Frequency distribution (%)
Tinea capitis	20	14	13.86
Tinea barbae	4	0	0
Tinea corporis	46	23	22.77
Tinea pedis	9	5	4.95
Tinea manuum	8	4	3.96
Tinea unguium	7	3	2.97
Tinea faciei	7	0	0
Total	**101**	**49**	**48.51%**

Total of 49 of dermatophytes and non dermatophytes were isolated from 101 specimens. The frequency of dermatophytes and non dermatophytes isolated are shown in (Table 4) below. There were no individuals who had co-infections with more than one strain of either the dermatophytes or the non dermatophytes. The commonly isolated dermatophytes were *Trichophyton rubrum* (16%), *Trichophyton mentagrophyte* (14%), *Trichophyton terrestre* (12%) and *Trichophyton violaceum* (10%) respectively (Table 4). There was a significant variation in the prevalence of the different dermatophytes was at ($P = 0.011$).

Table 5: Prevalence of dermatophytes isolated from clinical the samples.

Isolate Type	Frequency	Prevalence (%)
A. vanbreuseghemii	1	2.04
A. versicolar	1	2.04
C. keratinophilum	4	8.16
E. floccosum	4	8.16
M. canis	1	2.04
M. gypseum	2	4.08
S. brevicaulis	2	4.08
T. concentricum	1	2.04
T. interdigitale	1	2.04
T. mentagrophyte	7	14.29
T. rubrum	8	16.33
T. schoenleinii	1	2.04
T. terrestre	6	12.24
T. tonsurans	3	6.12
T. verrucosum	2	4.08
T. violaceum	5	10.20
Total	**49**	**100**

Chi square 27.33; P Value =0.011

From the total 101 patients that were observed and 49 of them were infected with either dermatophytes or non dermatophytes. The gender was 51 females and 49 males, (Table 6) which were divided into three groups based on their ages. 0-12 years the pediatric were 8, then adolescent from the ages 13-18 were 32 and the rest 61 patients were adults. 61% (62/101) of the samples collected were KOH positive (when examined for fungal elements using potassium hydroxide) and the rest 38% (39/101) were negative for fungal elements KOH negative. When these samples were cultured on to the Sabouraud dextrose agar, 57% (58/101) were positive and the rest 42% (43) were culture negative.

Table 6: Age, gender and specimen distribution amongst the patients suspected with dermatomycoses.

Dermatophytes	female	male	Child	adults
C. keratinophilum	2	2	4	0
A. vanbreuseghemii	0	1	1	0
M. canis	1	0	1	0
T. concentricum	1	0	1	0
T. interdigitale	1	0	0	1
T. schoenleinii	0	1	0	1
T. verrucosum	1	1	1	1
M. gypseum	1	1	1	1
T. tonsurans	2	1	2	1
E. floccosum	1	3	1	3
T. violaceum	1	4	5	0
T. terrestre	3	3	4	2
T. mentagrophyte	4	3	3	4
T. rubrum	6	2	6	2
Scopulariopsis brevicaulis	1	1	2	0
Aspergillus versicolar	1	0	1	0
TOTAL	**26**	**23**	**34**	**15**

The gender and age of the infected patients with dermatophytes

Trichophyton rubrum were commonly isolated in females who were under the age of eighteen and the rest was male who adults. Five children under the age of eighteen showed the highest isolation of *Trichophyton violaceum* these were isolated from tinea capitis (3) and (2) tinea corporis. The infestation was high in female (3) as compared to male (2) for Tinea corporis. *Trichophyton mentagrophytes* was isolated more in female who were adults, they were isolated from the head and feet, its frequency was lower in children. *Trichophyton terrestre* and *Chrysosporium keratinophilum* were isolated more from children (4) both of equal ratio of male to female compared to adults were it was lest isolated.

Trichophyton verrucosum was isolated from the body of a female child and *Trichophyton schoenleinii* was isolated from the leg of an adult male. *Trichophyton interdigitale* was isolated from the feet of an adult female whereas *Trichophyton tonsurans* were isolated from the body of a female child and the other one from the head of a female adult. *Epidermophyton floccosum* was isolated from three males who were adults and one female child; two were isolated from the body from hand and feet. *Microsporum canis* was isolated from the head of a female child. *Microsporom gypseum* were isolated from a male adult from the head and from the body of a female child. *Aspergillus versicolar* was isolated from the body of a female adult and *Scopulariopsis brevicaulis* was isolated from the body of a male child.

4.1: CHARACTERIZATION OF THE DERMATOPHYTES

4.1.1: Taxonomy of the dermatophytes isolated from the patients.

The suspected dermatophytes were cultured onto the SDA (Plate 1a, b); and slide culture was prepared and stained with lacto-phenol cotton blue (Plate 1c).

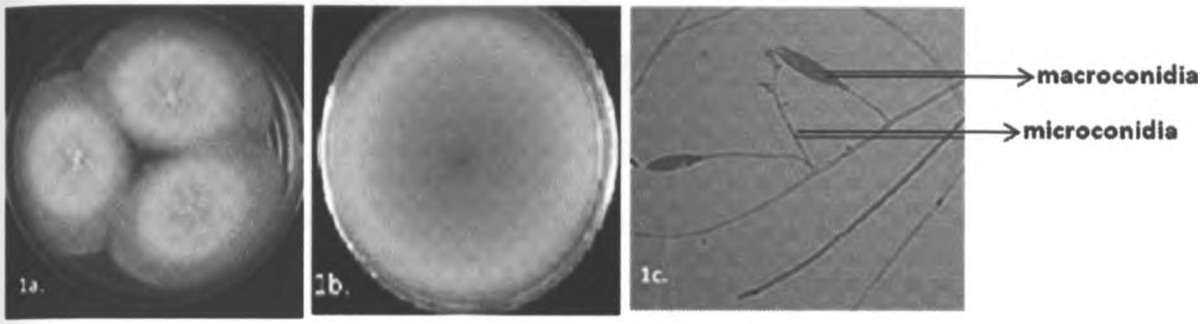


Plate 1a, 1b, 1c. *Microsporum gypseum* colonial and microscopic features.

(a). Typical colonial appearance of *Microsporum gypseum* on SDA frontal view.

(b). Typical colonial appearance of *Microsporum gypseum* on SDA reverse view.

(c). Microscopic features showing the appearance of microconidia and macroconidia of *Microsporum gypseum.*

On Sabouraud dextrose agar (SDA) *Microsporum gypseum*, morphological features included; flat topography with a radiating margin with powdery texture and front cinnamon colour, with buff reverse (plate 1a and 1b).Microscopic features included: Macro conidia formed in clusters, with rough and thin walled, cylindrically shaped, measuring 7.5µm to 11.5µm by 25 to 60 µm by 50 to 55 µm with 4 to 6 septa. The shape and branching of *Microsporum gypseum* macro and microconidia on LPCB (Plate 1c).

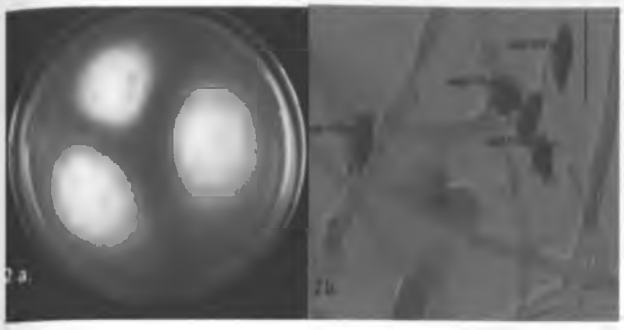


Plate 2a, 2b. *Chrysosporium keratinophilum* colonial and microscopic features.

(a). Typical colonial appearance of *Chrysosporium keratinophilum* on SDA frontal view.

(b). Microscopic appearance *Chrysosporium keratinophilum* showing large smooth macro conidia emerging from swollen stalk

The colonial appearance of *Chrysosporium keratinophilum* on SDA was yellowish white in front, with the topography heaped at the center as shown in (plate 2a). The microscopic features after staining LPCB: (Plate 2b) the conidia were broadly pear shaped (pyriforn), 6µm to 9µm in length forming alueriоconidia. The terminal and lateral conidia were sessile and non septate (Plate 2b).

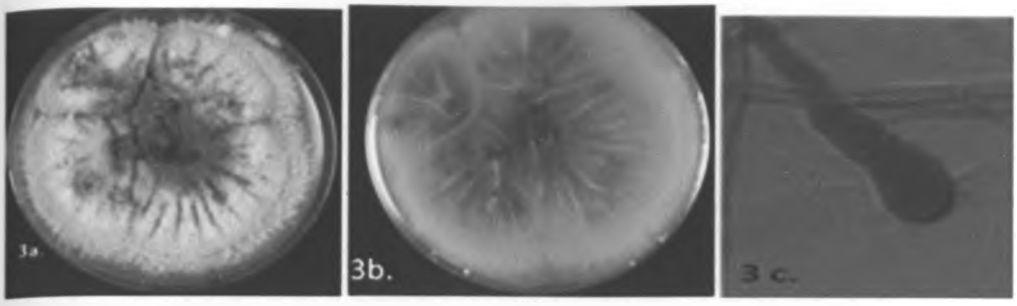


Plate 3a, 3b, 3c. *Epidermophyton floccosum* colonial and microscopic features.

(a). Typical colonial morphology of *Epidermophyton floccosum* frontal view

(b). Typical colonial morphology of *Epidermophyton floccosum* back view.

(c). Macroconidia with a clubbed shaped end.

From the top view, colonial morphology of *Epidermophyton floccosum* on SDA was yellowish green, with grainy appearance bearing small white tufts of cottony white material at the center with colourless submerged edge while the reverse was pale brown, (Plate 3a). The reverse of the plate was buff coloured with ridges as shown in (Plate 3b).The lacto phenol stained conidia were blunt and club shaped with the apical area swollen (Plate 3c).These conidia were 3 cells long and the surface was smooth with a banana shaped appearance as shown in (Plate 3c).

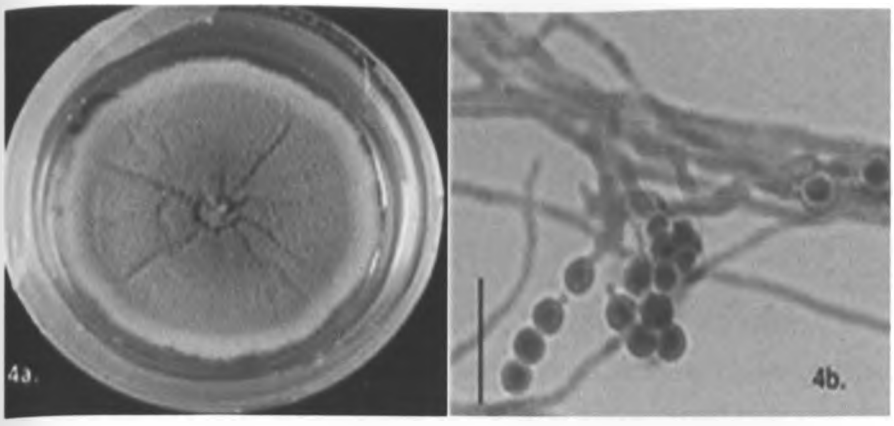


Plate 4a, 4b. *Scopulariopsis brevicaulis*, colonial and microscopic features.

(a). Typical colonial morphology of *Scopulariopsis brevicaulis*.

(b). Macroconidia that are rough walled forming lemony shaped chains

The colour of the colony of *Scopulariopsis brevicaulis* on SDA was brownish in colour, while the texture was velvety to powdery with a flat topography as shown in (Plate 4a). The conidia were rough walled forming lemony shaped chains shown in (Plate 4b).

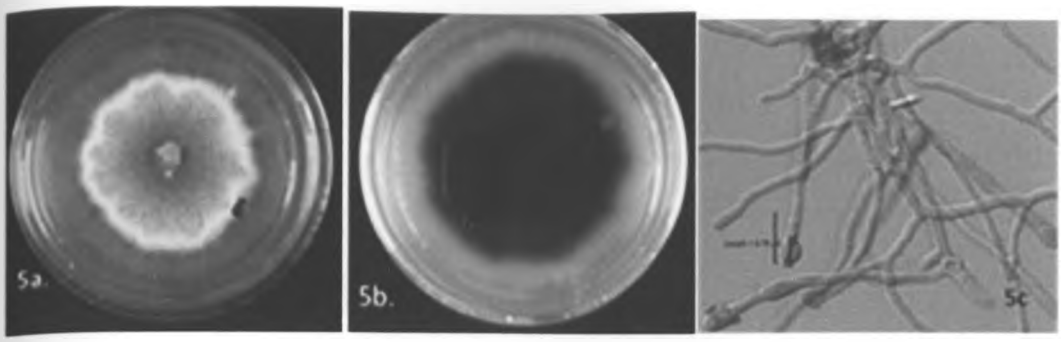


Plate 5a, 5b. *Trichophyton violaceum* colonial and microscopic features.

(a).Typical colonial morphology of *Trichophyton violaceum* on SDA frontal view.

(b).Typical colonial appearance of *Trichophyton violaceum* on SDA reverse view.

(c). Microscopic morphology of the microconidia which are clubbed shaped.

The colonial appearance of *Trichophyton violaceum* on SDA was irregular and wrinkled with a heaped center. The texture was glabrous, while the front and the reverse colour was dark purple red. The microconidia were club shaped as shown in (Plate 5c).

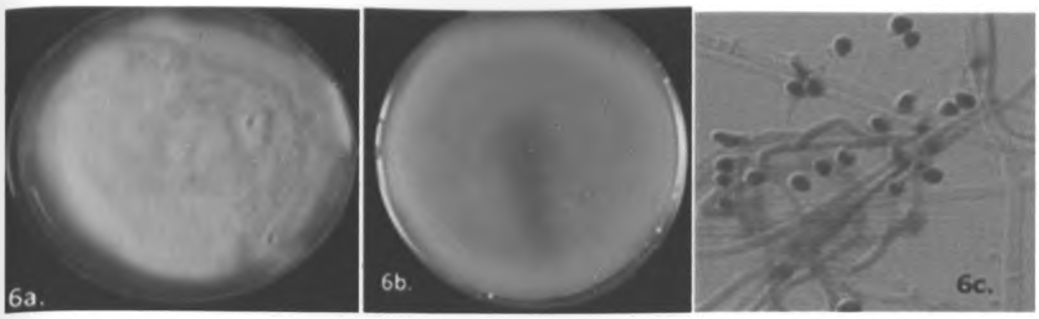


Plate 6a, 6b, 6c. *Trichophyton mentagrophyte* colonial and microscopic features.
(a). Typical colonial morphology of the *Trichophyton mentagrophyte* on SDA frontal view.
(b). Typical colonial morphology of the *Trichophyton mentagrophyte* on SDA reverse view.
(c). Round microconidia formed in clusters ("en grape").

The colonial morphology of the *Trichophyton mentagrophyte* on SDA was white in colour with a cottony to velvety texture as shown in (Plate 6a). The topography of the colony was flat in while the reverse was tan to pale yellow as shown in (Plate 6b). When stained with LPCB, the microconidia were abundant and round in shape with a length of 3µm and were borne in grape-like cluster with spiral hyphae as shown in (Plate 6c).

4.2:MOLECULAR CHACRACTERIZATION

PCR

DNA was extracted, and the internal transcribed spacer (ITS) region of the isolated strains was amplified by universal primers; V9G and LS266, as shown in (Plate7).

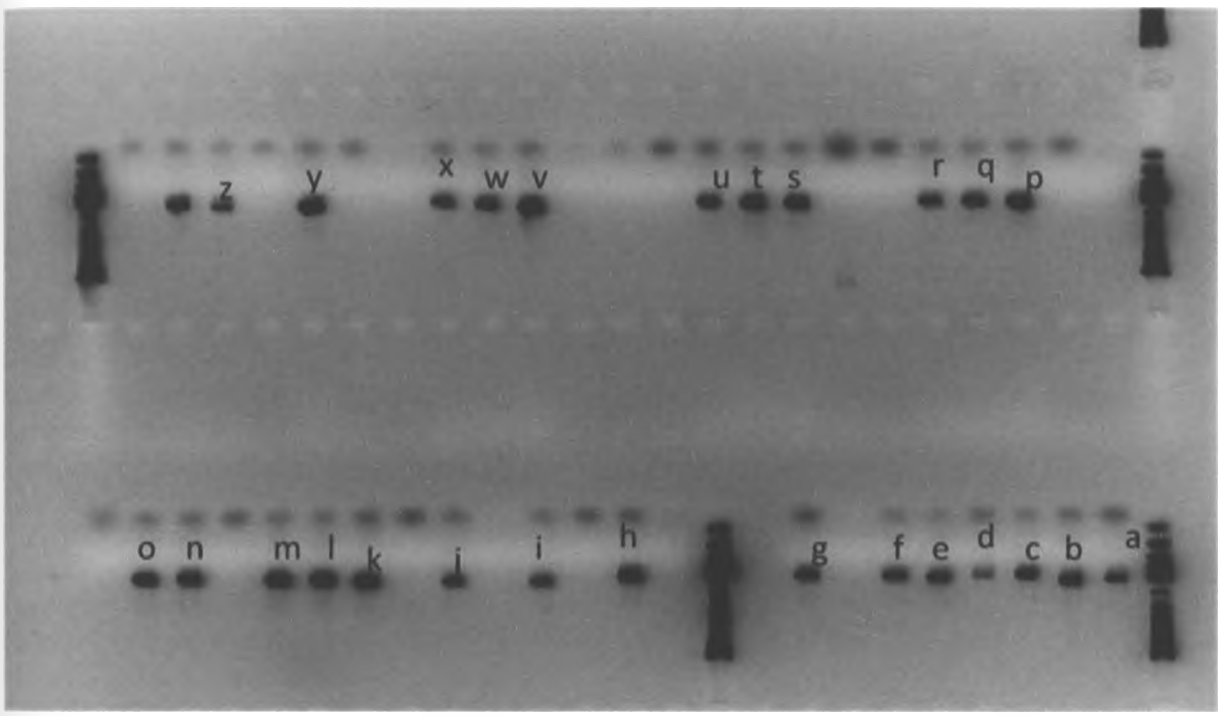
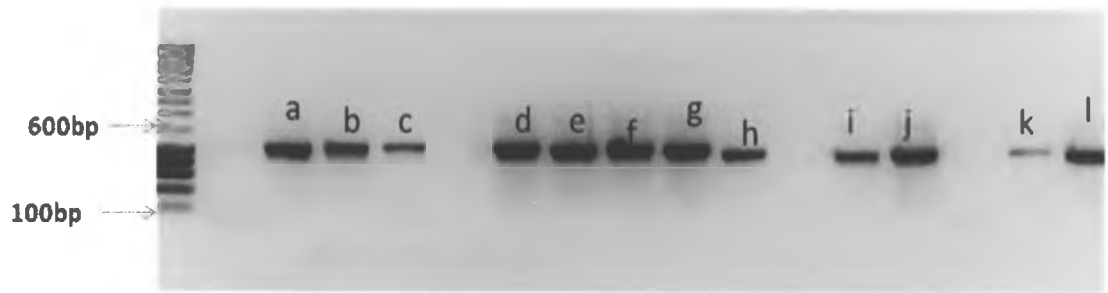


Plate 7.The gel electrophoresis of different kinds of species after(PCR): a -g represents *Arthroderma vanbreuseghemii*; m, l, k, p represents *Chrysosporium keratinophilum;* h, i, j, o, n represents *Trichophyton species;* b, e, f, z-*Trichophyton mentagrophyte;* y represents *Scopulariopsis brevicaulis;* w and x represent *Microsporum gypseum.*

Plate 8, below shows the length of the different dermatophyte in base pairs. The dermatophytes had a range of 500 to 600 base pairs.



Line **l**	Promega 100 base pairs kbn maker ladder
Line **a**	*Trichophyton verrucosum*
Line **b**	*Trichophyton rubrum*
Line **c**	*Microsporum gypseum*
Line **d**	*Trichophyton mentagrophyte*
Line **e**	*Trichophyton mentagrophyte*
Line **f**	*Trichophyton mentagrophyte*
Line **g**	*Trichophyton mentagrophyte*
Line **h**	*Arthroderma vanbreuseghemii*
Line **i**	*Arthroderma vanbreuseghemii*
Line **j**	*Chrysosporium keratinophilum*
Line **k**	*Microsporum canis*
Line **l**	*Chrysosporium keratinophilum*

Plate 8: Agarose gel electrophoresis of PCR products of the amplified ribosomal dermatophyte DNA by LS266 and V9G primers.

4.2.1: Phylogenetic tree

Cluster analysis (Neighbor-Joining method) was used to produce a phenogram and to show the genetic relationships of the populations studied (Figure 3). In this study the 12 isolates sequenced were used to generate phylogenetic tree as shown in (Figure 3).

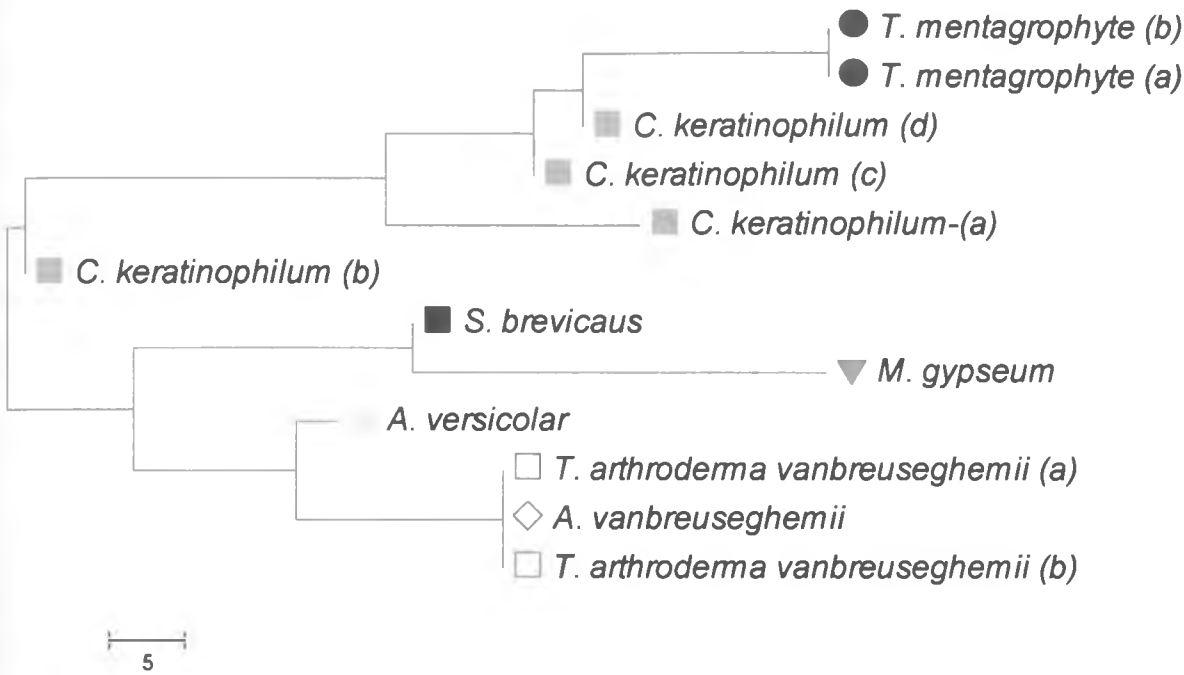


Figure 3: The evolutionary relationships of 12 sequenced strains of both dermatophytes as well as non dermatophytes.

The optimal tree with the sum of branch length = 81.1 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

There were two main clusters representing two main common ancestors. Two groups of isolates belonging to the same strains formed monocluster .i.e. the two *Trichophyton mentagrophyte* _ a and *Trichophyton mentagrophyte* _b while the three *Arthroderma vanbreuseghemii* _a, *Arthroderma vanbreuseghemii* _ b and the *A. vanbreuseghemii*, formed the second cluster. 4 *Chrysosporium keratinophilum* isolates did not form a mono-cluster.

4.3: ANTIFUNGAL DRUG PROFILE

Azoles activity against Dermatophytes isolated

Amongst the Azoles resistance was highest in Fluconazole, as shown in (Table 7) below. *Trichophyton schoenleinii* was the most resistant to all the azoles (Posiconazole, Voriconazole Itraconazole) with an MIC greater than 16 µg/ml, and for Fluconazole the MIC was 64 µgm/l. The only effective azole against *Trichophyton schoenleinii* was Isaconazole with an MIC of greater than >2 µg/ml.Itraconazole had an MIC range of 0.125- >16 µg/ml, Voriconazole the range of 0.063->16 µg/ml Posiconazole 0.125->16 µg/ml and Isaconazole was 0.016-2 µg/ml. Isaconazole was therefore most effective drug against the *Trichophyton* species with an MIC_{50} of 0.5 µg/ml in which 50% of the species were inhibited

Table 7: MIC ranges of the non dermatophytes and dermatophytes tested against Azoles

Type of antifungal	Tested Range	MIC $_{50}$	MIC $_{90}$	No of isolates tested
Fluconazole	>64-2	>64	>64	44
Itraconazole	>16-0.125	2	16	44
Voriconazole	>16-0.063	1	4	44
Isaconazole	>8-0.015	0.5	2	44
Posiconazole	>16-0.125	1	1	44

MIC, minimum inhibitory concentrations; MIC_{50} and MIC_{90} MIC at which 50% and 90% of the isolates tested are inhibited respectively.

All the *Trichophyton s*pecies were resistant to Fluconazole and their MIC values were greater than 64µg/ml with the exception of *Trichophyton mentagrophytes* and *Trichophyton verrucosum* which both had an MIC range of 8-64µg/ml.

Trichophyton mentagrophytes were more susceptible to Posiconazole (POS) with an MIC range of 0.5-1µgm/l, and Voriconazole (VOR).with an MIC range of 0.5-4 µg/ml respectively.*Trichophyton verrucosum* was resistant to Fluconazole with an MIC of >64 µg/ml (Table 8).*Trichophyton schoenleinii* species was resistant to Posiconazole, Voriconazole, and Itraconazole (ITR) all with an MIC > 16 µg/ml. It was susceptible to Isaconazole with an MIC < 2 µg/ml.

There was change in the MIC reading at day 4 and day 7, (Table 8). *Trichophyton violaceum* was resistant to Fluconazole with an MIC >64 µg/ml. Voriconazole was the most potent with an MIC range 0.0063-2 µg/ml against *Trichophyton violaceum.* Posiconazole showed an MIC range of 0.125-1 µg/ml compared to Itraconazole which had an MIC range of 0.25-16 µg/ml, (Table 8).

Table 8: The antifungal minimum inhibitory concentration (MIC) values for the dermatophytes and non dermatophytes tested.

Species	No	MIC parameter	Day four					Day seven				
			ISO	FLU	ITR	VOR	POS	ISO	FLU	ITR	VOR	POS
T. mentagrophyte	7	Range	1 - 2	8 - 64	2 - 16	0.5 - 4	0.5 - 2	0.25 - 2	8 - 64	0.5 - >16	0.125 - 4	0.5 - 1
T. schoenleinii	1	Range	2	>64	›16	16	16	›2	›64	›16	›16	›16
T. terrestre	1	Range	2	›64	2	4	1	›2	›64	16	4	2
T.interdigitale	1	Range	0.25	64	0.5	0.25	0.5	0.25	64	0.5	0.25	0.25
T. verrucosum	2	Range	0.063	32 - >64	1	0.125 - 0.5	0.25 - 0.5	0.031 - 0.5	64	1 - 0.125	0.25 - 1	0.25 - 1
T. violaceum	5	Range	0.008 - 2	64	0.125 - 4	0.031 - 2	0.125 - 1	0.016 - 2	32 - 64	0.25 - 16	0.063 - 2	0.125 - 1

NG-No growth; MIC-Minimum inhibitory concentration; FLU-Fluconazole
POS-Posiconazole; ITR-Itraconazole; VOR-Voriconazole ,ISO-Isaconazole

The *Trichophyton rubrum* was susceptible to all the azoles. There was a difference in activity between day 3 and day 7. At day 3 the MIC of all the azoles was high at 0.5-3 µgm/ml, as compared to day 7, whereby the MIC range changed to 0.125- 16 µg/ml with Itraconazole showing resistance. Isaconazole was the most potent drug followed by Posiconazole and Itraconazole respectively.

All the azoles were active against the *Trichophyton interdigitale* species with an MIC range of 0.25-0.5 µg/ml with Fluconazole showing resistance MIC > 64 µg/ml.

Among the Triazoles, Isaconazole was the most potent drug with an activity of MIC range of 0.0063 µgm/l; Voriconazole and Posiconazole had the same activity of MIC range 0.25-1µg/ml. Itraconazole had an MIC range of 0.125-<16 µg/ml (Table 8).

Microsporium gypseum was resistant to Fluconazole with MIC< 64 µg/ml, while *Microsporium canis* was susceptible to Fluconazole with an MIC of 2 µg/ml. Among the azoles activity against *Microsporium canis*; Isaconazole and Voriconazole were the most potent drug with MIC of 0.125 µg/ml; Itraconazole and Posiconazole had the same activity of MIC range 0.25µg/ml (Table 9).

Azoles activity against *Microsporium gypseum*; Posiconazole and Voriconazole were the most potent drug with MIC of 0.5-8 µg/ml; Itraconazole showed an MIC range of 2-16 µg/ml and Isaconazole had an activity of MIC range 1-2µg/ml respectively.

Epidermophyton floccosum were resistant to Fluconazole with an MIC of 64 µg/ml. The azoles activities against *Epidermophyton floccosum* were; Itraconazole and Voriconazole had an MIC range of 0.5-8 µg/ml, Isaconazole had a MIC range of 0.5-2 µg/ml and Posiconazole recorded MIC < 1 making it the most effective drug against *Epidermophyton floccosum* (Table 9).

Table 9: The antifungal minimum inhibitory concentration (MIC) values for the dermatophytes and non dermatophytes tested.

Species	No	MIC Parameter	day three					day seven				
			ISO	FLU	ITR	VOR	POS	ISO	FLU	ITR	VOR	POS
E. floccosum	4	Range	1 - 2	8 - >64	0.031 - 8	2 - 2	1 - 1	0.5-2	>64	0.25 - 8	0.5 - 8	1 - 1
T.rubrum	8	Range	0.125 - 0.5	16 - >64	0.5 - 3	0.25 - 0.5	0.5 - 1	0.03- 0.5	16 - 64	0.125 - 16	0.125 - 0.5	0.125 - 1
A. vanbreuseghemii	1	Range	1	>64	1	8	1	2	>64	2	4	1
A.versicolar	1	Range	1	16	1	0.5	0.5	0.5	32	1	0.5	1
C. keratinophilum	4	Range	0.25 - 1	16 - >64	0.5 - 8	0.125 - 1	0.25 - 1	0.5-1	32 - >64	0.5 - >16	0.5 - 1	0.25 - 1
M.canis	1	Range	NG	NG	NG	NG	NG	0.125	2	0.25	0.125	0.25
M.gyspeum	2	Range	0.25 - 2	64 - >64	0.5 - 16	0.25 - 4	0.25 - 2	1-2	64 - 64	2 - 16	0.5 -8	0.5 - 4

NG-No growth; MIC-Minimum inhibitory concentration; FLU-Fluconazole l;SO-Isaconazole
POS-Posiconazole; ITR-Itraconazole; VOR-Voriconazole

Among the non dermatophyte group, *Aspergillus versicolar* was slightly resistance to Fluconazole with MIC 32 µg/ml, while *Chrysosporium keratinophilum* was resistant with MIC range of 32->64µg/ml. Among the azoles activity against *Aspergillus versicolar*; Itraconazole and Posiconazole showed the same activity of MIC of 1 µg/ml Isaconazole and Voriconazole were the potent drugs with the same activity of MIC range 0.5 µg/ml (Table 9).

The azoles activity against *Chrysosporium keratinophilum*; Voriconazole and Isaconazole showed the same activity with MIC range of 0.5-1 µg/ml; Posiconazole was most the potent drug with of MIC < 0.25µg/ml and Itraconazole had an MIC range of 0.5- >16 µg/ml.

Amongst the slow growing *Trichophyton*, the *Trichophyton violaceum* and *Trichophyton rubrum* the MIC for Fluconazole was 64 µg/ml, on day 3 and the 7th day the MIC ranges changed to 32-64 µgm/l though it was still resistant (Table 9).

CHAPTER FIVE

5.0: DISCUSSION

Characterization of dermatophytes by conventional and molecular methods

Culture played a crucial role in aiding in the identification of dermatophytes and by complimenting direct microscopy. Sabouraud agar medium was used as the reference medium for primary isolation of the dermatophytes. However the media presented a lot of challenges due to the fact that it did not favour the sporulation of the dermatophytes therefore leading to the use of potato dextrose agar and Malt extract agar to enhance the sporulation and to aid in identification (Robert *et al.*, 2008).This method of identification was tedious because cultures had to be examined at least twice a week since some of the morphological characteristics appeared briefly, such as presence of coremia in *Trichophyton rubrum*. Borelli's lactrimel agar (BLA) and potato dextrose agar (PDA) have been used to enhance sporulation for the majority of dermatophytes, but also in the production of pigments (wine red for *T. rubrum*, ochraceous-yellow for *Microsporum canis*).

Alternate culture medias' have been developed for the isolation of dermatophytes; dermatophyte test medium (DTM) (Taplin *et al.*, 1969) and Dermatophyte Identification Medium (DIM). Based on the alkaline by-products generated during growth of dermatophytes, DTM was commercialized by Remel Inc. Difco and is available as plates or slants from Becton-Dickinson, the colour of this medium changes to deep red. However, some non-pathogenic fungi give false-positive results, or in case of contamination by bacteria results in false positive. Furthermore, the colour indicator may alter the growth and microscopic appearance of fungal colonies.

The inadequacy of DTM for presumptive identification of dermatophytes, led to the development of a new medium DIM by increasing the cycloheximide content (from 0.5 to 4 g/l) and incubation temperature from 30°C to 37°C thus achieving the recovery rate of dermatophytes. However, conflicting results have been reported by (Gromadzki *et al,.*2003) with a high number of false-negative and false-positive results after incubation at 37°C. Gromadzki noted that when arthrodermataceous fungus, (*Coccidioides immitis*) was cultured in DIM: there was colour at 37°C and good mould form was observed. These results were in contrast with the results of an earlier study, in which *C. immitis* was reported not to change the medium colour and no growth was seen at 37°C (Salkin *et al,* .1997*)*. It should be that *C. immitis* is a highly infectious fungal pathogen that often forms white colonies, resembling the colonies of dermatophytes, and its false positivity on DIM may pose a hazard for laboratory personnel (Kane *et al,.* 1999). Gromadzki study also evaluated the DIM association with significantly high false-negative results with common dermatophytes like *T. rubrum* and *T. tonsurans* at 37°C, but the results improved when the incubation temperature was at 30°C. In the case of primary dimorphic pathogens, the number of false-positive results was reported to be slightly lower when the incubation temperature was increased. Thus, DIM is presented to have high false negatives and false positives at its recommended formulations and usage, thus limiting its usefulness for presumptive identification of dermatophyte.

Some of the cultures from the study remained negative despite being positive on direct microscopy (potassium hydroxide positive). These false negative cultures was a results of insufficient amount of material and the specimen had little amount of fungal elements for it to grow.

Another explanation as to why there was false positive in culture was the fact the patient may have been on anti fungal treatment before sampling. Antifungal drugs are usually retained for a longer period of time in the horny layer of the epidermis and may inhibit the growth the of the pathogen (Raymond *et al,* 2008).The use of culture was also found to be an effective and convenient method in rapid diagnosis, when one has gained experience and with the distinguished characteristics features of the dermatophytes in culture.It is interesting to note while their was a 12.3% concordance between the two techniques, culture and microscopy combined had 13.95% positive predictive value (PPV). This is the proportion of patients with positive test results who were correctly diagnosed; as so with an undefined 0% negative predictive value (NPV) defined as the proportion of patients with negative test results who were correctly diagnosed as so.

Using conventional methods, technicians and biologists may encounter some difficulties. Indeed, sensitivity of direct examination may be insufficient due to the clinical sample or to the technique used, and the isolation and identification at the species level of some dermatophytes may be hampered by, the lack of commercially available specific culture media and the low discriminating power of already proposed biochemical or physiological tests.

Genotyping by sequencing was used not only to identify accurately the strains but also to construct phylogenetic tree. The variation in the slightly low sensitivity (50%) and the undefined specificity (0%) could be due to i) the in ability of culture and microscopy to identify some strains into their definite species (example the conventional culture and microscopy might have not been developed to identify different developmental stages as observed in the case of two

Arthroderma vanbreuseghemii which were identified as *Trichophyton verrucosum* and *Trichophyton tonsurans* that both belong to the same genus) or ii) in both the two techniques an element of human error cannot be entirely ruled out considering the there were four isolates identified as *Chrysosporium keratinophilum* by sequencing but was identified as *Trichophyton terrestre* by microscopy and culture. When isolated from the cutaneous specimen it grew well on media forming microconidia; that is not easily recognizable macroscopically and microscopically as it resembled typical dermatophytes. This could have been the reasons for both the low sensitivity and specificity seen with culture and microscopy. iii) Sequencing only a short segment of the organisms' genome was likely to introduce errors or to identify an organism erroneously.

There were 37 isolates that were identified by culture and microscopy but could not be amplified based on the region of interest (coded for by the two primersV9G and LS266). Thus the inability of sequencing only a representative genomic region to accurately identify an organism cannot be entirely dismissed. Phylogeny of dermatophytes from ITS 12 sequences dermatophytes. It was found that the following dermatophytes formed clusters a which included two mono clusters of A and B of *Trichophyton* complex and *Arthroderma vanbreuseghemii* complex.

The ITS1 and ITS2 sequences of 12 clinical samples were also determined. Each morphologically identified strain was shown to have the same ITS1 and ITS2 base pairs sequences. These corresponded to blast and the database found at the Centraalbureau voor Schimmelcultures (CBS). The four strains of *Chrysosporium keratinophilum* sequences had the same ITS base pairs which were novel. From the phylogenetic tree, *Chrysosporium*- b could have been the primitive type isolate and the other three *Chrysosporium* types were its derivative.

This could be due to the shift in base pairs either by deleting or addition, also due to the sequence analysis which resembled the *Chrysosporium keratinophilum*; in that they were genealogically similar but distant from each other. It could also be hypothesized that the *Chrysosporium* species could be new species that have been isolated from human causing Tinea capitis.

Using the ITS DNA sequences from the 12 sequenced isolates, it was possible to describe the phylogeny of the 12 sequenced dermatophytes.

This phylogenetic relationship was based on the ITS DNA sequences alignment. From the studies done on the analysis of RFLP and mtDNA; *Trichophyton mentagrophyte* and *Arthroderma vanbreuseghemii* were found to belong to the same clade and they shared a common ancestor which was reflected in this study too.

Studies done by Hsin *et al.,* 2007, showed the sequences of *Arthroderma behamie*, *Trichophyton verrucosum* and *Trichophyton mentagrophytes* joined to form a unique cluster B-b, and this was explained by high variability of their ITS 1 sequences. Hsin showed that they were able to clearly differentiate the three *Trichophyton* species (*T. violaceum, T. soudanense, T. rubrum*) by the analysis of the ITS1, which is also reflected in this study.

The significance of the ITS 1 region taxonomy has been established by correctly aiding classification of sequenced dermatophytes by bar coding. Work done by Summerbell 2005, accomplished one of the major aims of DNA barcoding, which was, facilitating identification.

The author noted that most twentieth century molecular identification approaches that relied on comparison of nucleic acid or protein band migration rates were vulnerable to coincidence (non-homologous bands migrating at essentially the same rate) and other factors limiting their resolution. The very sharp specificity of sequences has thus become the unquestioned molecular identification gold standard in recent years.

The main difficulty to using a uniform basic barcoding procedure for rapid identification of fungi is that it is a major challenge to find DNA extraction and PCR procedures, as well as primer pairs that will reliably yield suitable high-quality, long sequence reads in rapid cycle sequencing with a wide range of fungi These sentiments are reflected in this study: by the fact that it was only possible to get 12 sequenced isolates from forty samples.

The fact that dermatophytes posse' species specific sequences in the ITS 1 and ITS2 regions and to reach the correct sequence editing was necessary on some base pairs to get correct base pairs.
The advantage of bar coding (species specific) has been found to be able to differentiate phenotyipcal atypical dermatophytes like *T. rubrum* and *T. violaceum*. Studies done by Hsin *et al.*, 2007, were able to differentiate clinical isolates were misidentified by conventional methods. *T. violaceum* was identified as *T. soudanense* after sequencing.
These results reflected the ambiguity of *Trichophyton* species. He pointed out the morphological features reported for *T. soudanense* which indicated the reflexive hyphae (Laenone 2002) were not exclusive characteristics' for this species.

The same findings were encountered in this study whereby the staining of *Trichophyton terrestre* by LPCB showed the conidia morphology and topography culture characteristics resembling that of *C.keratinophilum*. Summerbell 2007 was able to clearly distinguish the two *Trichophyton* species by recognizing the presence of ITS barcode sequences whereby there was a single nucleotide polymorphism and repeated adenine bases in *T. tonsurans* and *T. equinine*.

It should be noted that ITS1 regions would be preferably be used in identifying the *Trichophyton* species (*T. violaceum*, *T. rubrum* and *T. soudanense*) due to the fact that their ITS 2 region have the same identical sequence pattern.

The ITS 2 region is also preferred for differentiating members of the *Microsporium* complex due to the fact that they possess a single nucleotide polymorphism located at the ITS2 region.

The application of sequencing remains preliminary in insufficiently studied fungal groups where sequence types are not yet known to correspond to well-delimited species; or other biosystematically or ecologically relevant units, but in many groups. Sequences can now be straightforwardly used for species identification (Summerbell and De-Hoog 2007).

From this study, Tinea unguium infection was more pronounced in females than males. The reason for this trend could have been through the roadside manicure and pedicure frequented by most of the women included in this study. This was supported by the pathogenesis of the disease which is attributed by fungi on or around the nail plate directly from the skin or the nails of the infected persons and contaminated floors.

It can also be hypothised that when the women are doing chores and most of them do not wear protective clothing like gloves which is a luxury to them repeated exposure leads to the infection. From the literature it has been shown that nail injury and low immune status predisposes one to infection, with the invasion occurring from the lateral nail fold.

Tinea pedis was caused by *Trichophyton interdigitale,* which has been reported to reside in socks of asymptomatic and symptomatic socks and shoes of patients. Poverty is likely to be the main influence of this spread of dermatophysis because one cannot be able to afford to buy new foot ware; therefore second shoes and clothes is the only option or from donation. When the patients received or purchased such clothes they do not wash and disinfect them properly due to high cost of water which is scarce. *Microsporium canis* accounted for the 2% percent of the total fungi isolated in this study.

This species was isolated from a female child. This infection may be due to the children frequenting places were infected dogs and cats have been lying hence cross infection during playing. And also the fact these pets rarely enter inside of most homes in urban areas but they share compounds, play grounds, verandahs that increased the rate of infection of these species.

Studies indicate that it is a common agent of ring worm in the western world (Ajello L 1960). Studies done in Kenya, among school going children indicated that 2% of the children were infected with *Microsporium canis* (Chepchirchir *et al.,* 2009) which correlated with this study. *Trichophyton mentagrophytes* was found to be the second common dermatophyte infection and from the study it accounted for the 14% of the total isolates.

Due to poverty in the slums and in the permanent homes sharing of households and personal items is the norm for survival which in turns increases infections. The species is reported to be endemic in Africa (Summerbell 2003).Another interesting find from this study was the isolation of *Chrysosporium keratinophilum* species which is predominately saprobic moulds. The species are found in the soil associated with keratinous substrates for example hooves, fur, feathers, skin scales, (Lynne sigler 1997). From this study four (8%) *Chrysosporium keratinophilum* species were isolated.

The ecology of this species is rarely known due to the fact that is rarely isolated from skin scrapings. This fact might change because from this study as it accounted for 8% of the total isolates. Another theory for this may be due to the fact that the species is evolving from being saprophyte and crossing over to the humans as non dermatophytes. *Trichophyton schoenleinii* was isolated from a child with tinea capitis who was HIV positive.

Trichophyton rubrum and *Trichophyton mentagrophyte* were also the etiological agents of Tinea capitis which was dominating. It has been reported that the epidemiology of Tinea capitis in males is higher than females before puberty (5:1) which reverses after puberty (Seghal *et al.*, 1985).

Antifungal drug profile

Evaluation of the drugs susceptibility profile revealed that, amongst the Azoles, Posiconazole was the most effective followed by Isaconazole and Itraconazole. This showed the same correspondence to studies done by Gupta 2005 and Barcheisi *et al.*, 1999.

All of the dermatophytes were resistant to Fluconazole which is not the drug of choice for treating dermatophytes. From this study all the *Epidermophyton* and *Trichophyton* species were resistance to Fluconazole with the exception of *Microsporium canis*. FCZ showed the lowest activity of all antifungals tested. However, *T. rubrum*, one of the most frequent species causing chronic diseases with frequent remissions and relapses, was more susceptible to Fluconazole than other species, such as *T. mentagrophytes*, *M. canis*, and *M. gypseum*. From this study, the results agree with those of other authors' studies by Fernandez-Torres *et al*,. 2002, demonstrated that susceptibility to FCZ varies greatly among the species. In the non dermatophytes group resistance to Fluconazole was also recorded and this was confirmed by the recommendations stipulated out by CLIS.

When conducting standardized antifungal susceptibility testing the following factors should be put in mind: type of media, a specific incubation time, specific incubation temperature, detailed protocol with reference to the initial inoculum size and an MIC endpoint determination that is applicable to all dermatophytes. Media plays a significant role by influencing the quantity conidia of produced, reflected by the fact that potato dextrose and Sabouraud dextrose agar medias favoured limited conidia production.

Studies done by Jessup *et al.,* 1999, showed that when *Trichophyton rubrum* was seeded on to oatmeal cereal agar prompted large production of conidia (≥26 conidia per field) as compared to 1-15 conidia per field observed with potato dextrose agar. This is very critical because a very small amount of conidia portrays low MIC values, resulting poor management of the mycoses.

The ability to perform the antifungal drug profile accurately will also be affected because the inoculums load for the initial testing will be insufficient. Another supporting fact is that *T. rubrum* is an important pathogen responsible for a significant number of dermatophytes infections (for example in this study 16% of the isolates were *T. rubrum). T. rubrum* has been found to be a major causative agent for superficial dermatophytes for example in onchmycosis and it has been documented to account for as many as 70% of all the dermatophytes infections (Weitzman and Summerbell 1995) and also due to the fact that *T. rubrum* is associated with frequent relapses following cessation of antifungal therapy.

Norris *et al.*1999 tested the slow growing dermatophytes (*Trichophyton rubrum*) and recommended that the reading should be taken after the third day. From the study, there was a difference in activity between day 3 and day7. These results were in agreement with those done (Fernandez-Torres *et al.,* 2002.)

The treatment of Dermatophytes in Kenya (unpublished data) the prescribed treatment of choice is Clotrimazole locally known as candid B with steroids or Fluconazole: and from published work and from the data it was found that there is much resistance. However, it will be necessary to obtain more clinical data to confirm if this good *in vitro* efficacy is predictive for clinical outcome.

CHAPTER SIX

6.0: CONCLUSION

Results from this study indicate that there is a lot to be done in the field of dermatophytes infection. It was found out that, the molecular characterization and identification of the dermatophytes was the suitable method to properly distinguish different kind's dermatophytes. The practicality on the ground tends is difficult to conduct due lack expertise and it is expensive to carry out especially due to requirement of screening dermatophytes. The conventional method of identification of dermatophytes needs to be strengthened because it is the cost effective was of identification in most of the laboratory in Kenya. Even though it can be quite laborious and time consuming, it is the cheaper option available in the developing countries.

There is need to establish a data collection for dermatophytes species collected from Kenya, detailing their physiological, biochemical, morphology on different kinds of medias and their molecular characteristics. This is due to the fact that most the identification is based from reference strains isolated from different continents like Europe and Asia Northern as compared to southern part of Africa. With such a data base it will be easier to document the evolving of fungal dermatophytes species from saprophytes to pathogenic in humans. This is supported with the isolation of *Chrysosporium* genus from this study.

Strain typing of dermatophytes has a number of other potential clinical and epidemiological applications. Superficial and cutaneous fungal infections due to dermatophytes are increasingly being treated with a new generation of systemic triazoles and allylamines antifungal agents. Treatment courses of either continuous or pulsed therapy typically last for several months, increasing the potential for acquisition of resistance to some of these compounds. If resistant

isolates do emerge, typing methods will allow these strains to be characterized and their occurrence and distribution to be monitored. The ability to type dermatophytes could also provide new insights into the epidemiology, population biology and pathogenicity of these fungi.

The role of gender should not be ruled out in management of dermatophytes infections, from the study it has been shown that some etiological agents of Tinea are more prone to a specific type of gender compared to the other. Age should also a factor because some of the infection is more prominent in children as compared to adults.

Civil education on proper disinfection of second hand clothes that have been purchased should be carried out to reduce and manage the infection dermatophytes. Similarly Hair salons and beauty parlors should be encouraged to properly sanitize their equipments and towels. Poverty reduction and upgrading the slum areas will significantly contain dermatophytes infection.

From the study Isaconazole and Posiconazole antifungals had the best activity against the dermatophytes as compared to Fluconazole. The use of Fluconazole as treatment of dermatophytes should be discouraged because it has been documented to show resistant to most filamentous fungi such as dermatophytes. Even though there are no standard methods for conducting *in-vitro* antifungal drugs profiles; more correlation of *in- vivo* and *in-vitro* drug susceptibility profiles studies should be done, using both laboratory and clinical approach to improve treatment. According to world health organization (WHO), the treatment of dermatophytes accounts for about 5 billion dollars annually. The antifungals are toxic and have bad side effects, it is for this reason that self medication should be avoided and the public should be educated on the value of having the correct diagnosis of dermatophyte infections thus the correct antifungal for the treatment and management of the disease.

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