ISOLATION IDENTIFICATION AND IN VITRO ANTIFUNGAL SUSCEPTIBILITY OF DERMATOPHYTES

DISSERTATION

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PSG INSTITUTE OF MEDICAL SCIENCES & RESEARCH, PEELAMEDU, COIMBATORE - 641 004

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Certificate

PSG INSTITUTE OF MEDICAL SCIENCE & RESEARCH
PEELAMEDU, COIMBATORE – 4.

CERTIFICATE

This is to certify that the dissertation work entitled "Isolation, Identification and In vitro antifungal susceptibility testing of dermatophytes" submitted by Dr.Sowmya.N, is work done by her during the period of study of her post graduation in Microbiology from June 2008 to March 2010 in our institution. This work is done under the guidance of Dr.B.Appalaraju, Professor and Head, Department of Microbiology, PSG IMS & R.

Dr.B.Appalaraju,
Prof & Head
Department of Microbiology,
PSG IMS & R

Dr.S.Ramalingam
Principal
PSG IMS & R

Place: Coimbatore
Date: 10/12/2010

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Introduction

INTRODUCTION:

Fungi are eukaryotic organisms, which multiply sexually and asexually by the production of spores. Medical interest in fungi has increased as more and more fungi are associated with pathogenic infections. Medical mycology is the study of epidemiology, ecology, pathogenesis, diagnosis and therapeutic modalities of fungal infections in human beings[1]. The incidence and prevalence of fungal infections is increasing in both developed and developing countries due to underlying predisposing factors such as immunocompromised situations, use of corticosteroids, immunosuppressive agents, anticancer drugs, HIV-positivity, etc[1]. The fungi can cause a wide variety of superficial and systemic infections. Superficial fungal skin infections are more common in the hot and humid climate in the tropical and subtropical countries like India[2].

Cutaneous infection in man includes a wide variety of diseases in which the skin and its appendages the hair and nail are involved. The majority of these infections are caused by homogenous group of keratinophilic fungi called the dermatophytes[3]. They have the capacity to invade keratinized tissues (skin, hair and nail) of humans and animals to produce an infection, dermatophytosis, commonly referred as ringworm. Dermatophytosis is a clinical entity caused by the members of the anamorph genera *Trichophyton, Microsporum* and *Epidermophyton*[3].

Infection is generally restricted to the cutaneous portion, because of the inability of the fungi to penetrate the deeper tissues or organs of immunocompetant hosts[4, 5]. The organisms colonize the keratin tissues and inflammation is caused by host response to metabolic by-products. The diagnosis can be done with the help of history, physical examination, microscopy and culture.

The clinical presentation though typical of ringworm infection is very often confused with other skin disorders particularly due to topical application of steroid ointments and creams, leading to further misdiagnosis and mismanagement[6]. Hence there arises a need for correct, efficient and rapid laboratory diagnosis of dermatophytes.

Dermatophytosis responds well to topical antifungal therapy. But local therapy may be inappropriate for extensive lesions of nail and scalp. In recent years, number of safe and highly effective antifungal agents has been introduced into clinical practice such as terbinafine, itraconazole, fluconazole, ketoconazole and voriconazole. However their activity against a wide spectrum of dermatophyte species has not yet been fully investigated[7]. Inadequate use or inappropriate dosage of drugs contributes to the failure in eliminating the disease agent completely, encouraging growth of the most resistant strains, which results in greater difficulty in treating these infections[8].

The CLSI method (M-38-A2) for antifungal susceptibility testing of filamentous fungi gives the standardized procedure for performing the antifungal susceptibility for filamentous fungi, including the testing method for dermatophytes, but lacks clinical implication [9,10]. Hence there arises a need to have a reliable and reproducible method for testing antifungal susceptibility and to identify the resistant pathogens as early as possible.

Aim & Objectives

AIM:

To study the prevalence and antifungal susceptibility pattern of dermatophytes isolated from clinical samples in Coimbatore.

OBJECTIVES:

1. Identification and characterization of different species of dermatophytes from 300 clinically defined cases of ring worm infections based upon their morphological features studied by microscopic, culture and biochemical techniques.
2. Comparison of Sabouraud dextrose agar and Dermatophyte test medium for the primary isolation of dermatophytes from the clinical samples.
3. Performance of in vitro antifungal susceptibility using broth micro-dilution method (CLSI M38-A2) and determination of the MIC range of the clinically isolated dermatophytes.

Review of Literature

REVIEW OF LITERATURE:

The fungal infections of the epidermis and its appendages can be divided into two groups – Dermatophytosis and Dermatomycoses [1]. The former is caused by a group of fungi called as dermatophytes which includes three anamorph genera *Trichophyton, Microsporum and Epidermophyton.* The term dermatomycoses refers to the infections produced by non-dermatophytic fungi such as *Candida* species, *Scytalidium* species, *Fusarium* species, *Scopulariopsis* species and others [3].

Historical review:

The history of human medical mycology started with the discovery and incrimination of etiologic agents of dermatophytosis. In 1835 Robert Remak, a Polish born student at the University of Berlin first observed peculiar microscopic structures appearing as rods and buds in crusts from favic lesions [11]. Remak's observations were cited in a doctoral dissertation by Xavier Hube in 1837 [12]. Johann L.Schonlein in 1839 described these filaments as molds and considered plants as source of origin [3, 11].

In 1842 Remak, inoculated himself with the materials from favus and proved them to be infectious and he also named it as *Achorion schoenlenii* in honor of his mentor [13]. In 1845, David Gruby, a Hungarian born Physician in Paris described the causative agents of favus both clinically and microscopically [14, 15]. He named the etiological agent of the ectothrix infection of hair as *Microsporum audounii* and of endothrix as *Herpes tonsurans.* [16, 17]

In 1853, Robin reviewed the early literature and described clearly several types of dermatophytes in his book, *Historie naturelle des vegetaux parasites* [11]. Domenico Majocchi described the variant of tinea corporis in 1883 and he named the disease as "Granuloma tricofitico" which is popularly now known as Majocchi's

granuloma[1]. From 1892 to 1938, Raymond Sabouraud did his studies on dermatophytes and published his monumental work, *Les Teignes* in 1910. In his work he classified dermatophytes into four genera namely *Achorion, Epidermophyton, Microsporum and Trichophyton* based on the clinical aspects of the disease combined with cultural and microscopic characteristics of the fungi [17, 18].

In 1925, Block et al. reported "trichophytin" activity of crude polysaccharide containing extracts [19]. In 1934, Chester Emmons modified taxonomic scheme of Sabouraud and other scientists' classification of dermatophytes. He eliminated the genera *Achorion* and recognized only three genera *Trichophyton, Microsporum* and *Epidermophyton* based on their spore morphology and other accessory structures [20].

The nutritional requirements and physiological characteristics of dermatophytes were studied by Benham, Silva, George, and Camp in 1950[21,22]. In 1958, Gentles reported successful treatment of dermatophytes in guinea pigs by oral administration of Griseofulvin [23]. The discovery of teleomorphs (sexual forms) of *Trichophyton ajelloi* in 1959 by Dawson and Gentles using Hair bait technique lead to rapid discovery of such forms in many dermatophytes [24]. In 1969, Taplin and co-workers developed Dermatophyte test medium (DTM) to isolate and distinguish dermatophytes from fungal or bacterial contaminants in cutaneous lesions, based on their pH alteration [25].

Until 1986, the teleomorphic forms of dermatophytes were grouped into two genera – *Arthroderma* and *Nannizzia*, based on their peridial hyphae. In 1986, Weitzman et al. demonstrated that *Arthroderma* and *Nannizzia* were the same based on their phylogenetic analysis [26]. In 1980s, discovery of azole derivatives and allied

group of antifungal drugs had significant impact in the management of dermatophytes.

EPIDEMIOLOGY AND ECOLOGY

Ecology: Dermatophytes are classified into three groups based on their natural habitats and host preferences as 1) Anthropophilic – whose only host is humans; 2) Zoophilic – whose normal host is animals but infect humans; and 3) Geophilic – soil saprophytes which can become pathogenic to humans and animals [11].

Some species of soil saprophytes (Geophilic) gradually evolved to parasitize the keratinous tissues of animals living in contact with the soil (Zoophilic), hence losing their ability to survive in soil anymore. Among the zoophilic species those that came into contact with humans, gradually lost their ability for animal keratin and became anthropophilic[27].

In the process of evolution from soil saprophytes to zoophilic and anthropophilic parasites, changes have occurred in their reproductive system. Conidial production has gradually decreased from geophilic to anthropophilic and most have lost their sexual reproductive cycle. The decrease in conidial production is more evident among the anthropophilic species. For example, a zoophilic variant of *T.mentagrophytes var. mentagrophytes* produces conidia more abundantly than anthropophilic variant of *T.mentagrophytes var. interdigitale.* Anthropophilic species such as *Microsporum audounii, Trichophyton rubrum and Trichophyton schoenleinii* rarely produce macroconidia. These conidia survive in the environment for a prolonged period of time [28].

The distinction between geophilic and zoophilic dermatophytes is based on detailed ecological analysis and may not be obvious in small-scale studies. Zoophilic and geophilic species in general tend to form lesions that are more inflammatory than those formed by anthropophilic, but are more likely to resolve spontaneously [3].

Epidemiology:

Epidemiology is important in infection control and public health issues related to the different types of dermatophytes. Some species of dermatophytes are prevalent throughout the world (Cosmopolitan species), while some are restricted to certain geographical areas[29]. The cosmopolitan species establish themselves in new geographical areas when the carriers move from the original endemic areas. The migration of labor, troop movements, emigration and other travel played important role in spreading these fungi [30].

Table 1. Current synopsis of dermatophyte species and congeners: ecological classification, host preference, and endemicity [7]

Anthropophilic species (Areas of endemicity)	**Zoophilc species (Typical host)**	**Geophilic species**
Epidermophyton floccosum (Cosmopoliton)	*Microsporum canis* (cats,dogs)	*Epidermophyton stockdale*
Microsporum audouinii (Africa)	*Trichophyton equinum* (Horse)	*Microsporum cookie*
Microsporum ferrugineum (East Asia and Europe)	*Microsporum gallinae* (fowl)	*Microsporum gypseum*
Trichophyton concentricum (S.E.Asia)	*Microsporum persicolor* (vole)	*Microsporum nanum*

Anthropophilic species (Areas of endemicity)	**Zoophilc species (Typical host)**	**Geophilic species**
Trichophyton megninii (Portugal,Sardinia)	*Trichophyton Simii* (monkey,fowl)	*Microsporum Praecox*
T.mentagrophytes (Cosmopoliton)	*Trichophyton mentagrophytes* (rodents,rabbits,dogs)	*Microsporum recemosum*
Trichophyton rubrum (Cosmopoliton)	*Trichophyton verrucosum* (cattle,sheep)	*Trichophyton ajelloi*
Trichophyton tonsurans (Cosmopoliton)		*Trichophyton terrestre*
Trichophyton violaceum (North Africa, Middle East)		
Trichophyton yaoundei (Central Africa)		
Trichophyton schoenleinii (Cosmopoliton)		

Classification:

The etiologic agents of dermatophytosis are classified into three anamorphic (asexual or imperfect) genera, *Epidermophyton*, *Microsporum*, and *Trichophyton* belonging to the class Hyphomycetes of the family Deuteromycota (Fungi Imperfecti). The descriptions of the genera essentially follow the classification scheme of Emmons [20] on the basis of conidial morphology and formation of conidia.

The characteristic features of the three genera and their type specific species are as follows:

***Epidermophyton* species:** The type species is *Epidermophyton floccosum*. Colonies are usually slow growing, greenish-brown or khaki coloured, raised and folded in the centre, with a flat periphery and submerged fringe of growth. Microscopic morphology shows characteristic smooth, thin-walled macroconidia, 20 to 60 by 4 to 13 µm in size, usually abundant and borne singly or in clusters growing directly from the hyphae. No microconidia are formed. This genus has only two known species to date - *Epidermophyton floccosum and Epidermophyton stockdale*, and the former is pathogenic.

***Microsporum* species:** The type species is *Microsporum audouinii*. The macroconidia may have thin to moderately thick walls and 1 to 15 septae and range in size from 6 to 160 by 6 to 25 µm characterized by the presence of rough walls which may be asperulate, echinulate, or verrucose. Microconidia are sessile or stalked and clavate and usually arranged singly along the hyphae or in racemes.

Microsporum canis: They produce ectothrix infection of hair and show a bright greenish-yellow fluorescence under wood's ultra-violet light. Colonies are flat, spreading and white to cream-colored, with a dense cottony surface and usually have a bright golden yellow to brownish yellow reverse pigment. Macroconidia are typically spindle-shaped with 5-15 cells, verrucose, thick walled and often have a terminal knob.

Microsporum gypseum: Invaded hair shows an ectothrix infection but do not fluoresce under wood's ultra-violet light. Colonies are usually flat, spreading and suede-like to granular, with a deep cream or tawny-buff to pale cinnamon coloured

surface and a yellow-brown reverse pigment. Macroconidia are ellipsoidal, thin-walled, verrucose and 4-6 celled.

Microsporum audouinii: Infected hair fluoresce bright greenish-yellow under wood's ultra-violet light. Colonies are flat, spreading, and grayish-white to light tan-white in colour, and have a dense suede-like to downy surface, suggestive of mouse fur in texture. Key features include the absence of conidia, poor or absence of growth on polished rice grains, inability to perforate hair in vitro.

Microsporum ferrugineum: Invaded hair shows an ectothrix type of infection with greenish-yellow fluorescence under wood's ultra-violet light. Key mycological features include distinctive "bamboo" hyphae.

***Trichophyton* species:** The type species is *Trichophyton tonsurans*. Macroconidia, when present, have smooth, usually thin walls and one to 12 septae which are borne singly or in clusters, and may be elongate and pencil shaped, clavate, fusiform, or cylindrical. They range in size from 8 to 86 by 4 to 14 µm. Microconidia are more abundant than macroconidia which may be globose, pyriform or clavate, and sessile or stalked. They are borne singly along the sides of the hyphae or in grape-like clusters.

Trichophyton tonsurans: They may be suede-like to powdery, flat with a raised centre or folded, often with radial grooves. The colour may vary from pale-buff to dark-brown. The reverse colour varies from yellow/reddish-brown to deep mahogany. Numerous microconidia varying in size and shape from long clavate to broad pyriform, are borne at right angles to the hyphae, which often remain unstained by lacto phenol cotton blue. Very occasional smooth, thin-walled, irregular, clavate macroconidia may be present on some cultures.

Trichophyton rubrum: Colonies are flat to slightly raised, white to cream, suede-like to downy, with a yellow-brown to wine-red reverse. Most cultures show scanty to moderate numbers of slender clavate to pyriform microconidia. Macroconidia are usually absent, however closterospore-like projections may be present in some mounts.

Trichophyton mentagrophytes: Colonies are generally flat, white to cream in colour, with a powdery to granular surface. Reverse pigmentation is usually a yellow-brown to reddish-brown colour. Numerous single-celled microconidia are formed, often in dense clusters. Microconidia are hyaline, smooth-walled, and are predominantly spherical to subspherical in shape; however occasional clavate to pyriform forms may occur. Varying numbers of spherical chlamydoconidia, spiral hyphae and smooth, thin-walled, clavate shaped multicelled macroconidia may also be present.

Trichophyton equinum: Colonies are usually flat, but some may develop gentle folds or radial grooves, white to buff in colour, suede-like to downy in texture. Cultures usually have a deep-yellow submerged fringe and reverse which later becomes dark red in the centre. Microscopically, abundant microconidia which may be clavate to pyriform and sessile or spherical and stalked are formed laterally along the hyphae. Macroconidia are only rarely produced, but when present are clavate, smooth, thin-walled and of variable size.

Trichophyton violaceum: Colonies are very slow growing, glabrous or waxy, heaped and folded with a deep violet colour. Occasional non-pigmented strains may occur. Hyphae are relatively broad, tortuous, much branched and distorted. No conidia are usually seen, although occasional pyriform microconidia have been observed on enriched media.

Table 2. The anamorphic species of the dermatophytes with the name of their discoverers [7]

Epidermophyton (Sabouraud 1907)
E.floccosum (Langeron et Milochevitch 1930)
Microsporum (Gruby 1843)
M.audounii (Gruby 1843)
M.canis (Bodin 1902)
M.equinum (Geuguen 1904)
M.ferrugineum (Ota 1921)
M.fulvum (Uribure 1909)
M.gallinae (Grigorakis 1929)
M.gypseum (Guiart et Grigorakis 1928)
M.nanum (Fuentes 1956)
M.persicolor (Guiart et Grigorakis 1928)
M.praecox (Ajello et McGinnis 1987)
M.recemosum (Borelli 1965)
M.vanbreuseghemii (Friedman et Brinkman 1962)
Trichophyton (Malmesten 1845)
T.concentricum (Blanchard 1895)
T.equinum (Gedoelst 1902)
T.kanei (Summerbell 1989)
T.maginii (Blanchard 1896)
T.mentagrophytes (Blanchard 1896)
T.raubitschekii (Kane,Salkin,Weitzman,Smitka 1981)
T.rubrum (Sabouraud 1911)
T.schoenleinii (Langeron et Milochevitch 1930)

T.simii (Stockdale,Mackenzie et Austwick 1965)
T.soudanense (Joyeux 1912)
T.tonsurans (Malmsten 1845)
T.verrucosum (Bodin 1902)
T.Violaceum (Bodin 1902)
T.yaoundei (Cochet et Doby Dubois 1957)

Table 3. The teleomorph - anamorph states of the dermatophytes and related species [7]

Teleomorph	Anamorph
Arthroderma	*Microsporum, Trichophyton*
A.benhamiae	*T.mentagrophytes*
A.fulvum	*M.fulvum*
A.grubyi	*T.mentagrophytes*
A.gypseum	*M.gypseum*
A.incurvatum	*M.gypseum*
A.obtusum	*M.nanum*
A.otae	*M.canis var.canis,M.canis var distortum*
A.persicolocr	*M.persicolor*
A.simii	*T.simii*
A.racemosum	*M.racemosum*
A.vanbreuseghemii	*M..vanbreuseghemii*

PATHOGENESIS:

Among the many fungi that produce disease in man, only the dermatophytes show evolution towards a parasitic mode of existence and dependence on human infection for survival [32]. There are various hypothesis to support these features – 1) Evolution in the soil of specialized group of fungi with keratinolytic ability. 2) Association with furred animals and ability to produce transient lesions – Eg: *T.ajelloi* and *M.gypseum*. 3) Adaptation to growth in the living keratinizing zone. 4) Development of adaptation and equilibrium to the host. 5) Development of specialized methods for reproduction and successful dissemination Eg: Arthroconidia.

The dermatophytes show a high degree of specificity to keratinized tissues. While these fungi are well adapted to parasitize the horny layer of the epidermis, hair and nail, they were unable to invade other organs of the body in normal patients. Intravenous injection of *Microsporum* conidia does not produce an infection of the internal organs; rather the fungi become localized in the skin and produce infection only in areas previously damaged by scarification [33]. Dermatophyte growth is quite sensitive to temperature. Normal body temperature inhibits the growth of most of the strains and species. The optimum temperature for growth is at 28-30°C [3].

The three main steps involved in the pathogenesis of dermatophytic infections are: 1) Adhering to and invading the superficial skin. 2) Growth on hardened keratinized substances. 3) Overcoming host's immune response.

Adhering to and invading the superficial skin: The kinetics of adherence to the skin or nail surface was investigated in several *Trichophyton* and *Microsporum* species using different experimental models and microscopy techniques [34]. Very less detail is known about the factors that mediate adhesion of dermatophytes. Zurita and

Hay [35] observed that maximum adherence of the arthroconidia of *Trichophyton species* to keratinocytes in suspension occurred within 3–4 hours while that of *Microsporum canis* arthrospores occurred by 2 hours [36]. The supporting factors might be a) Carbohydrate-specific adhesins, expressed on the surface of microconidia [37]. b) Fibrillar projections – on the skin surface, long and sparse fibrils connect fungal arthroconidia to keratinocytes and to each other. c) Dermatophytic-secreted proteases which could facilitate or even be necessary for efficient adherence. Eg: subtilisins, metalloproteases, and dipeptidyl-peptidases [38].

Growth on hardened keratinized substance: These fungi secrete multiple serines, metallo - endoproteases (subtilisins and fungalysins) and keratinases which are aimed at the digestion of the keratin network into digestible oligopeptides or amino acids. The importance of dermatophytic keratinolytic proteases for pathogenicity is thus well established. Nevertheless, they cannot act before disulfide bridges are reduced within the compact protein network that constitutes the keratinized tissues. This was recently shown to be dependent on a sulfite efflux pump encoded by the Ssu1 gene [39]. Sulfite excretion by this transporter allows sulfitolysis of proteins, rendering them accessible for proteases.

Overcoming host's immune response: Infections by dermatophytes induce a specific immune response, with humoral and cellular components. The efficient and protective response against dermatophytosis is a cell-mediated immune response of the delayed type hypersensitivity (DTH), characterized by the action of macrophages as effector cells and by some key cytokines like interferon-c (IFN-c). The immune response varies according to the dermatophyte species and pathophysiological status of the host. They include lymphocyte inhibition by cell wall mannans, macrophage

function alteration, differential activation of keratinocytes and differential secretion of proteases [34].

IMMUNOLOGY:

Dermatophyte colonization is characteristically limited to the dead keratinized tissue of the stratum corneum and results in either a mild or intense inflammatory reaction. Both humoral and cell-mediated reactions eventually eliminate the fungus, preventing invasion into the deeper viable tissue. This array of defense mechanisms thought to be active against dermatophytes consists of α 2-macroglobulin keratinase inhibitor, unsaturated transferrin, epidermal desquamation, lymphocytes, macrophages, neutrophils, and mast cells [40].

There are two major classes of dermatophyte antigens: glycopeptides and keratinases. The protein portion of the glycopeptides preferentially stimulates cell-mediated immunity (CMI), whereas the polysaccharide portion preferentially stimulates humoral immunity. Keratinases, produced by the dermatophytes to enable skin invasion, elicit delayed-type hypersensitivity (DTH) responses when injected intradermally into the skin of animals [41].

Although the host develops a variety of antibodies to dermatophyte infection, i.e., IgM, IgG, IgA, and IgE, they apparently do not help in the elimination of infection [42]. Rather, the development of CMI which is correlated with DTH is usually associated with clinical cure and ridding the stratum corneum off the offending dermatophyte [41]. In contrast, the lack of CMI or defective CMI prevents an effective response and predisposes the host to chronic or recurrent dermatophyte infections. The classical studies of Jones and coworkers in human volunteers suggested that CMI is the major immunologic defense in clearing dermatophyte

infections [43]. Although there are no serological kits commercially available to specifically detect and identify antibodies to dermatophytes, studies of dermatophyte antigens by monoclonal antibodies indicate a potential use of such reagents in the immuno-identification of dermatophytes [44].

CLINICAL MANIFESTATIONS

The infections caused by dermatophytes (ringworm) have been named according to the anatomic locations involved by appending the Latin term designating the body site after the word tinea. The *Trichophyton* species usually infect skin, hair and nail. The *Microsporum* species infect skin and hair but not nail. The *Epidermophyton* species infect skin and nail but not hair. The most common clinical manifestations are described below:

Tinea capitis:

Tinea capitis is a dermatophyte infection of the scalp, eyebrows and eyelashes caused by the species of the genera *Microsporum* and *Trichophyton*. The infection may range from mild erythema and a few patchy areas of scaling with dull gray hair stumps to a highly inflammatory reaction with folliculitis, kerion formation, and extensive areas of scarring and alopecia, sometimes accompanied by fever, malaise, and regional lymphadenopathy. In scalp, both the skin surface and hairs are involved. Infection of the hair may be described as ectothrix (sheath of arthroconidia formed on the outside of the hair shaft) or endothrix (arthroconidia formed within the hair shaft)[7]. The current predominant cause of ectothrix is *Microsporum audouinii* and *Microsporum canis* and that of endothrix is *Trichophyton violaceum* and *Trichophyton tonsurans* [11].

The dermatophytid (id) reaction may occur in tinea capitis which is an allergic manifestation of infection at a distal site and the lesions are devoid of organisms. A group of dense, itchy and painful vesicles appear in the trunk in case of tinea capitis, whereas in fingers and palms in case of tinea pedis [11].

Tinea barbae:

Tinea barbae, an infection of the bearded area, may be mild and superficial or a severe inflammatory pustular folliculitis, the latter form is more commonly caused by the zoophilic dermatophytes *Trichophyton verrucosum*, *Trichophyton mentagrophytes*[11].

Tinea favosa:

Favus (Latin: Honeycomb) is a clinical entity characterized by the occurrence of dense masses of mycelium and epithelial debris which form yellowish, cup shaped crusts called scutula in the hair follicle, with hair shaft in the centre of the raised lesion. The common causative organism is *Trichophyton schoenleinii* [11]

Tinea corporis:

It is the most common type of manifestation in tropical and subtropical countries. Tinea corporis is a dermatophyte infection of the glabrous skin (trunk, shoulder and limbs) most commonly caused by *Trichophyton* and *Microsporum* species. Lesions vary from simple scaling with erythema and vesicles to deep granulomas. The most common species are *Trichophyton rubrum* and *Trichophyton mentagrophytes* [11].

Tinea imbricata:

Tinea imbricata is geographically restricted form of Tinea corporis caused by *Trichophyton concentricum*. It is characterized by polycyclic, concentrically arranged rings of papulosquamous patches of scales scattered over most of the body surface[11].

Tinea cruris (Jock's itch):

Tinea cruris is the dermatophyte infection of the intertriginous areas with high moisture like groin, perineum and perianal region, and is generally pruritic. The lesion is characterized by sharply demarcated, raised, erythematous margin and thin dry epidermal scaling. The most common causative agents are *Trichophyton rubrum, Trichophyton mentagrophytes* and *Epidermatophyton floccosum* [11].

Tinea manum:

Tinea manum refers to those infections in which the interdigital areas and the palmar surfaces are involved .The majority of infections are due to *Trichophyton rubrum, Trichophyton mentagrophytes* and *Epidermatophyton floccosum* [7].

Tinea pedis:

Tinea pedis is the dermatophyte infection of the feet involving the toe webs and the soles. It is also known as the athelete's foot. The most common causative agent is *Trichophyton rubrum* [7].

Tinea unguinum:

Tinea unguinum is the dermatophyte invasion of the nail plates. This has to be well differentiated from onychomycosis, which is actually the infection of the nails

by non-dermatophytic fungi and yeasts. The disease tinea unguinum is of two types – i) Leukonychia mycotica – superficial invasion which is restricted to patches or pits on the surface of nails and ii) Invasive in which the lateral or distal edges of the nails are involved. The most common causative agent is *Trichophyton mentagrophytes* [11].

LABORATORY DIAGNOSIS:

A definitive diagnosis of dermatophytic infection needs to be done before the initiation of antifungal therapy because of the long duration of the treatment, its high cost and potential side effects of the drugs. The difficulty in the diagnosis of dermatophytosis primarily lies not only in the absence of standardized collection of clinical specimens and of mycological techniques, but also on the commercial unavailability of most of the reagents needed. Name, sex, age and ethnic origin are standard requirements. History of recent stay or travel overseas, a contact with animals may also be relevant [45]

Specimen collection:

The specimen should be collected in a sufficient amount from the edge of the infected area, which corresponds to the active zone of the lesion. To improve the efficiency of mycological examination, samples should be obtained before any local or systemic antifungal treatment [45]. The samples collected for the isolation of dermatophytes is skin, nails and hair. It is recommended to clean the area with 70% alcohol before sampling to remove contaminants such as bacteria [46]. For good visualization of skin scales, nail and hair samples collected, they may be collected in sterile dark colored papers.

Skin:

A lesion of the glabrous skin (ringworms and intertriginous lesions) with an active inflammatory border is scraped from the edge entirely with sterile scalpel blade or glass slide. When lesions are highly inflammatory and/or oozing, scrapping is followed by swabbing. Additionally, the use of vinyl tape skin stripping has been proposed to improve the patient's compliance during sampling, particularly with young children or in sensitive areas [47]. But an additional conventional sample is required for culture.

Nail:

Methods for collecting the infected nails differ according to the site of infection. In case of distal infections nail samples are obtained by clipping followed by scraping of the nail bed with a small curette or a scalpel blade. When proximal subungual infection is suspected, it is essential to spare the healthy upper table before collecting material from the infected lower table of the nail. [48]

Hair:

The whole scalp is first examined under filtered ultraviolet (Wood's lamp). Hair roots and crusts are plucked from the infected area or from the edge in case of large lesions, particularly when these elements are glowing under UV lamp, and suppurating lesions are swabbed [45].

Microscopic examination & culture:

There are three sets of observation generally useful for the identification of dermatophytes [3] –

1. Colony obverse: Note the color, consistency and topography.

2. Colony reverse: Look for pigment production.

3. Microscopic morphology: Observe the microconidia and macroconidia; observe their size, shape, arrangement and hyphal structures.

Direct microscopy:

Direct microscopy, although falsely negative in 5 to 15% of the cases in ordinary practice, is a highly efficient screening technique [3]. Correct visualization of the fungal elements requires the dissociation of collected material. The specimens are therefore submitted to clearing reagents that allows digestion of the keratin. With plenty of reagents and various combinations available, 10–20% potassium hydroxide (KOH) with or without 40 % dimethyl sulfoxide (DMSO) is the most commonly used [49]. It is one of the simplest and cheapest techniques that allows an immediate observation and is particularly suitable for nails. However, as keratin is rapidly digested by KOH, an immediate examination is required. This limitation may be overcome by the use of chloral-lactophenol which allows clearing without heating. Various stains may be associated to the clearing agents are used such as, Chlorazol black E (CBE), Periodic acid-Schiff (PAS) stain, Blue–Black Ink permanent and Congo red.

The detection of fungal hyphae and spores is greatly facilitated by the use of fluorochromes such as Calcofluor white, Blankophor P Flussig or Uvitex 2B. These distilbene derivatives, which are usually dissolved in sodium sulfur bind to chitin, a polymer of N-acetyl-D-glucosamine which is one of the main polysaccharides of the fungal cell wall [50]. Calcofluor white (CW) is the most convenient fluorochrome

allowing a rapid and accurate diagnosis of dermatomycosis. Fungal elements in calcofluor-stained specimens appear blue when using a fluorescence microscope equipped with a 330–380 nm excitation filter and an emission filter of 420 nm. Abdelrahman et al. [51] demonstrated that sensitivity of direct examination for the detection of dermatophytes and non-dermatophyte pathogenic fungi in nails and skin scrapings was significantly higher with calcofluor than with KOH (88% and 72%, respectively, P = 0.0116). CW is also considered as the ‘‘gold standard’’ in the study of Weinberg et al. [52], the only limitation being that it requires a fluorescence microscope.

Culture Medium:

Culture is a valuable tool in adjunct to direct microscopy, which helps in the identification up to species level. The most commonly used culture medium is Sabouraud dextrose agar containing antibiotics (Chloramphenicol + cycloheximide). Cycloheximide is incorporated into the medium as a semi selective agent to reduce the growth of nondermatophytic saprophytic fungi, where Chloramphenicol reduces the bacterial contaminants [45].

The Dermatophyte test medium provides an alternative for the isolation. Due to the alkaline by-products generated during the growth of the dermatophytes, the color of the medium changes to deep-red [1-3]. Enzymatic Digest of Soybean Meal provides nitrogen and vitamins required for the growth of the organism. Dextrose is included as an energy source. Phenol Red is the pH indicator used to detect acid production. The supplements, Cycloheximide, Gentamicin and Chlortetracycline aid in selectivity of Dermatophyte Test Medium. However there are some studies showing false-positives and false-negative reports from Dermatophyte test medium [53].

Various media such as Casamino acids – erythritol albumin agar or Bromocresol purple casein yeast extract agar (BCP) [54] are available for culture of dermatophytes. Sporulation can be induced by growing them on Potato dextrose agar (PDA), oat meal agar, Borelli's lactrimel agar (BLA), Baxter's medium, Takashio medium, malt agar or water agar [55].

Biochemical and Physiological tests:

Urease activity:

Some dermatophytes are able to hydrolyze high concentrations of urea. Commercially available Urea–indole broth or Christensen's urea agar medium can be used. With urease-producing strains, urea is split into ammonia, resulting in a pH change to alkaline and therefore change in the color of the phenol red containing medium from a straw yellow to pink after two (urea broth) or six (Christensen's agar) days of incubation at 27°C. [56] Urease test is positive for *Trichophyton mentagrophytes, Trichophyton tonsurans and Epidermophyton flocossum,* but negative for *Trichophyton rubrum.*

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In vitro hair perforation test:

In vitro, some dermatophytes are able to penetrate and invade hair shaft by producing specialized perforating organs, while other species attack the hair by simple peripheral erosions. The short strands of sterilized hair are deposited in petri dishes with 25 ml of sterile distilled water containing 2–3 drops of 10% yeast extract. The fungal growth deposited onto the hair is examined under microscope by mounting in

lacto phenol cotton blue [57]. Hair perforation test is positive for *Trichophyton mentagrophytes* while negative for *Trichophyton rubrum*.

Bromocresol Purple milk glucose test:

All dermatophytes raise the pH of the medium by breaking down the proteins to alkaline products. They use the milk protein as casein base and glucose as carbon source. It also indicates the organic acid production by many contaminating bacteria and yeasts and hence helps to correlate and interpret the false positive results produced in the urease tests. The medium is made as a slant and a small quantity of the inoculum is placed in the middle of the medium which is incubated at 25°C for 7 to 10 days and a change in the pH of the medium is noted. The dermatophytes will either not produce any color change or change it into deep blue color, shifting the pH to alkaline side. Whereas contaminants produce an acidic pH giving rise to yellow color in the medium [58].

Vitamin requirements and Nutritional tests:

Nutritional requirements are determined by comparing the growth on control medium and on medium enriched with a specific vitamins or amino acids. Inositol, thiamine, nicotinic acid and histidine have been tested in casein agar-based media or ammonium nitrate agar to study the vitamin or amino acid requirement [57]. Nutritional test media are commercially available (Trichophyton agars 1–7, Difco), but they are used only in specialized laboratories.

Other valuable methods:

Volatile organic compound profiles, determined by gas chromatography-mass spectrometry, have been evaluated as a potential tool for species identification in

dermatophytes[59]. Studies are being conducted where experimental infections are produced in guinea pigs or rabbits to demonstrate the pathogenesis and antifungal susceptibility of dermatophytes [60]

Molecular methods:

Identification of dermatophyte species is essentially based on macroscopic and microscopic observations of their morphological features. However, the identification is complicated and laborious due to the morphological similarity, variability and polymorphism shown by dermatophytes. Thus accurate identification is time consuming and requires a significant level of knowledge and technological expertise. The present molecular biology-based techniques make it possible to identify dermatophytes up to species level and to discriminate between isolates at the strain level [61].

The mitochondrial DNA has frequently been used for phylogenetic studies and or identification of dermatophytes using restriction fragment length polymorphism (RFLP) analysis [62]. Randomly amplified polymorphic DNA (RAPD) methods have frequently been used for phylogenetic analysis and identification of dermatophytes by Graser et al. [63]. Turin et al reported a PCR-based identification of dermatophytes targeting 18S rDNA and internal transcribed spacers (ITS) regions, in which DNA samples, purified from fungi or clinical specimens, were amplified by three primer sets[64]. The Non – transcribed spacer (NTS) region is recommended as a target for the study of strain by Mochizuki et al [65] .Some used genes such as chitin synthase I gene (CHS1) or DNA topoisomerase II gene (TOP2) as a target for species identification of dermatophytes [66]. Baek et al directly purified DNA from nail powder

of patients with onychomycosis and subjected it to PCR targeting the 18S rRNA gene followed by RFLP analysis[67].

Savin et al reported a multicenter evaluation of a PCR-enzyme-linked immunosorbent assay (ELISA)-based diagnostic kit for diagnosis of onychomycosis and showed the good performance of this technique for rapid diagnosis of dermatophytic onychomycosis[68]. Kano et al purified DNAs from skin and hair of infected dogs, cats, and rabbits and amplified it using a universal primer set specific to the chitin synthase I gene of dermatophytes[69]. In almost all studies of species identification of dermatophytes using PCR and PCR-RFLP, the products or restriction fragments were visualized using agarose gel or polyacrylamide gel electrophoresis followed by ethidium bromide staining. The molecular approaches for dermatophyte identification continue to develop with more scientists working on them.

TREATMENT OF DERMATOPHYTIC INFECTIONS:

Treatment of dermatophyte infection involves primarily oral and/or topical formulations of azoles or allylamines, particularly itraconazole and terbinafine. Topical medications applied once or twice daily are the primary treatment indicated for tinea corporis/cruris, and tinea pedis/manuum. Use of oral antifungals may be practical where the tinea involvement is extensive or chronic, or where application of a topical is not feasible like tinea unguium and in some cases of tinea capitis. Recently, topical amorolfine and ciclopirox formulations have been approved for use in milder tinea unguinum cases.

Relapse of infection remains a problem, particularly with tinea pedis/unguium. Appropriate follow-up and education of patients on proper foot hygiene are also important components in providing effective therapy [70].

Commonly used drugs [70]:

Terbinafine: An allylamine, fungicidal which inhibits the squalene epoxidase that leads to ergosterol deficiency and accumulation of squalene.

Itraconazole: It's a trizole and a fungistatic. It inhibits fungal lanosterol 14 -α demethylase which leads to ergosterol deficiency.

Fluconazole: It's a bis – triazole and fungistatic. It inhibits fungal lanosterol 14 -α demethylase which leads to ergosterol deficiency.

Ketoconazole: It's an imidazole which is fungistatic at a lower concentration and fungicidal at a higher concentration. Inhibits C ^{14} demethylation of cell membrane sterols.

Griseofulvin: It's a spiro – benzofuran and is fungistatic. It distrupts fungal mitotic spindle and arrests cell division.

Ciclopirox: The mechanism of action of ciclopirox is poorly understood. However, loss of function of certain catalase and peroxidase enzymes, as well as inhibition of the membrane transfer system by interrupting the Na^+ K^+ ATPase has been implicated as the mechanism of action.

Topical antifungal applications [70]:

A variety of topical agents are available in the form of gels, creams and and shampoo formulations. A majority of these agents belongs to azole and allylamine families. Cure rates of tinea corporis / tinea cruris / tinea pedis are high with regular topical applications for two to four weeks. Most adverse effects of topical applications are at the site of application and they are mild and transient [71].

Systemic antifungal drugs:

Five main systemic agents are available: terbinafine, itraconazole, fluconazole, griseofulvin, and ketoconazole. Oral formulations of itraconazole and terbinafine are the most common drugs used for tinea unguinum. Griseofulvin plays a large role in tinea capitis treatment, though its use has been superseded in other areas by itraconazole and terbinafine. The oral antifungal medications may be associated with severe hepatic toxicity, rare serious skin events such as Stevens–Johnson syndrome, and possible drug–drug interactions due to metabolism through the cytochrome P-450 system [72,73].

Table 4. A detailed updated description on antifungal treatment for dermatophytes [70] (FDA approved Indications).

Disease	Terbinafine	ItraconaZole	FluconaZole	Ketoconazole	Griseofulvin	Topicals
Tinea pedis / manum	Cream & 1%solution: apply twice daily 1–4 weeks Oral: 250 mg/day 2 weeks	Oral: 200 mg bid for 1 week	Oral: 150 mg once weekly for 2–6 weeks	2% Cream: apply once daily for 6 weeks Oral: 200–400mg/day for 4 weeks	Microsize: 1 g/day Ultra microsize 660 or 750 mg/day for 4–8 weeks	Ciclopirox: 0.77% cream &gel twice daily for 4 weeks
Tinea corporis /cruris	Cream & solution: apply twice daily for 1–4 weeks Oral: 250 mg/day for 2–4 weeks	Oral: 200 mg/day for 1 week	Oral: 150–300 mg once weekly for 2–4 weeks	2% Cream: apply once daily for 2 weeks Oral: 200–400 mg/ day for 4 weeks	Microsize:500 mg/day Ultra microsize: 330–375mg/day for 2–4 weeks	Ciclopirox 0.77% cream And gel twice daily for 4 wks
Tinea unguinum	Oral: 250 mg/day Toenail: 12–16 weeks Fingernail: 6 weeks	Oral: Pulse therapy:200 mg bid for 1 week, followed by 3 itraconazole free weeks Toenails: 3 pulses Fingernails only:2 pulses	Oral: 150 or 300 mg once weekly for 6–12 months (Toe nail: approx.9–15 months; Finger nail:approx. 4–9 months	Oral: 200–400 mg/ day for 6 months Not used Due to hepato Toxicity risk	Microfine preparation: 500 mg daily for 6–12 months	Amorolfine 5% lacquer

Table 5. The treatment for tinea capitis (FDA approved instructions)

Body Weight (kg)	Terbinafine (Continuous) 2–4 weeks	Itraconazole(Continuous) 2–4 weeks	Itraconazole(pulse) 1–3 pulses	Fluconazole (continuous) 1-3pulses for 20 days	Fluconazole (pulse) 6–12 weeks	Griseofulvin (continuous) 6-12 weeks
< 10kg	5mg/kg/day	5mg/kg/day	5mg/kg/day	6mg/kg/day	6mg/kg/day	15-25 mg/kg/day
10-20kg	62.5mg qid	100 mg qid	100 mg qid	-	-	-
21-30kg	125 mg qid	100 mg qid	100 mg qid	-	-	-
31-40kg	125 mg qid	100 mg qid	100 mg qid	-	-	-
41-50kg	250 mg qid	200 mg qid	200 mg qid	-	-	-
>50kg	250 mg qid	200 mg qid	200 mg bd	-	-	-

Mechanism of Resistance [74]:

Only few reports have addressed the drug resistance mechanism in dermatophytes and most of them have been described in *Trichophyton rubrum.* There has been only one clinically confirmed case of terbinafine resistance in dermatophytes, where six sequential *Trichophyton rubrum* isolates from the same patient were found to be resistant to terbinafine and cross-resistant to other squalene epoxidase (SE) inhibitors. The MICs of terbinafine for these strains were >4 µg/ml, whereas they were <0.0002 µg/ml for the susceptible reference strains [75]**.** The in vitro resistance of an isolate can be classified as either intrinsic or acquired. Intrinsic resistance allows all normal members of a species to tolerate particular drug. Acquired resistance is a term used when a resistant strain emerges from a population that was previously drug sensitive.

Table 6. The putative mechanism of drug resistance in dermatophytes

Drugs	Putative resistance mechanism
Terbinafine	Modification of target enzymes by mutation Increased drug efflux Stress adaptation Over-expression of salicylate mono-oxygenase(drug degradation)
Fluconazole	Increased drug efflux Stress adaptation
Ketoconazole	Increased drug efflux Over-expression of lanosterol -14 ά- demethylase
Amphotericin B	Increased drug efflux Stress adaptation
Griseofulvin	Increased drug efflux Stress adaptation

ANTIFUNGAL SUSCEPTIBILITY TESTING:

Antifungal susceptibility testing remains an area of intense interest. Although antifungal susceptibility testing remains less well developed and utilized than antibacterial testing, they can be used for drug discovery and epidemiology. The approved methodology for testing of yeasts was published by NCCLS (M27-A) in 1997 [76]. The need extended beyond testing for *Candida species* alone, with development of resistance among various filamentous molds [72,73]. Following the principles established for testing yeasts, a proposed standard method entitled “Reference method for broth

dilution antifungal susceptibility testing of conidium forming filamentous fungi" was published as NCCLS M38-P [77] in 1998.

A significant difference between M27-A and M-38 is the inoculum size and the end point (defined as the lowest drug concentration producing a prominent reduction in growth) determination [78]. The standard broth micro dilution M38-A2 method has been developed and adopted by the Clinical and Laboratory Standards Institute (CLSI) for testing antifungal susceptibility of filamentous fungi, including the dermatophytes in 2008 [79]. In recent years several studies on the in vitro susceptibility of dermatophytes to antifungal drugs have been done and the results have shown considerable variation. In developing this method for antifungal susceptibility testing of dermatophytic fungi, many variables need to be considered, like the medium for production of conidia, the inoculum size, temperature and duration of incubation, medium, and endpoint determination.

Norris et al. [80], in an attempt to standardize optimal conditions for dermatophyte susceptibility testing, selected potato dextrose agar for production of conidia, RPMI 1640 medium, 35°C for 4 days as temperature and time of incubation respectively and an inoculum of 10^3 conidia/ml as the most appropriate. Fernandez – Torres et al [81, 82] proposed some modifications which also found to improve the outcome of the results. The temperature of incubation was 28ºC with the incubation duration increased from 4 to 10 days and inoculum concentration of 10^4 conidia/ml. Jessup et al. [83] demonstrated that oatmeal and rice agar is good media to enhance sporulation.

The end point determination is highly variable for the drugs tested against dermatophytes. For Amphotericin B the end point concentration is that which prevents

any discernible growth (100% inhibition) [79]. For fluconazole, flucytosine and ketoconazole, end points are 80% or more reduction in the growth compared to the growth in the growth control well (drug free medium) [79].

Most MIC for ciclopirox and griseofulvin are ≤ 1 µg /ml for the dermatophytes [79, 84]. Most MIC for terbinafine is ≤ 0.25µg /ml, but MIC ≥ 0.5 µg /ml have been reported for Trichophyton rubrum [85]. Correlation of MIC with clinical outcome has yet to be decided which gives a strong hope for further fruitful research in this field. Thus effective control of dermatophytes will necessarily involve the development of a new generation of potent broad spectrum antifungals with selective action against new targets in the fungal cells.

Materials & Methods

MATERIALS AND METHODS:

A total of 300 specimens were collected from the clinically suspected cases of dermatophytic infections from the patients attending the inpatient and outpatient department in PSG Hospitals from the period September 2009 to August 2010. Human ethical clearance for the study was obtained from the Institutional ethical committee prior to collection of samples. The samples were collected after explaining the procedure to the patients and getting their consent in writing. The copies of both the forms are enclosed in the annexure.

Collection and transportation of Samples[45]:

Skin Scrapings: The lesion was disinfected with 70 % alcohol and with the help of sterile blunt end of the scalpel or with sterile glass slide; the infected area was scraped from the centre to the periphery.

Nails: After disinfection with alcohol, the debris from beneath the distal end of the nail were scraped with sterile scalpel or close clipping of the distal end of the nail with nail clippers were done.

Hair: The infected hair were removed with epilating forceps and never by cutting, because this fails to remove the area most likely to harbors the fungus.

Samples were collected and transported in a sterile black paper. This was to avoid exposure to moisture and prevent the growth of contaminants (Fig 1).

Processing of Samples:

The skin and hair samples were taken directly for microscopy and culture. The nail clippings were taken in sterile petri plates and with the help of sterile scalpel were cut into fine pieces.

Direct microscopy:

For direct microscopy the samples collected were screened for the presence of fungal elements by 20% Potassium hydroxide (KOH) with 40% Dimethyl Sulfoxide Mount (DMSO) mixed in equal proportion [2].

20% KOH & 40% DMSO mount:

1. A drop of 20% KOH with 40% DMSO was placed in the clean slide and the skin and hair samples were added to it and covered with a clean cover slip.
2. The slide was not passed through flame and was screened for the presence of fungus after five minutes.
3. In case of nails, two to three drops of 20% KOH and 40% DMSO were added to a sterile test tube and a portion of finely divided nail was added to it and left for overnight at room temperature. The microscopic examination was done on the next day.
4. Each slide was thoroughly examined for the presence of filamentous, septate, branched hyphae with or without arthrospores crossing the margins of the squamous epithelial cells of the skin and in nails (Fig 2).

5. In case of hair, type and arrangement of the spores, the involvement of hair shaft were noticed to name it as ectothrix or endothrix type of infection.

Culture:

For primary isolation of dermatophytes following media were used:

A. Sabouraud Dextrose Agar (SDA) with antibiotics – Chloramphenicol and Cycloheximide.
B. Dermatophytes Test Medium (DTM).

The medium used for subculture to induce conidia formation was Potato dextrose agar.

CULTURE AND COLONY MORPHOLOGY

The SDA and DTM were inoculated in duplicate; one incubated at 25°C - 30°C and other at 37°C for 3 weeks. SDA was taken as standard media for primary isolation and compared with Dermatophyte Test medium (DTM) (Fig 3). Identification of dermatophytes was confirmed by gross morphology of growth, typical microscopic characteristics, supplemented with slide culture, hair perforation test, urease test and growth characters on Bromocresol Purple agar (BCP).

Growth was slow observed on an average of 10 days incubation. When the growth became evident on the primary isolation medium (SDA), the colony morphology and the pigment production on the obverse and reverse was observed. The growth of dermatophytes in Dermatophyte test medium was observed as change in the colour of the medium to red. It denoted the shift in the pH to alkaline due to the dermatophytes . The colonies were transferred on to Potato dextrose agar for enhancement of sporulation and for maintaining the stock culture.

LACTO PHENOL COTTON BLUE MOUNT

1. A Lacto phenol cotton blue mount was made from the colony to study the microscopic characteristics.
2. A drop of lacto phenol cotton blue was placed on the slide and a small portion of the colony was teased with the teasing needle and placed in the mount and was observed under the microscope.
3. The presence of macroconidia, macroconidia and the hyphal arrangements were noted.

SLIDE CULTURE

1. The mycelial strands were transferred to slide culture to study the morphology more clearly.
2. A small portion (around one centimeter area) of the Potato dextrose agar was cut in a square fashion and was placed in a sterile glass slide which in turn was placed over a sterile U-shaped plastic tube. The settings were kept inside a sterile glass Petri plate.
3. A small portion of the fungal colony was taken from the original culture and was inoculated on all the four sides of the square block of the agar. The slide culture was incubated at 30°C until a good growth of hyphal elements was observed.
4. Then a lacto phenol cotton blue mount was done from the slide culture and the morphology of the mycelial forms was observed.
5. The main characters observed were – the presence or absence of micro conidia and macro conidia, the nature of their walls (Smooth walled or rough walled),

their arrangements, the presence of other features like spiral hyphae, antler hyphae and others.

BROMOCRESOL PURPLE AGAR

1. Bromocresol purple agar was inoculated in the centre of the slant with a small amount of the inoculum.
2. A growth control tube of Sabouraud's dextrose agar was also inoculated with BCP agar. Both tubes were incubated at 30°C for 10 days.
3. Growth rates were recorded at 7 days, after comparing with the growth control. Rates were recorded as slow, restricted or profuse when compared to the growth control medium.
4. pH of the medium was recorded at 7 and 10 days. The readings were noted as Unchanged (sky blue colour); alkaline (blue to blue purple; acidic (yellow).
5. The dermatophytes produce no or alkaline changes in the medium, while bacteria and some saprophytic fungi produce acidic changes in the medium (Fig 4).

CHIRSTENSEN'S UREASE TEST

1. The Christensen's urease agar with 1% glucose was commonly used for this test.
2. A small amount of the inoculum was transferred to the middle of the slant in a tube and is incubated for 7- 14 days at 25°C.
3. Positive result was indicated by the change in colour to pink; negative results were seen as unchanged yellow – orange colour (Fig 5).

HAIR PERFORATION TEST

1. Hair from prepubertal age group, preferably from neonates was collected. They were cut into small pieces and sterilized by autoclave.
2. A sterile glass plate was taken and about 25 ml of distilled water was added. Three to four drops of sterile yeast nitrogen base was added.
3. The sterilized hair samples were placed in the distilled water with the help of sterile forceps.
4. The colony of the dermatophytes was taken and introduced into the sterile water, just touching the hairs.
5. They were allowed to incubate at room temperature without disturbing for 14 to 21 days.
6. After the incubation time, the hair was examined under LPCB mount.
7. Any wedge shaped projection in the shaft of the hair was considered as positive (Fig 6).

ANTIFUNGAL SUSCEPTIBILTY TESTING [75]

The aim of doing antifungal susceptibility testing was to find the Minimum Inhibitory concentration (MIC) ,which is defined as the lowest concentration of the drug that causes a specified reduction in visible growth of the organism in a broth or agar dilution method. The antifungal agents used to find the MIC for Dermatophytes were Amphotericin B (Himedia), Fluconazole (Himedia), Ketoconazole (Himedia), Ciclopirox (Sigma Aldrich), Terbinafine (Sigma Aldrich) and Griseofulvin (Sigma Aldrich).

Table 7 showing the preparation of Antifungal stock solution:

Drugs	Solvent	Diluent	Desired concentration (µg/ml)
Amphotericin B	DMSO	RPMI 1640	16 to 0.0313
Ciclopirox	DMSO	RPMI 1640	32 to 0.06
Griseofulvin	DMSO	RPMI 1640	16 to 0.0313
Ketoconazole	DMSO	RPMI 1640	16 to 0.0313
Terbinafine	DMSO	RPMI 1640	0.5 to 0.001
Fluconazole	Water	RPMI 1640	64 to 0.125

1. The amount of drug and diluents needed were calculated from the below given formulae:

$$\text{Weight (mg)} = \frac{\textbf{Volume (ml) X Concentration (µg/ml)}}{\textbf{Potency ((µg/mg)}}$$

Or

$$\text{Volume (ml)} = \frac{\textbf{Weight (mg) X Potency ((µg/mg)}}{\textbf{Concentration (µg/ml)}}$$

2. The concentrations of drugs to be tested for dermatophytes were: Amphotericin B 16 to 0.0313 µg/ml; Ketoconazole 16 to 0.0313µg/ml Ciclopirox 32 to 0.06 µg/ml; Terbinafine 0.512 to 0.001 µg/ml; Griseofulvin 64 to 0.125 µg/ml; Fluconazole 64 to 0.125 µg/ml.

3. The dilutions of the agents were prepared 100 times the final strength with the solvent and then diluted 1:50 times with the growth medium. The method to prepare serial dilutions of the drug is enclosed in the annexure.

Growth medium Preparation:

1. The completely synthetic medium Rose Parker Memorial Institute – 1640 (RPMI-1640) from Hi-media was used as a growth medium in antifungal susceptibility testing. The medium was buffered to the pH of 7.0 ± 0.1.
2. The buffer used was Phosphate buffer saline (0.067M) with pH of 7.0 which was sterilized by autoclave.
3. In 1000ml of Phosphate buffer saline 9.6 grams of RPMI 1640 was dissolved. The final solution was sterilized by filtration through membrane filter.

Inoculum preparation:

1. The fungal colony to be tested was grown in Potato dextrose agar to induce the conidia formation. After 7 – 10 days of incubation with well grown hyphae, the culture was taken for testing.
2. Around 5 ml of sterile saline was added to the culture tube and was scraped gently with a sterile loop allowing the conidia to enter into the saline.
3. With the help of sterile pipettes, about 3 ml of the saline with conidia was transferred into a sterile screw cap tube.
4. The tube was then vortexed for 30 seconds to one minute. The tube was allowed to stand at room temperature for 5 to 10 minutes for the heavier particles to settle down.

5. The supernatant portion alone was taken (around 2 ml) and diluted again with 1 ml of sterile saline.
6. Around 10 µl of the diluted solution was transferred to the Neubauer's chamber and the conidia were counted in the four corner squares. The average of the cells in all the four corner squares was calculated.
7. 100 µl of the diluted inoculum was transferred to 5 ml of RPMI-1640 solution.
8. The final concentration of the conidia was made to be $1 – 3 \times 10^{3}$ cfu/ml.

Inoculation in RPMI – 1640 medium:

1. The inoculation was done in sterile 96 – well microtitre plate with flat bottom.
2. Each well was inoculated with 100 µl of the conidial suspension in RPMI 1640.
3. 100µl of the diluted drugs were added correspondingly to each well.
4. The growth control well was inoculated with only 200 µl of diluted conidial suspension with the growth medium without any antifungal agent.
5. The sterility control well was inoculated with 200 µl of the growth medium alone without any conidium.
6. All microtitre plates were incubated at 37°C without agitation and evaluation was done after four days of incubation.

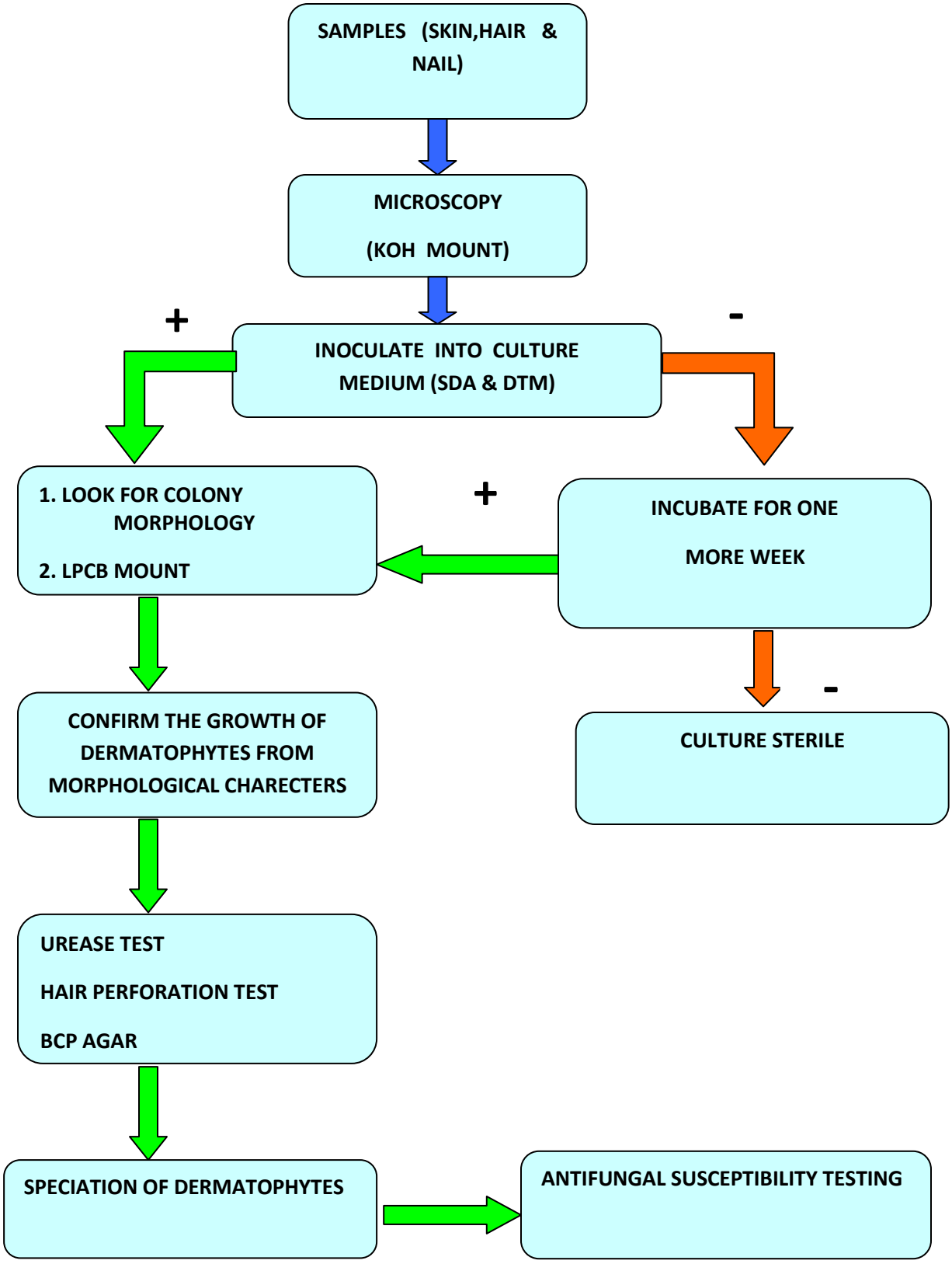
Control: *Trichophyton mentagrophytes* ATCC MYA – 4439 was used as the control in performing the antifungal susceptibility testings.

Interpretation of results:

For Amphotericin B end points were typically well defined and the MIC was easily read as the lowest drug concentration that prevents any discernible growth (100%). Trailing endpoints were not encountered with Amphotericin B.

For all other drugs (Fluconazole, Ketoconazole, Ciclopirox, Griseofulvin and Terbinafine) end points were not typically as well defined as that of Amphotericin B. It was taken as 80% or more reduction in growth when compared to growth in the control well (drug free medium).

Diagnostic Algorithm for processing of samples



Results

RESULTS:

A total of 300 specimens were collected from clinically suspected cases of dermatophytic infections from the patients attending the Inpatient and Outpatient department in PSG Hospitals between the periods September 2009 to August 2010.

There were 165 (55%) males and 134 (45%) females included in the study. The positive rate among males was 42.4 % (72) and among females was 42.5% (57) (Fig.7). Of the 21(7%) samples collected in the group of 1-14 years of age, 13 (4.3%) were positive. Twenty nine (9%) were positive in the age group between 15 – 28 years. Similarly the number of patients positive in the age group of 29 – 42 years , 43-56 years and > 57 years were 52(17.3%), 18 (6%)and 17 (5.6%) respectively (Fig.8).

Contact history with animals was positive for 35 (11.6 %).Twenty one of them were culture positive (60%) (Fig.9). Out of 300 samples collected, 103(34.33%) samples were microscopically positive and 147(49%) were positive by culture. This includes 129(43%) dermatophytes and 18(6%) non- dermatophytic fungi (Table 9).

Of the 241(80.33%) skin samples collected, 90 (30%) were positive for dermatophytes. Thirty three (11%) of the 45(15%) nail samples were positive. Six (2%) out of the 15 (6%) hair samples were positive (Fig.10). Majority of the patients had tinea corporis followed by tinea cruris. Of the 90 positive skin samples 39 (43.33%) were tinea corporis, 22 (24.44%) were tinea cruris, 12 (13.33%) were tinea pedis, 7 (7.77%) were tinea manum, 7(7.77%) for tinea capitis and 3(3.33%) were tinea facium (Fig 11).

Comparison of Sabouraud's dextrose agar and Dermatophyte test medium for the primary isolation of dermatophytes from clinical samples was done. Of the 129(43%)

culture positive, 126 (42%) were positive in Dermatophyte test medium and 122(40.6%) were positive in Sabouraud's dextrose agar (Table 10).

The macroscopic and microscopic features of each isolates were observed (Fig 12 - 24). The isolates from the culture medium were subjected to Lacto phenol cotton blue mount, slide culture, hair perforation test, urease test, bromocresol purple agar for identification up to species level.

Table 8. The results of various biochemical tests performed for the identification of dermatophytes

	Hair perforation test	Urease test	Bromocresol purple agar
Trichophyton mentagrophytes	Positive	Positive	Alkaline
Trichophyton rubrum	Negative	Negative	Alkaline
Trichophyton tonsurans	Negative	Positive	Alkaline
Trichophyton equinum	Negative	Positive	Alkaline
Trichophyton meginii	Negative	Positive	Alkaline
Trichophyton ajelloi	Positive	Positive	No change
Trichophyton violaceum	Negative	Positive	No change
Trichophyton kanei	Negative	Positive	No change

Microsporum gypseum	Positive	Positive	No Change
Microsporum ferrugineum	Negative	Negative	No change
Epidermophyton flocossum	Negative	Positive	Alkaline

The majority of the isolates were *Trichophyton mentagrophytes* followed by *Trichophyton rubrum*. Out of 129 positive 50 (38.75%) were *Trichophyton mentagrophytes*, 35 (27.13%) were *Trichophyton rubrum*, 15 (11.6%) were *Trichophyton tonsurans*, 9 (6.9%) were *Epidermatophyton floccosum,* 7 (5.4%) were *Trichophyton equinum*, 4 (3.1%) were *Microsporum gypseum,* 3 (2.3%) were *Trichophyton meginii* and *Trichophyton violaceum* each, 1 (0.7%) was *Trichophyton ajelloi, Trichophyton kanei,* and *Microsporum ferrugineum* each (Fig 25).

The most common causative agents of tinea corporis was *Trichophyton mentagrophytes* (53.8%) followed by *Trichophyton rubrum* (20.5%) (Fig.26). Majority of tinea cruris were caused by *Trichophyton rubrum* (31.8%) (Fig 27). Ectothrix infections were observed with *Microsporum gypseum* (50%), *Trichophyton mentagrophytes* (30%) and *Trichophyton rubrum* (20%) (Fig 28). *Trichophyton mentagrophytes* was found to be the most predominant agent than the others in causing tinea manum, tinea unguinum and tinea pedis (Table 11).

As per the guidelines given in CLSI document M38-A2, the antifungal susceptibility testing was done on 129 dermatophytes by broth micro dilution method

(Fig 29). The MIC for *Trichophyton mentagrophytes* for Amphotericin B varied from 0.5 – 8 µg/ml, Fluconazole 1 – 8 µg/ml, Ketoconazole 0.0313-1µg/ml, Ciclopirox 0.125-2µg/ml, Terbinafine 0.001-0.008µg/ml and Griseofulvin 0.25-0.5µg/ml. In case of *Trichophyton rubrum*, the MIC values for Amphotericin B is 2 – 8 µg/ml, Fluconazole 0.125 – 2 µg/ml, Ketoconazole 0.25 -1µg/ml, Ciclopirox 0.125-2µg/ml, Terbinafine 0.001-0.008µg/ml and Griseofulvin 0.25-0.5µg/ml. For *Trichophyton tonsurans* the MIC values were within the following range: Amphotericin B 1 – 8 µg/ml, Fluconazole 1 – 4 µg/ml, Ketoconazole 0.313- 0.25 µg/ml, Ciclopirox 1-2µg/ml, Terbinafine 0.001-0.004µg/ml and Griseofulvin 0.125-0.5µg/ml. For *Trichophyton equinum* the MIC values were as follows : Amphotericin B 4 – 8 µg/ml, Fluconazole 2 µg/ml, Ketoconazole 0.25 µg/ml, Ciclopirox 0.5 - 1µg/ml, Terbinafine 0.004µg/ml and Griseofulvin 0.25µg/ml. For *Trichophyton meginii* the MIC values were as follows : Amphotericin B 4 – 8 µg/ml, Fluconazole 0.25 – 0.5µg/ml, Ketoconazole 0.25 µg/ml, Ciclopirox 2 µg/ml, Terbinafine 0.004µg/ml and Griseofulvin 0.25µg/ml**.**

In case of *Trichophyton ajelloi* the MIC values were as follows : Amphotericin B 4 µg/ml, Fluconazole 1 µg/ml, Ketoconazole 1 µg/ml, Ciclopirox 0.5 µg/ml, Terbinafine 0.002 µg/ml and Griseofulvin 0.5µg/ml**.** For *Trichophyton violaceum* the MIC values were as follows : Amphotericin B 2 - 4 µg/ml, Fluconazole 0.25 – 0.5 µg/ml, Ketoconazole 0.5 µg/ml, Ciclopirox 1 µg/ml, Terbinafine 0.004 µg/ml and Griseofulvin 0.25 – 1 µg/ml**.** For *Trichophyton kanei* the MIC values were as follows : Amphotericin B 4µg/ml, Fluconazole 4 µg/ml, Ketoconazole 0.5 µg/ml, Ciclopirox 1 µg/ml, Terbinafine 0.004 µg/ml and Griseofulvin 0.5 µg/ml**.** For *Microsporum gypseum* the MIC values were Amphotericin B 0.5 -2 µg/ml, Fluconazole 2 - 4 µg/ml,

Ketoconazole 0.25 - 0.5 µg/ml, Ciclopirox 0.125 - 1 µg/ml, Terbinafine 0.004 µg/ml and Griseofulvin 0.125 - 0.5 µg/ml**.** For *Microsporum ferrugineum* the MIC values were Amphotericin B 4 µg/ml, Fluconazole 8 µg/ml, Ketoconazole 1 µg/ml, Ciclopirox 2 µg/ml, Terbinafine 0.5 µg/ml and Griseofulvin 0.008 µg/ml **.** For *Epidermophyton flocossum* the MIC values were Amphotericin B 2 - 4 µg/ml, Fluconazole 1 - 2 µg/ml, Ketoconazole 1 - 2µg/ml, Ciclopirox 0.25 – 0.5 µg/ml, Terbinafine 0.001 – 0.004 µg/ml and Griseofulvin 0.5 - 1 µg/ml**.**

The range of MIC was within the normal susceptibility range of the standard ATCC fungal strains mentioned in CLSI document M-38 A2. The results of antifungal susceptibility testing were shown on the Table 12. for all the species isolated in our study.

Fig 1. Sample collection in a sterile black paper

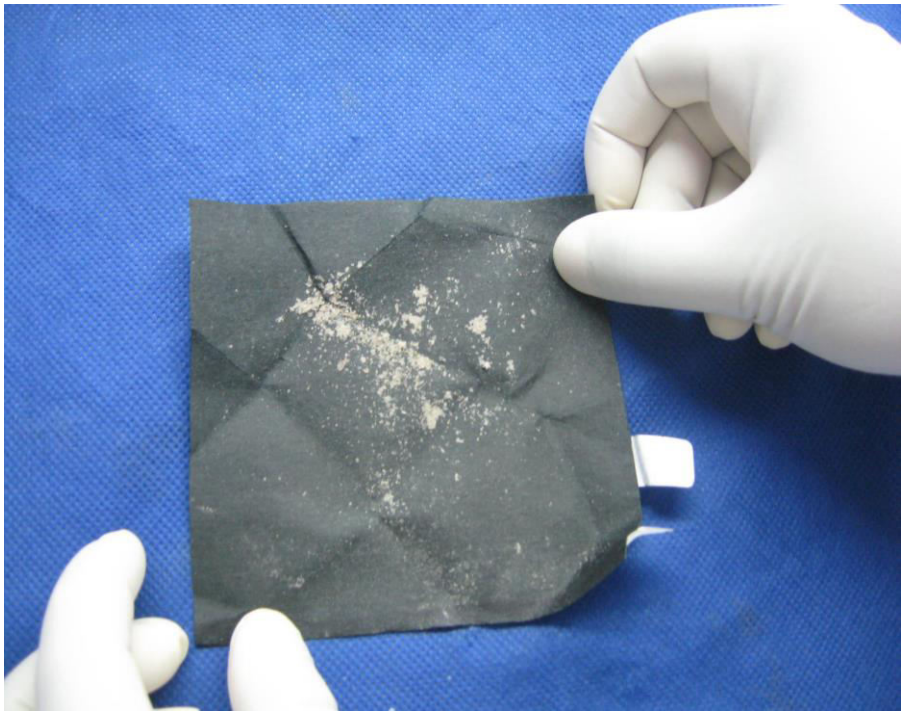


Fig 2. Potassium hydroxide mount showing fungal elements

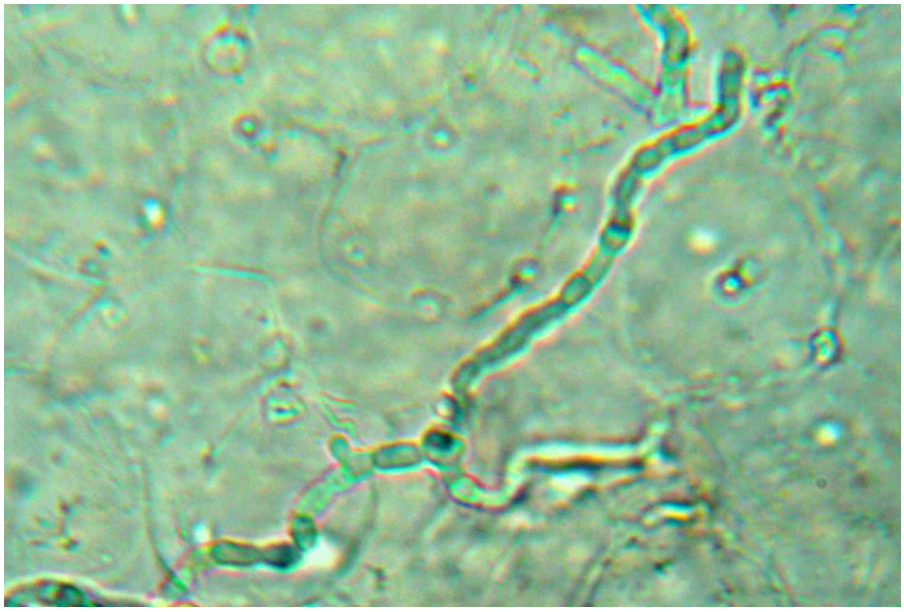


Fig 3. Growth of dermatophytes on Dermatophyte test medium and Sabouraud dextrose agar

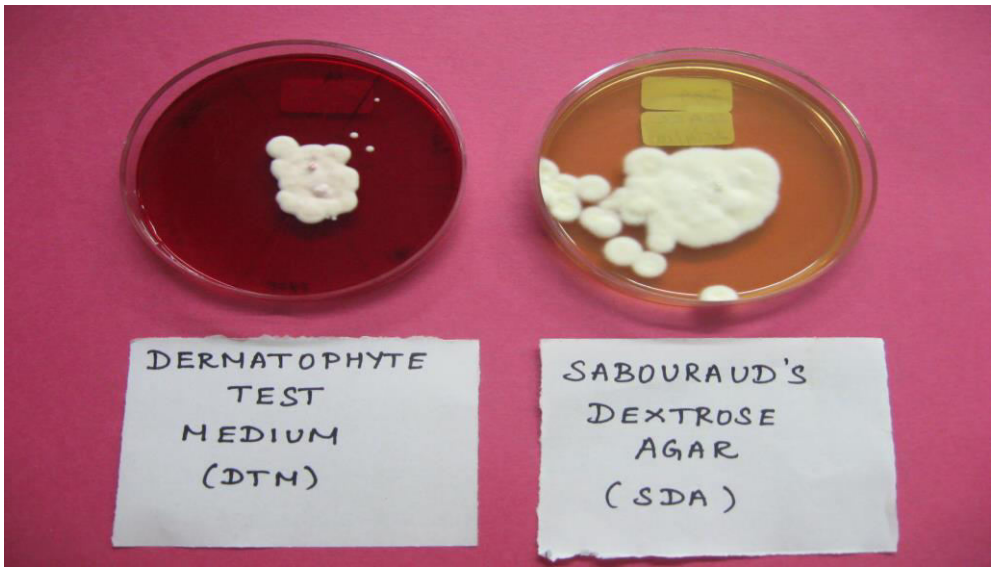
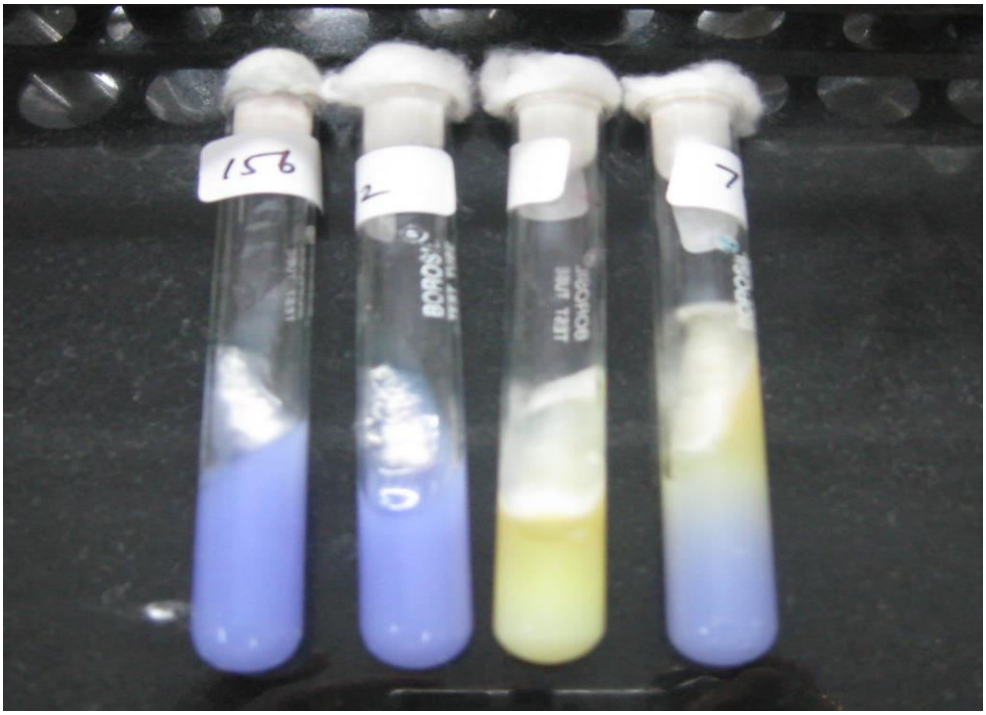


Fig 4. Bromocresol purple Agar showing alkaline and acidic reaction



Alkaline **Acidic**

Fig 5. Positive and negative urease test for dermatophytes

Positive **Negative**

Fig 6. Positive Hair perforation test with wedge shaped projections

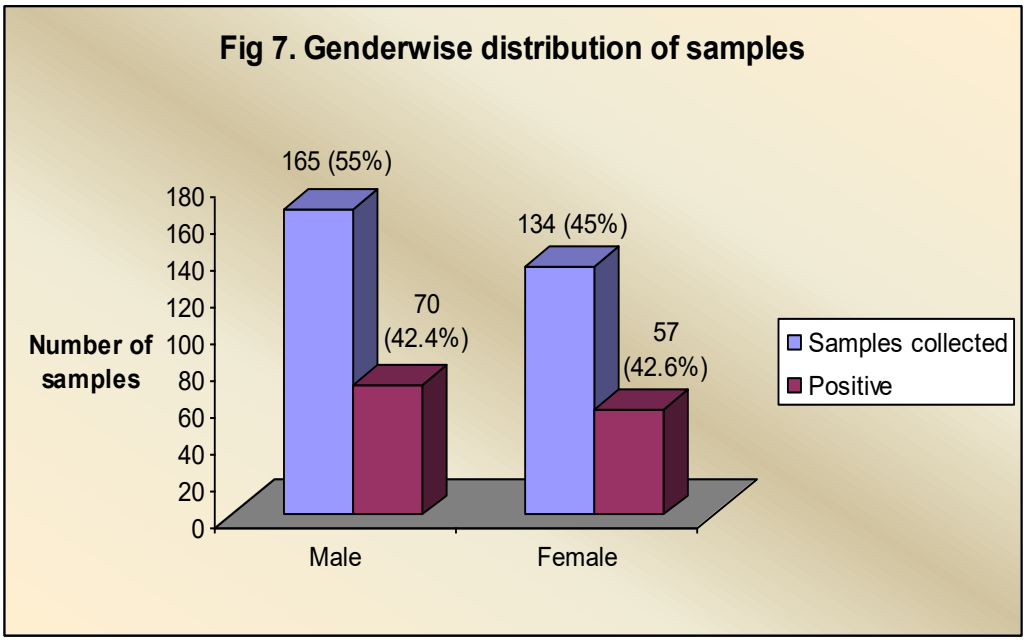


Fig 7. Genderwise distribution of samples
165 (55%)
70
(42.4%)
134 (45%)
57
(42.6%)
Number of samples
180
160
140
120
100
80
60
40
20
0
Male
Female
Samples collected
Positive

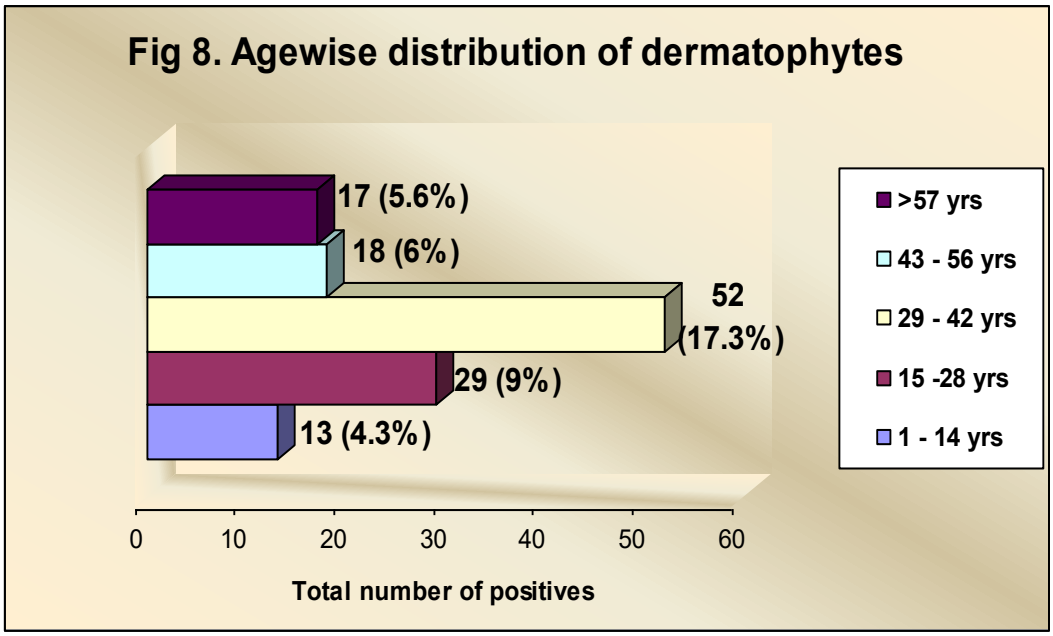


Fig 8. Agewise distribution of dermatophytes
17 (5.6%)
18 (6%)
52
(17.3%)
29 (9%)
13 (4.3%)
>57 yrs
43 - 56 yrs
29 - 42 yrs
15 -28 yrs
1 - 14 yrs
0
10
20
30
40
50
60
Total number of positives

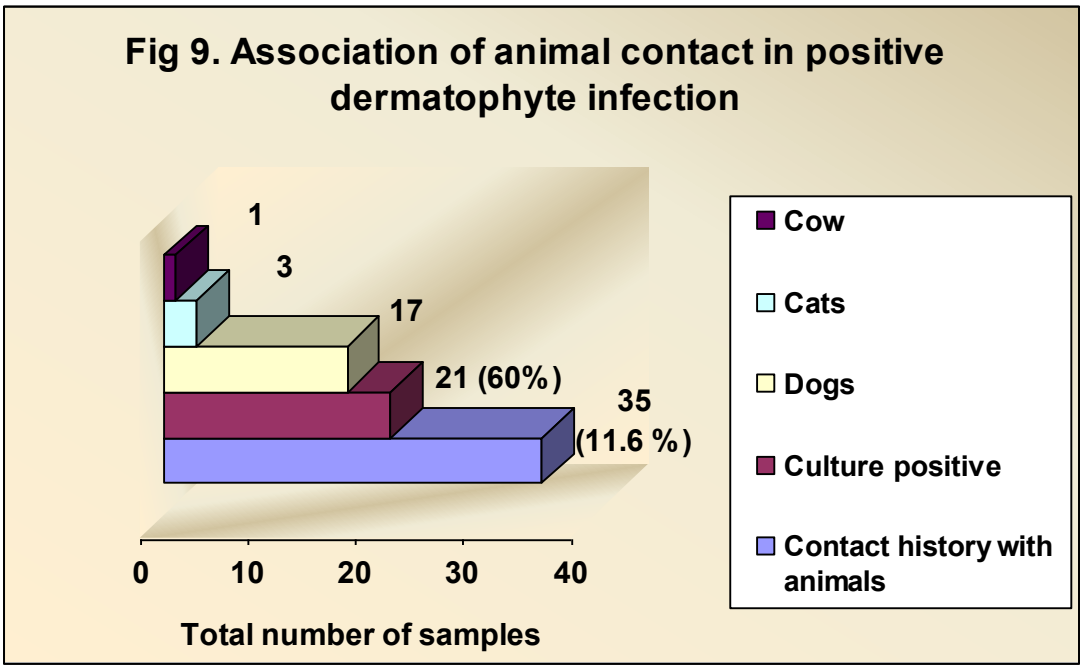


Table 9. Comparison of results of microscopy and culture of positive isolates among dermatophytes infection

Total number of cultures positive			**147 (49%)**
Total number of cultures positive for dermatophytes			**129 (43%)**
Total number of cultures positive for non dermatophytic fungi			**18 (6%)**
	Microscopy +	**Microscopy -**	
Culture +	**97 (32.3%)**	**32(10.6%)**	**129 (43%)**
Culture -	**6 (2%)**	**165(55%)**	**171 (57%)**
Total Number	**103 (34.3%)**	**197(65.6%)**	**300**

Fig 10. Distribution of Samples & the number of samples yielded dermatophytes in the study

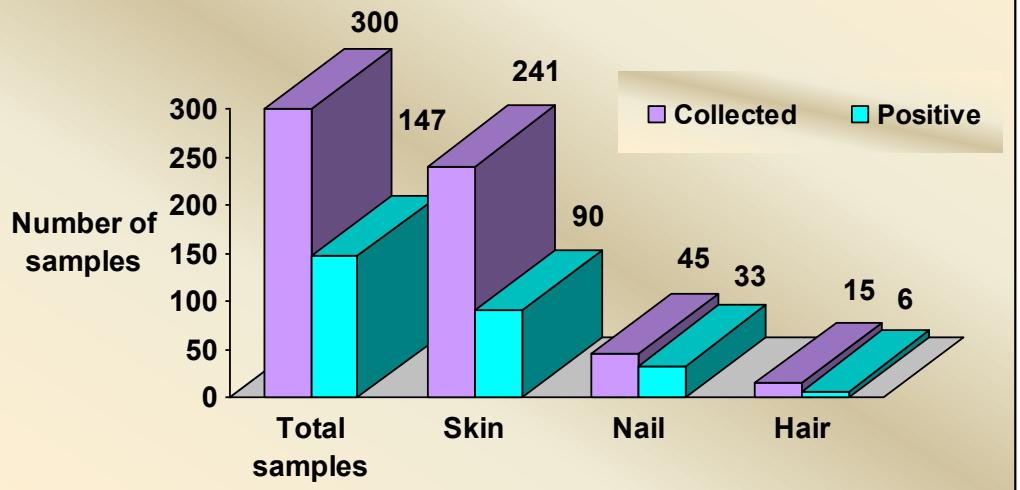


Fig 11.Distribution of positive skin samples

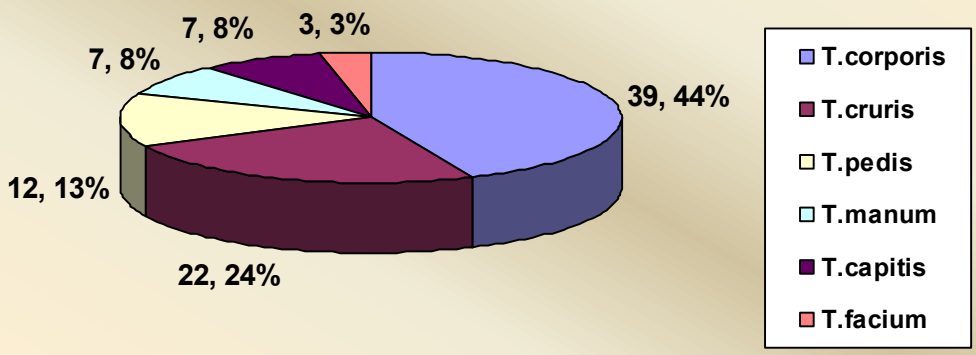


Table 10. Comparison of Sabouraud dextrose agar and Dermatophyte test medium in the primary isolation

Total number of cultures positive for dermatophytes			**129 (43%)**
Culture positive by DTM			**126 (42%)**
Culture positive by SDA			**122 (40.6%)**
SDA	**DTM**	**Number**	
+	**+**	**122**	
-	**+**	**4**	
+	**-**	**0**	

Fig 12. Obverse and reverse view of *Trichophyton mentagrophytes*

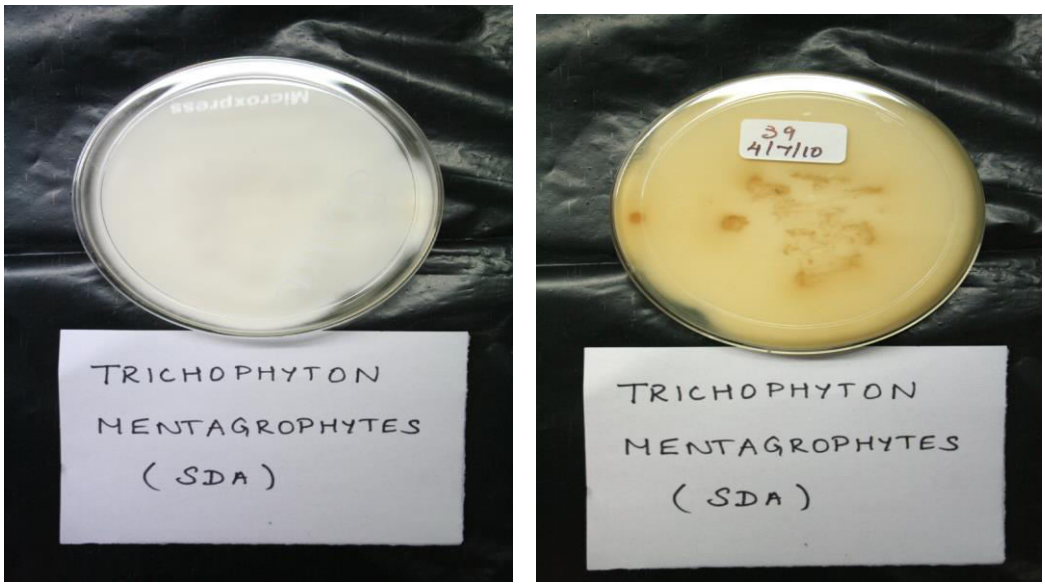


Fig 13. Lacto phenol cotton blue mount of *Trichophyton mentagrophytes* showing spiral hyphae

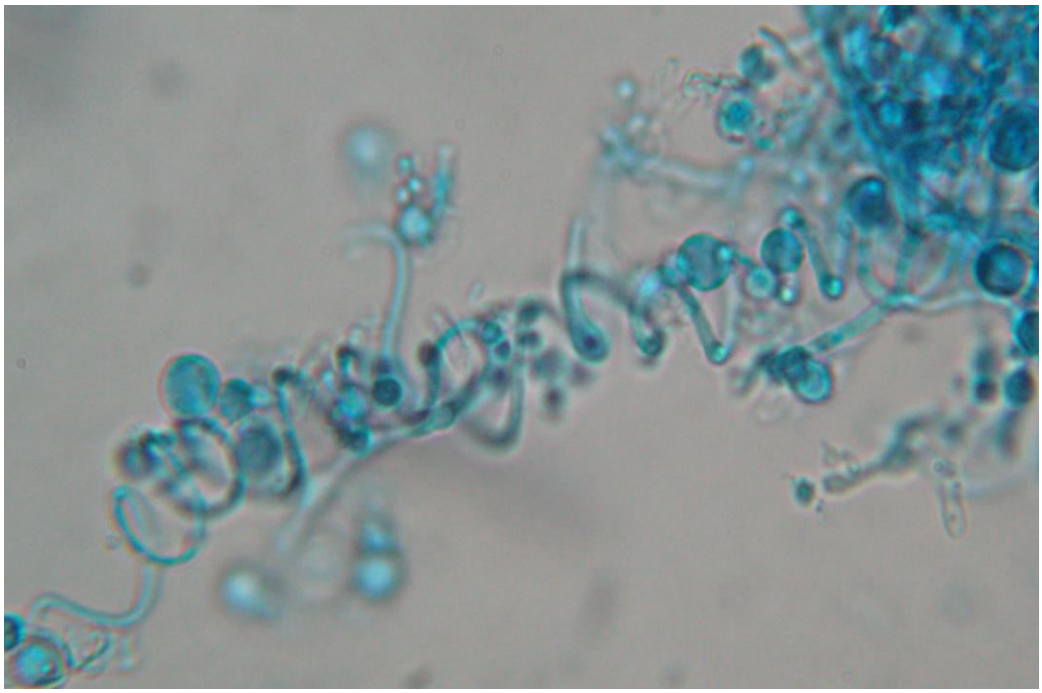


Fig 14. Obverse and reverse view of *Trichophyton rubrum*

Fig 15. Lactophenol cotton blue mount of *Trichophyton rubrum* showing pyriform microconidia

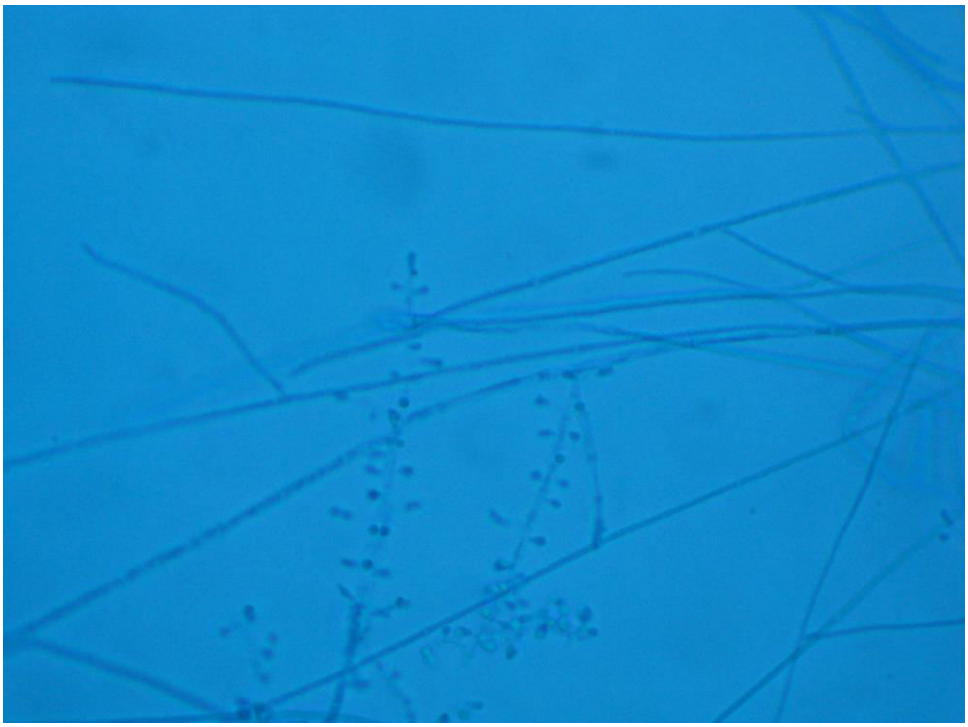


Fig 16. Obverse and reverse view of *Epidermophyton flocossum*

Fig 17. Lacto phenol cotton blue mount of *Epidermophyton flocossum*

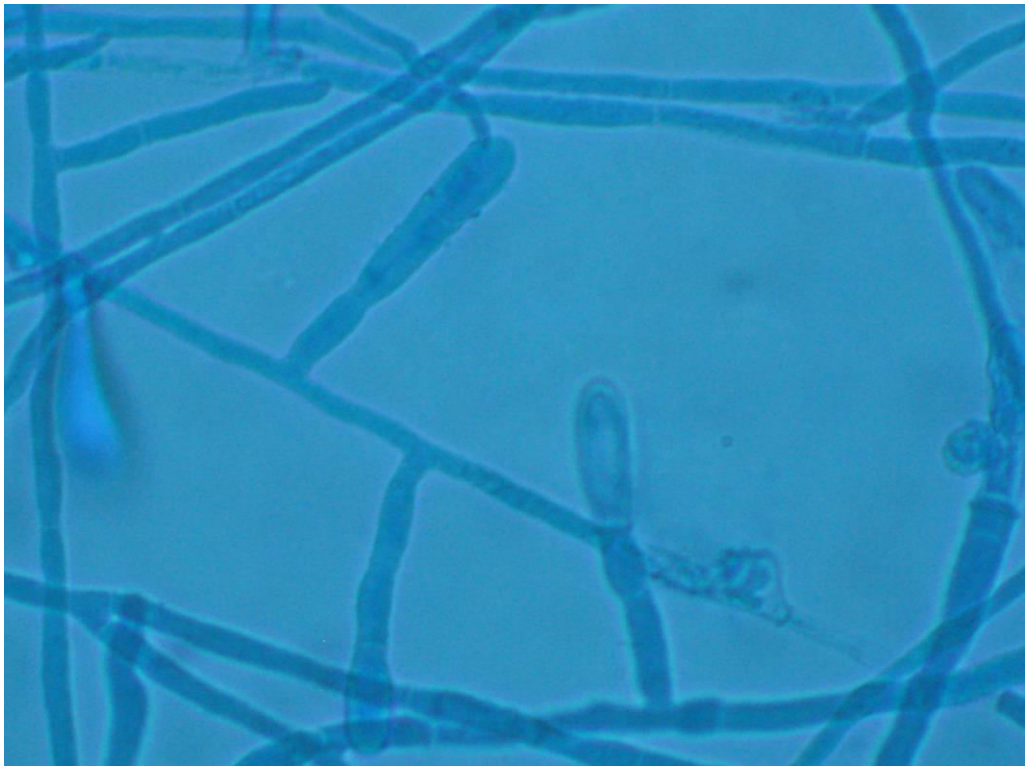


Fig 19. Obverse and reverse view of *Microsporum gypseum*

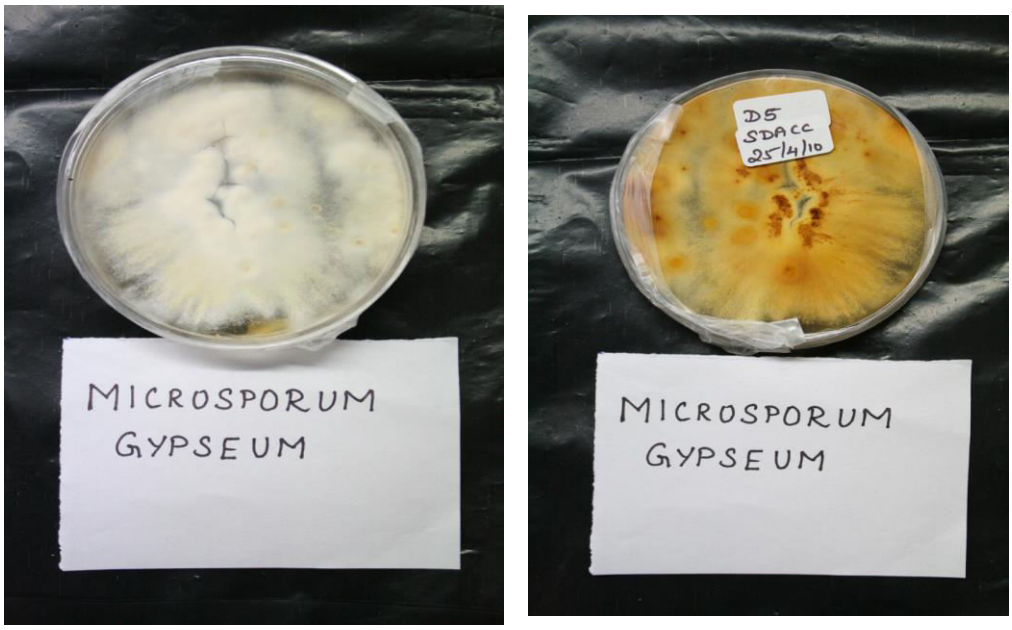


Fig 20. Lacto phenol cotton blue mount of *Microsporum gypseum* showing ellipsoidal macroconidia

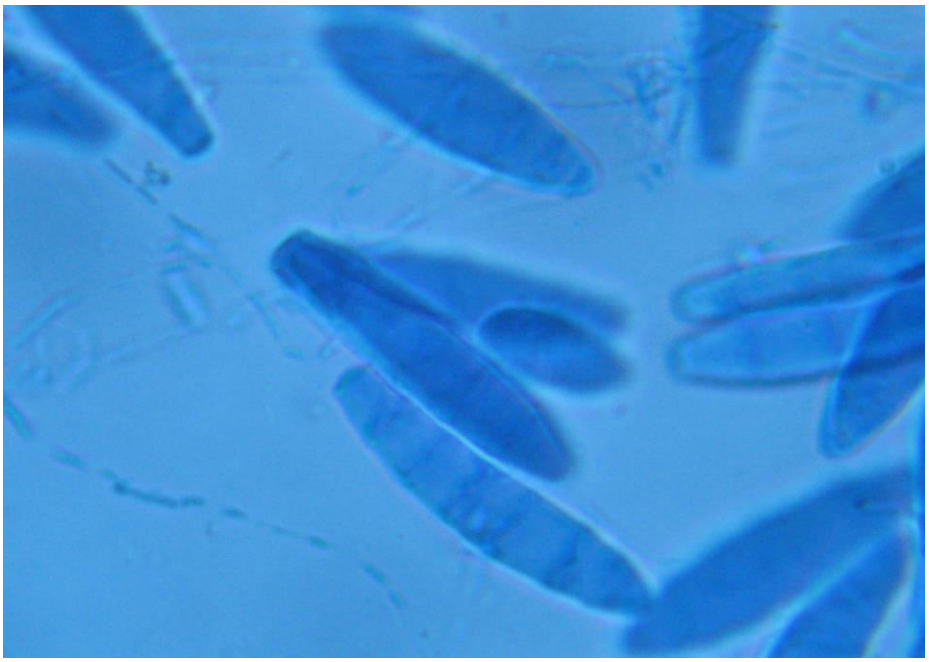


Fig 21. Obverse and reverse view of *Trichophyton ajelloi*

Fig 22. Obverse and reverse view of *Microsporum ferrugineum* and LPCB mount showing bamboo hyphae

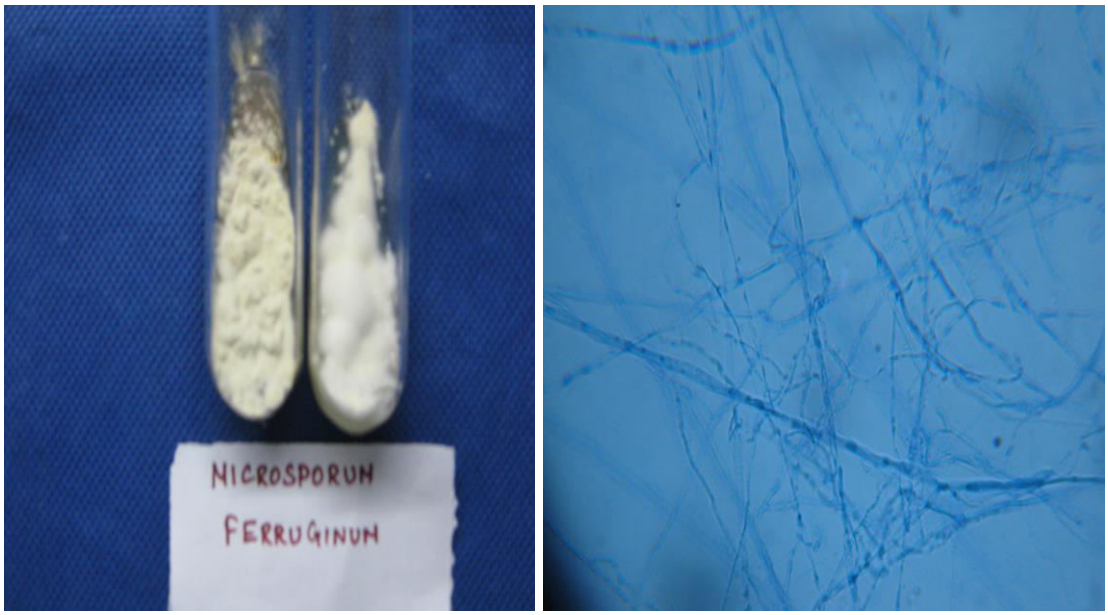
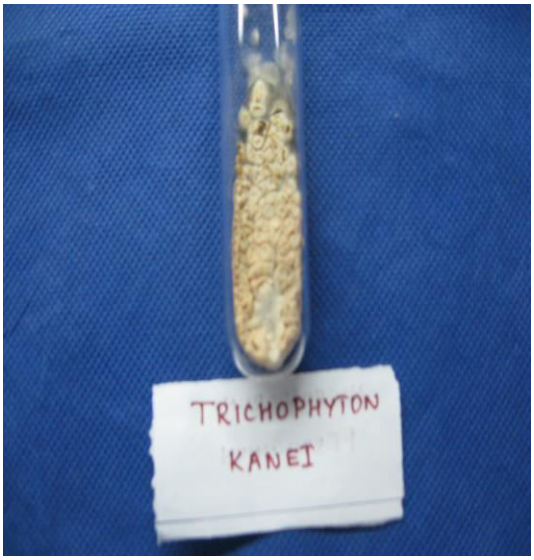
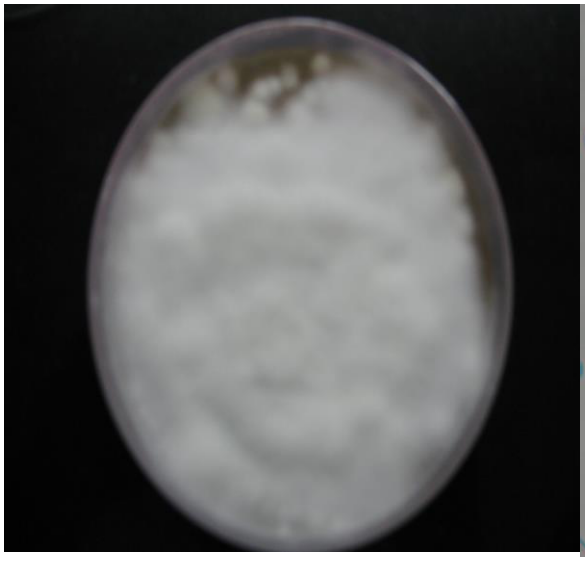


Fig 23. Culture of *Trichophyton kanei* & its LPCB mount



Breaking up into arthroconidia

Fig 24. Culture of *Trichophyton equinum* & its LPCB mount with clusters of microconidia



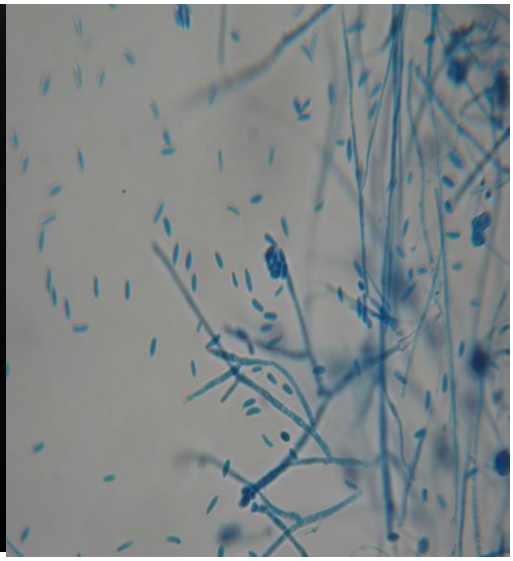


Fig 25. Species wise distribution of isolated dermatophytes

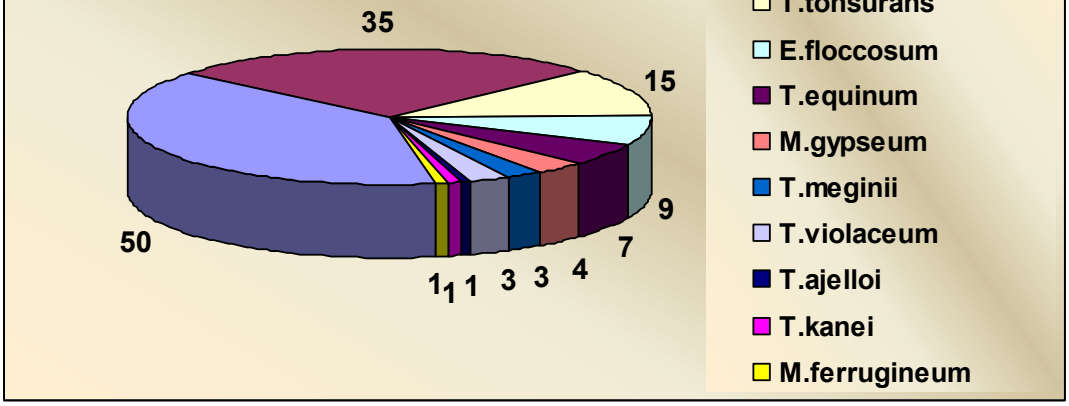


Fig 26. Causative agents of Tinea corporis

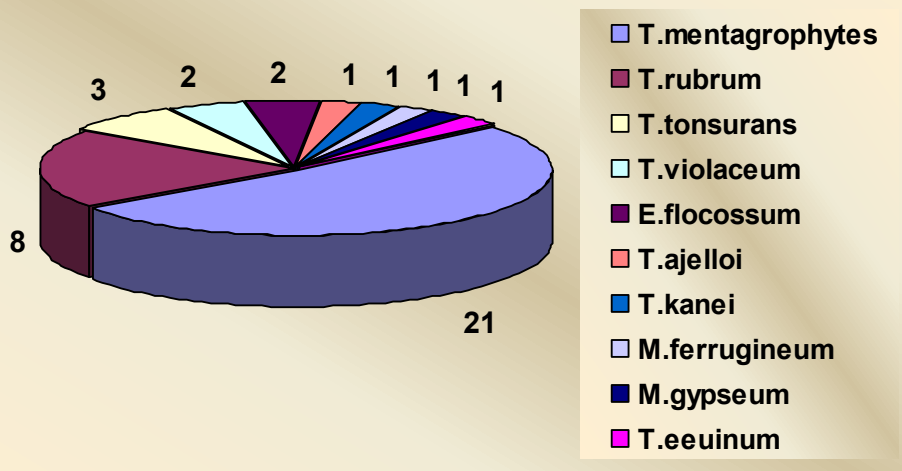


Fig 27. Causative agents of Tinea cruris

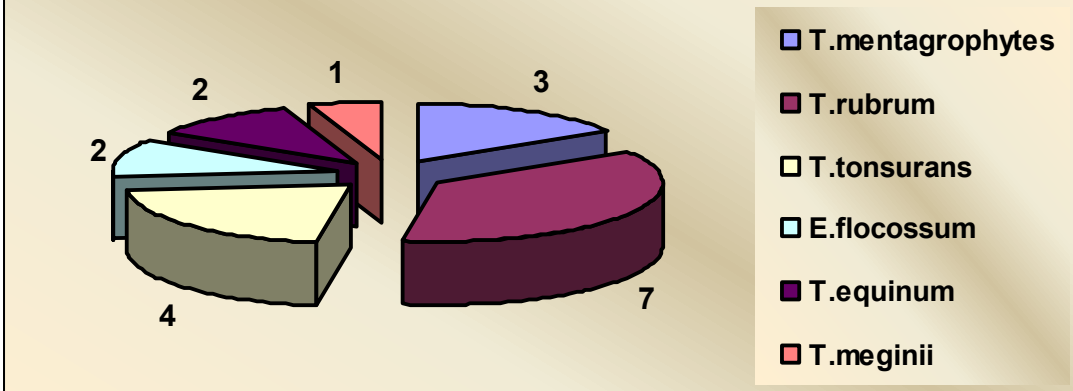


Fig 28. Dermatophytes isolated from Hair samples

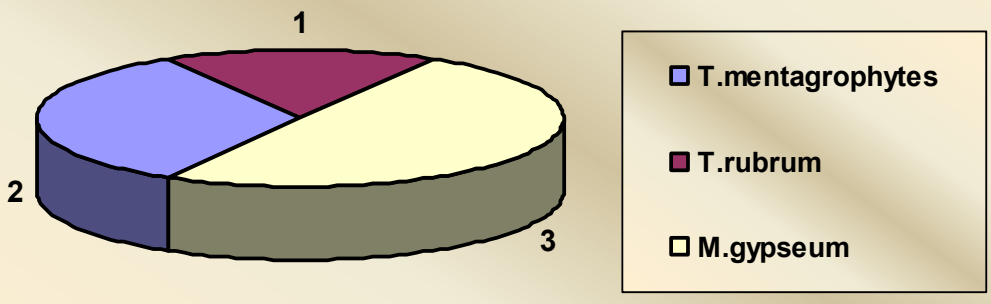


Table 11. Showing the causative agents of various dermatophytic infections isolated from our study

	Tinea pedis (12)	Tinea manum (7)	Tinea facium (3)	Tinea capitis (7)	Tinea unguinum (33)
T.mentagrophytes	5	3	2	1	13
T.rubrum	5	1	-	2	11
T.tonsurans	-	2	1	2	3
T.meginii	-	1	-	-	1
T.equinum	-	-	-	1	3
T.violaceum	-	-	-	1	-
E.floccosum	2	-	-	-	2

Table 12. Showing the MIC pattern of 129 isolates of dermatophytes obtained from the study

Drugs	Amphotericin B (16-0.0313 µg/ml)		Fluconazole (64- 0.125µg/ml)		Ketoconazole (16- 0.0313µg/ml)		Ciclopirox (32- 0.06µg/ml)		Terbinafine (0.5-0.001µg/ml)		Griseofulvin (16-0.0313µg/ml)	
Isolates	MIC range (µg/ml)	Mode	MIC range (µg/ml)	Mode	MIC range (µg/ml)	Mode	MIC range (µg/ml)	Mode	MIC range (µg/ml)	Mode	MIC range (µg/ml)	Mode
Trichophyton mentagrophytes	0.5 - 8	4	1 - 8	2	0.0313-1	0.5	0.125-2	1	0.001-0.008	0.004	0.25-0.5	0.5
Trichophyton rubrum	2 - 8	8	0.125 - 2	2	0.25 -1	0.5	0.125-2	1	0.001-0.008	0.004	0.25-0.5	0.5
Trichophyton tonsurans	1 - 8	4	1-4	2	0.313 - 0.25	0.25	1-2	1	0.001-0.004	0.004	0.125-0.5	0.125
Trichophyton equinum	4 - 8	4	2	2	0.25	0.25	0.5-1	1	0.004	0.004	0.25	0.25
Trichophyton meginii	4 - 8	4	0.25-0.5	0.5	0.25	0.25	2	2	0.004	0.004	0.25	0.25
Trichophyton ajelloi	4	4	1	1	1	1	0.5	0.5	0.002	0.002	0.5	0.5

	Amphotericin B		Fluconazole		Ketoconazole		Ciclopirox		Terbinafine		Griseofulvin	
	MIC range (µg/ml)	**Mode**	**MIC range (µg/ml)**	**Mode**	**MIC range (µg/ml)**	**Mode**	**MIC range (µg/ml)**	**Mode**	**MIC range (µg/ml)**	**Mode**	**MIC range (µg/ml)**	**Mode**
Trichophyton violaceum	**2 - 4**	**2**	**0.25-0.5**	**0.5**	**0.25-1**	**1**	**1**	**1**	**0.004**	**0.004**	**0.5**	**0.5**
Trichophyton kanei	**4**	**4**	**4**	**4**	**0.5**	**0.5**	**1**	**1**	**0.004**	**0.004**	**0.5**	**0.5**
Microsporum gypseum	**0.5 - 2**	**0.5**	**2-4**	**4**	**0.25-0.5**	**0.25**	**0.125-1**	**1**	**0.004**	**0.004**	**0.125-0.5**	**0.5**
Microsporum ferrugineum	**4**	**4**	**8**	**8**	**1**	**1**	**2**	**2**	**0.008**	**0.008**	**0.5**	**0.5**
Epidermophyton flocossum	**2 - 4**	**2**	**1-2**	**2**	**1-2**	**2**	**0.5-1**	**1**	**0.001-0.004**	**0.004**	**0.25-0.5**	**0.5**
***Trichophyton mentagrophytes* ATCC MYA- 4439**	**-**	**-**	**-**	**-**	**-**	**-**	**0.5-2**	**1**	**0.002 – 0.008**	**0.004**	**0.12-0.5**	**0.25**

Discussion

DISCUSSION:

Dermatophytes are pathogenic fungi of skin, hair and nail. These fungi utilizing the keratinous substrates belong to three genera, *Epidermatophyton, Microsporum* and *Trichophyton*. Although these fungi do not cause outbreaks or pandemics, the incidence of severe systemic fungal infections has increased significantly, mainly because of the explosive growth in patients with compromised immune system[11]. The reason for treatment failure is attributed to decrease in drug uptake, structural alterations in the target site, increase in drug efflux or in intracellular target levels[74]. Thus drug resistance in pathogenic fungi, including dermatophytes is gaining importance.

The present work is conducted to determine the clinical variants of dermatophytes in our region. About 300 samples were collected from the suspected cases of dermatophytic infections for our study. Out of which 147(49%) were positive for fungal growth. Dermatophytes were grown in 129 (43%) samples and the remaining 18 were non – dermatophytic fungi. Higher prevalence rates were observed in studies by Kannan et al (48.5%)[86] and Singh et al (44.61%)[2] Lower prevalence rates were also observed in studies by Rajesh et al 32.9%. [87] and Sharma et al (37.9%). Male to female positive ratio in our study was 2: 1.6 (64:52) which correlated with such similar study done in Calicut by Bindu et al (2.06:1) [88]. The reason for varied distribution of dermatophytes depends on several factors, such as life style, type of the population, migration of people, nature of geographical area and climatic conditions.

From a clinicomycological study done in Rajasthan, the prevalence rate is more in the age group between 31 – 40 years (23.33 %) [90]. In our study, higher prevalence (17.3%) of dermatophytosis was observed in the age group of 29 – 42 years. The higher incidence of fungal

infection in second to fourth decade could be due to greater physical activity and hence increased sweating.

Zoophilic species of dermatophytes show less inflammatory response in animals who are their normal hosts. The same when infect man is known to cause severe inflammatory response [3, 11]. The percentages of positive among those who have history of contact with animals were 60% in our study and the most common agent is *Trichophyton mentagrophytes*. Zoophilic dermatophytes were found in 421 (90.5%) patients in a prevalence study conducted in Bosnia. The most common zoophilic species being *Microsporum canis* (80.0%) followed by *Trichophyton mentagrophytes* 9.7%. [89]

Direct examination by microscopy is highly efficient screening technique as it allows the clinician to start treatment independent of the culture report. The percentage of samples positive by microscopy was 34.3%. Whereas, the rate of positivity by culture was 49% with 43% of them being dermatophytes and remaining 6% were nondermatophytic fungi. The present study shows that culture is more sensitive than microscopy in the diagnosis of dermatophytosis. Our study correlates with other studies by Singh et al where microscopy (40.76%) is proved to be less sensitive than culture (44.61%).[2]

Even though microscopy can be used as a screening procedure, culture is an essential tool to study different morphological characters of dermatophytes. The culture media used for the isolation of dermatophytes are Sabouraud's Dextrose Agar with Chloramphenicol and Cycloheximide and Dermatophyte Test Medium. The rate of positive culture by Sabouraud's Dextrose Agar with Chloramphenicol and Cycloheximide was 40.6% and by Dermatophyte Test Medium was 42%.This shows there is no statistically significant difference between the two medium for primary isolation from the clinical samples. Our results correlated well with the

study by Singh et al, where the two media were proved to be technically good with no statistical difference for the primary isolation [2].

Ringworm has diverse range of clinical manifestation in different areas of the body. The percentage of positive skin, nail and hair samples were 37.3%, 73.3% and 40% respectively. Many studies have found tinea corporis is the most common among dermatophytic infections [2, 86 –89.] Our study also proves that tinea corporis was the most common infection with the high positive rate (43.33%) followed by tinea cruris (24.44%). The most common isolate from the clinical samples in our study was *Trichophyton mentagrophytes* (38.75%) followed by *Trichophyton rubrum* (27.13%). But in most other studies the common isolate was *Trichophyton rubrum* followed by *Trichophyton mentagrophytes* [2, 86-89]. The reason for more prevalence of *Trichophyton mentagrophytes* in our region could be due to more labor class population with increased migration and climatic conditions.

Being the only one human pathogenic species, *Epidermophyton floccosum* is thought to have originated from tropics and subtropical areas [3]. It is a cosmopolitically distributed anthropophilic fungi that primarily causes tinea cruris. In our study *Epidermophyton floccosum* constituted 6.9% of the total dermatophytes. *Trichophyton tonsurans* is an anthropophilic species, which is worldwide in distribution causing tinea capitis and tinea corporis [11]. In our study 11.6% of the isolates were *Trichophyton tonsurans.* A zoophilic species which produces frequent infection in horses and few cases of skin and hair infections in man is *Trichophyton equinum*[91] which comprised of 5.4% of the total isolates.

Very few cases of tinea corporis and tinea capitis are reported in United States, Europe and Asia [3, 11] with *Trichophyton megninii as* the causative agent. In our present study 2.3% of the

isolates were *Trichophyton megninii*. *Trichophyton violaceum* which was isolated in 2.3% of our samples is an anthrophophilic species causing endemic infections in Europe, South America and Asia. [3, 11] *Trichophyton ajelloi* is a widely distributed geophilic fungi with notable incidence of human infections [3, 11] and was isolated from one skin lesion in our study. An anthrophophilic species reported by Summerbell et al in *Trichophyton kanei* [92] known to infect keratin tissues. Literature revealed no reports of this fungus in India while in our study one case of tinea corporis caused by *Trichophyton kanei* was noted.

Being one of the most commonly isolated geophilic species in causing human skin infections, *Microsporum gypseum* was isolated from 3.1% of our isolates. *Microsporum ferrugineum* is an anthropophilic species found in Asia, Africa, Europe and Russia [3, 11] reported to cause infections of skin and hair. One case of *Microsporum ferrugineum* was reported in our study.

In the last two decades the incidence of infections caused by dermatophytes and other fungi has increased considerably. The inadequate use of drugs contributes to the failure in eliminating the disease agent completely, encouraging growth of the most resistant strains. Clinically confirmed cases of drug resistant *Trichophyton rubrum* to terbinafine, azoles and griseofulvin were reported by Osborne et al [75] and Mukherjee et al [85]. Various other potential mechanisms of resistance were also proposed for other dermatophytic species [74].With an increasing variety of drugs available for the treatment of dermatophytoses, the need for a reference method for the testing of the antifungal susceptibilities of dermatophytes and to alert the emergence of resistance has become apparent. The antifungal susceptibility was done by broth dilution method with references to CLSI document M38-A2 [79].This document describes the detailed methodology to test antifungal susceptibility of various mycelia forms. The same has

been advocated to test dermatophytes also. The drugs evaluated by this method in this study are Amphotericin B, fluconazole, ketoconazole, ciclopirox, terbinafine and griseofulvin.

Trichophyton mentagrophytes ATCC MYA – 4439 is used as the control in performing the antifungal susceptibility testings. The MIC for the antifungal drugs tested for ATCC strain is as follows: Amphotericin B 0.0625 µg/ml, ketoconazole 0.25 µg/ml, fluconazole 0.125 µg/ml, ciclopirox 1 µg/ml, terbinafine 0.004 µg/ml, griseofulvin 0.25 µg/ml. The antifungal susceptibility testing for dermatophytes was done by Fernandez et al [10, 81], Ghannoum et al [84], Jessup et al [83] and Norris et al [80] with various antifungal drugs for different species of dermatophytes.

The results of antifungal susceptibility testing for eight different species(*Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton tonsurans, Trichophyton ajelloi, Trichopyton violaceum, Microsporum gypseum, Microsporum ferrugineum* and *Epidermophyton flocossum)* in our study correlated well with the results obtained by previous studies except for results of Amphotericin B. Higher MIC values were obtained for Amphotericin B in our study for Trichophyton mentagrophytes (0.5 – 8 µg/ml), and Trichophyton tonsurans (1 – 8 µg/ml). Whereas the studies by Fernandez et al [10,81] shows the MIC range for Amphotericin B in Trichophyton mentagrophytes and Trichophyton tonsurans as 0.125 - 1 µg/ml and 0.03 – 0.5 µg/ml respectively. However the patients were not treated with Amphotericin B to know the clinical outcome.

No previous studies were available for antifungal susceptibility of *Trichophyton equinum, Trichophyton meginii* and *Trichophyton kanei.* For *Trichophyton equinum* the MIC values were Amphotericin B 4 – 8 µg/ml, Fluconazole 2 µg/ml, Ketoconazole 0.25 µg/ml, Ciclopirox 0.5 -1µg/ml, Terbinafine 0.004µg/ml and Griseofulvin 0.25µg/ml. For *Trichophyton*

meginii the MIC values were Amphotericin B 4 – 8 µg/ml, Fluconazole 0.25 – 0.5µg/ml, Ketoconazole 0.25 µg/ml, Ciclopirox 2 µg/ml, Terbinafine 0.004µg/ml and Griseofulvin 0.25µg/ml**.** For *Trichophyton kanei* the MIC values were Amphotericin B 4µg/ml, Fluconazole 4 µg/ml, Ketoconazole 0.5 µg/ml, Ciclopirox 1 µg/ml, Terbinafine 0.004 µg/ml and Griseofulvin 0.5 µg/ml**.**

No resistant strains were isolated in our study with reference to the standard testing method. The differences in the MIC for some species when compared to the previous studies can be attributed to the sample size and lack of standardized MIC range as reference values. The management of dermatophytic infections needs personal hygiene, awareness of infection, proper diagnosis and appropriate medication.

Summary

SUMMARY:

1. A total of 300 patients with suspected dermatophytosis were studied.

2. Skin, nail and hair samples from the above patients were collected under sterile precautions.

3. Microscopic examination (KOH mount) of the samples from these patients showed the presence of hyphal elements in 103 (34.33%).

4. All the samples were simultaneously inoculated in Sabouraud dextrose agar(SDA) and Dermatophyte test medium(DTM). Fungal growth was observed in 147 (49%) of the samples with 129 (43%) of them being dermatophytes and remaining 18(6%) were non-dermatophytic fungi. There was no statistically significant difference between the two medium ($p< 0.01$) in primary isolation of dermatophytes.

5. Clinically 39 (43.33%) cases of tinea corporis, 22 (24.44%) of tinea cruris, 12 (13.33%) of tinea pedis, 7 (7.77%) of tinea manum, 7(7.77%) of tinea capitis, and 3 (3.33%) of tinea facium were positive for dermatophytes. Similarly, 33(11%) nail samples and 6 (2%) of hair samples also showed dermatophytic growth.

6. Trichophyton mentagrophtes (38.75%) was the predominant fungi isolated followed by Trichophyton rubrum (27.13%).

7. One case of tinea corporis caused by Trichophyton kanei, an anthrophophilic species was reported in our study. There were no reports of isolation of this fungus in India earlier.

8. All the dermatophytes isolated in our study were subjected to antifungal susceptibility testing by broth micro dilution method proposed by Clinical Laboratory Standard Institute (CLSI M-38 A2. 2008).

9. Trichophyton mentagrophytes ATCC MYA-4439 was used as control in our study.

10. The antifungal agents used in the study were Amphotericin B (Himedia), Fluconazole (Himedia), Ketoconazole (Himedia), Ciclopirox (Sigma Aldrich), Terbinafine (Sigma Aldrich) and Griseofulvin (Sigma Aldrich).

11. The MIC for *Trichophyton mentagrophytes* in our study were as follows: Amphotericin B 0.5 – 8 µg/ml, Fluconazole 1 – 8 µg/ml, Ketoconazole 0.0313-1µg/ml, Ciclopirox 0.125-2µg/ml, Terbinafine 0.001-0.008µg/ml and Griseofulvin 0.25-0.5µg/ml.

12. Incase of *Trichophyton rubrum*, the MIC values were: Amphotericin B 2 – 8 µg/ml, Fluconazole 0.125 – 2 µg/ml, Ketoconazole 0.25 -1µg/ml, Ciclopirox 0.125-2µg/ml, Terbinafine 0.001-0.008µg/ml and Griseofulvin 0.25-0.5µg/ml.

13. For *Trichophyton tonsurans* the MIC values were Amphotericin B 1 – 8 µg/ml, Fluconazole 1 – 4 µg/ml, Ketoconazole 0.313 - 0.25 µg/ml, Ciclopirox 1-2µg/ml, Terbinafine 0.001-0.004 µg/ml and Griseofulvin 0.125-0.5µg/ml.

14. For *Trichophyton ajelloi t*he MIC values were Amphotericin B 4 µg/ml, Fluconazole 1 µg/ml, Ketoconazole 1 µg/ml, Ciclopirox 0.5 µg/ml, Terbinafine 0.002 µg/ml and Griseofulvin 0.5µg/ml**.**

15. For *Trichophyton violaceum* the MIC values were Amphotericin B 2 - 4 µg/ml, Fluconazole 0.25 – 0.5 µg/ml, Ketoconazole 0.5 µg/ml, Ciclopirox 1 µg/ml, Terbinafine 0.004 µg/ml and Griseofulvin 0.25 – 1 µg/ml**.**

16. For *Trichophyton equinum t*he MIC values were Amphotericin B 4 – 8 µg/ml, Fluconazole 2 µg/ml, Ketoconazole 0.25 µg/ml, Ciclopirox 0.5 -1µg/ml, Terbinafine 0.004µg/ml and Griseofulvin 0.25µg/ml.

17. For *Trichophyton meginii t*he MIC values were Amphotericin B 4 – 8 µg/ml, Fluconazole 0.25 – 0.5µg/ml, Ketoconazole 0.25 µg/ml, Ciclopirox 2 µg/ml, Terbinafine 0.004µg/ml and Griseofulvin 0.25µg/ml**.**

18. For *Trichophyton kanei* the MIC values were Amphotericin B 4µg/ml, Fluconazole 4 µg/ml, Ketoconazole 0.5 µg/ml, Ciclopirox 1 µg/ml, Terbinafine 0.004 µg/ml and Griseofulvin 0.5 µg/ml**.**

19. For *Microsporum gypseum* the MIC values were Amphotericin B 0.5 -2 µg/ml, Fluconazole 2 - 4 µg/ml, Ketoconazole 0.25 - 0.5 µg/ml, Ciclopirox 0.125 - 1 µg/ml, Terbinafine 0.004 µg/ml and Griseofulvin 0.125 - 0.5 µg/ml**.**

20. For *Microsporum ferrugineum* the MIC values were Amphotericin B 4 µg/ml, Fluconazole 8 µg/ml, Ketoconazole 1 µg/ml, Ciclopirox 2 µg/ml, Terbinafine 0.5 µg/ml and Griseofulvin 0.008 µg/ml**.**

21. For *Epidermophyton flocossum* the MIC values were Amphotericin B 2 - 4 µg/ml, Fluconazole 1 - 2 µg/ml, Ketoconazole 1 - 2µg/ml, Ciclopirox 0.25 – 0.5µg/ml, Terbinafine 0.001 – 0.004µg/ml and Griseofulvin 0.5 - 1 µg/ml**.**

22. None of the isolates have showed abnormal MIC range (except for Amphotericin B) when compared to MIC of standard strain reported in literature. This suggests that all are susceptible to the antifungals used. However future studies are required to evaluate the effect of drugs upon clinical response.

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Appendix

APPENDIX:

1. **20% Potassium hydroxide and 40% Dimethyl ssulfoxide (KOH) mount**:

   Dimethyl sulfoxide (DMSO) - 40 ml.

   Sterile distilled water - 60 ml. 20 grams of Potassium hydroxide crystals are added to 40% DMSO and are mixed well to dissolve. Stored in a stopper bottle at room temperature.

2. **Preparation of Lactophenol cotton blue**:

   Phenol - 200 gm

   Cotton Blue - 0.5gm

   Glycerol - 400 ml

   Lactic Acid - 200 ml

   Distilled Water - 200 ml.

   Stored in a stopper bottle at room temperature.

3. **Preparation of culture media:**

   A) Sabouraud's Dextrose Agar (Himedia) with Chloramphenicol (Sigma Aldrich) and Cycloheximide (Sigma Aldrich)

   Compostion:

   Dextrose - 40 gm

   Neopeptone - 10 gm

   Agar - 20 gm

Distilled water - 1000 ml.

pH – 5.6 .

After autoclave add chloramphenicol (5 mg dissolved in 1 ml of 95% alcohol for every 100 ml of medium) and cycloheximide (50 mg dissolved in 1 ml of acetone for every 100 ml of the medium).Pour around 10 ml for each test tube. Pour around 15 ml for each plate.

B) Dermatophyte test medium (Himedia):

Base:

Peptic digest of Soyameal meal	- 10g/l
Glucose	- 10g/l
Phenol red	- 0.20 g/l
Agar	- 20 g/l
pH	- 5.5 ±0.2

Dissolve 20.1 grams of agar base in 500 ml of distilled water. Heat to dissolve and autoclave.

Dermatosupplement (per vial for 500 ml of medium)

Cycloheximide	- 250mg
Chlortetracycline	- 50 mg
Gentamicin	- 50 mg.

Rehydrate the contents of one vial in 5 ml of 50 % acetone. Mix well and add aseptically to 500 ml of sterilized and cooled base solution.Mix well and pour around 10 to 15 ml in sterile petriplate or in Mc Cartney bottle.

C) Potato dextrose agar (Himedia):

Potato infusion - 200 gms/l

Dextrose - 20 gms/l

Agar - 15 gms/l

Dissolve 39.0 grams of medium in 1000ml of distilled water. pH = 5.6 ± 0.2

Sterilize by autoclaving and pour about 10 ml per tube

D) Yeast Nitrogen base

Ingredients	Gms / Litre
Ammonium sulphate	5.00
L-Histidine hydrochloride	0.01
DL –Methionine	0.02
DL – Tryptophan	0.02
Biotin	0.000002
Calcium pantothenate	0.0004
Folic acid	0.000002
Inositol	0.002
Niacin	0.0004
p-Amino benzoic acid (PABA)	0.0002
Pyridoxine hydrochloride	0.0004
Riboflavin (Vitamin B2)	0.0002
Thiamine hydrochloride	0.0004
Boric acid	0.0005
Copper sulphate	0.00004
Potassium iodide	0.0001

Ferric chloride	0.0002
Manganese sulphate	0.0004
Sodium molybdate	0.0002
Zinc sulphate	0.0004
Monopotassium phosphate	1.000
Magnesium sulphate	0.500
Sodium chloride	0.100
Calcium chloride	0.100

Suspend 6.7 gms in 100 ml of distilled water and add 5 grams dextrose to it. The final pH is adjusted to be 5.4 ± 0.2.Final medium is sterilized by filtration.

E) Christensen's urea agar

Peptic digest of animal tissue - 1 gm

Dextrose - 1 gm.

Sodium chloride - 5 gms.

Disodium phosphate - 1.2 gms.

Monopotassium phosphate - 0.80 gm

Phenol red - 0.012 gm

Agar - 15 gms.

Distilled water - 1000 ml.

pH = 6.8 ± 0.2.Autoclaved at 10 lbs at 150° C for 20 minutes. Add sterile solution of 40 % urea and mix well. Pour in test tubes with a longer slant.

F) Bromocresol purple agar

STEP 1:

Agar - 7.5 gm

Distilled water - 100 ml.

Soak the agar in water for 15 minutes and autoclave at 121°C for 15 mts. Retain in water bath at 50°C until steps 2 and 3 get ready.

STEP 2:

Skim milk powder - 20 gm.

Bromocresol purple (1.6% stock solution in ethanol) - 0.5 ml

Distilled water - 250 ml.

Dissolve and autoclave at 10 lb/in^2 for 8 mts.

STEP 3:

D-glucose - 10 gm.

Distilled water - 500 ml.

Dissolve and autoclave at 10 lb/in^2 for 8 mts.

Mix all and adjust the pH to 6.6.Dispense aseptically in slants – 5ml per tube.

Uninoculated medium will be sky blue in colour.

4. RPMI medium: (HIMEDIA)

Constituent	**Grams/ L of water**	**Constituent**	**Grams/ L of water**
L-asparagine (freebase)	0.200	Biotin	0.0002
L-asparagine (anhydrous)	0.050	D-pantothenic	0.00025
L-aspartic acid	0.020	Choline chloride	0.003
L-cystine.2HCl	0.0652	Folic acid	0.001
L-glutamic acid	0.020	Myo-inositol	0.035
L-glutamine	0.300	Niacinamide	0.001
Glycin	0.010	PABA	0.001
L-histidine	0.015	Pyridoxine	0.001
L-hydroxyproline	0.020	Riboflavin	0.0002
L-isoleucine	0.050	Thiamine	0.001
L-leucin	0.050	Vitamin B12	0.000005
L-lysine	0.040	Calcium nitrate	0.100
L-methionine	0.015	Potassium chloride	0.400
L-phenylalanine	0.015	Magnesium sulfate	0.04884
L-poline	0.020	Sodium chloride	6.000
L-serine	0.030	Sodium phosphate	0.800
L-threonine	0.020	D-glucose	2.000
L-tryptophan	0.005	Glutathionine	0.001
L-tyrosine	0.02883	Phenol red	0.0053
L-valine	0.020		

5. Phosphate buffer saline:

Preparation of PBS (0.067M – M15) – pH - 7.0

SOLUTION I

Disodium hydrogen phosphate (anhydrous) - 9.47 gm

Distilled water - 1000ml

SOLUTION II

Potassium dihydrogen phosphate - 9.07 gm

Distilled water - 1000 ml

Mix 61.1 ml of solution I with 38.9 ml of solution II – pH – 7.0 just be for use. Sterilize by autoclaving. Store at 4°C.

9.6 grams of the RPMI 1640 powder is dissolved in 1000 ml of PBS and is filter sterilized.

6. Drug dilutions: <u>Amphotericin B</u>

Desired concrntration = 16 to 0.0313 µg/ml

Potency = 750µg/mg

Volume of DMSO = 1ml

Weight of the drug needed (mg) = $\frac{1\text{ml} \times 1600\mu\text{g/ml}}{750\mu\text{g/mg}}$

= 2.15 mg in 1 ml of DMSO

2.15 X 10 = 21.5 mg. Hence dissolve 21.5 mg of drug in 1 ml of DMSO – Master stock.100 µl of this Master stock is diluted with 900 µl of DMSO will give the final concentration as 2150 µg in 1 ml of DMSO – Junior stock.

Tubes	Tube 1	Tube 2	Tube3	Tube4	Tube5	Tube6	Tube7	Tube8	Tube9	Tube10
Source	Tube 0 = 1	Tube 1	Tube 1	Tube 1	Tube4	Tube4	Tube4	Tube7	Tube7	Tube7
Drug amount(ml)	-	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
RPMIsolvent(ml)	-	0.25	0.75	1.75	0.25	0.75	1.75	0.25	0.75	1.75
Intermediate conc (µg/ml)	1600	800	400	200	100	50	25	12.5	6.25	3.125
Add drugs from tubes of Row1(ml)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
RPMI (ml)	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Conc 1:5	32	16	8	4	2	1	0.5	0.25	0.125	0.0625

Add drugs from Row 2 to microtitre plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Add inoculum (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final conc 1:100 (µg/ml)	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313

Ketoconazole:

Desired conc = 16 to 0.0313 µg/ml

Potency = 1000µg/mg

Volume of DMSO = 1ml

Weight of the drug needed (mg) = $\frac{1\text{ml} \times 1600\mu\text{g/ml}}{1000\mu\text{g/mg}}$

= 1.6 mg in 1 ml of DMSO

For the convenience of measurement, we multiply the weight of the drug by 10.1.6 X 10 = 16 mg. Hence dissolve 16 mg of drug in 1 ml of DMSO – Master stock. 100 µl of this Master stock is diluted with 900 µl of DMSO will give the final concentration as 1600 µg in 1 ml of DMSO – Junior stock.

Tubes	Tube 1	Tube 2	Tube3	Tube4	Tube5	Tube6	Tube7	Tube8	Tube9	Tube10
Source	Tube 0 = 1	Tube 1	Tube 1	Tube 1	Tube4	Tube4	Tube4	Tube7	Tube7	Tube7
Drug amount(ml)	-	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
RPMI solvent (ml)	-	0.25	0.75	1.75	0.25	0.75	1.75	0.25	0.75	1.75
Intermediate conc (µg/ml)	1600	800	400	200	100	50	25	12.5	6.25	3.125
Add drugs from tubes of Row 1 (ml)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
RPMI (ml)	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Conc 1:5	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
Add drugs from Row 2 to microtitre plate (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Add inoculum (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final conc 1:100	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313

Ciclopirox:

Desired conc = 32 to 0.06 µg/ml

Potency = 970 µg/mg

Volume of DMSO = 1ml

Weight of the drug needed (mg) = $\frac{1\text{ml} \times 3200\mu\text{g/ml}}{970\mu\text{g/mg}}$

= 3.29 mg in 1 ml of DMSO = 3.3

For the convenience of measurement, we multiply the weight of the drug by 10.3.3 mg X 10 = 33 mg. Hence dissolve 33 mg of drug in 1 ml of DMSO – Master stock. 100 µl of this Master stock is diluted with 900 µl of DMSO will give the final concentration as 3300 µg in 1 ml of DMSO – Junior stock.

Tubes	Tube 1	Tube 2	Tube3	Tube4	Tube5	Tube6	Tube7	Tube8	Tube9	Tube10
Source	Tube 0 = 1	Tube 1	Tube 1	Tube 1	Tube4	Tube4	Tube4	Tube7	Tube7	Tube7
Drug amount(ml)	-	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
RPMI solvent (ml)	-	0.25	0.75	1.75	0.25	0.75	1.75	0.25	0.75	1.75
Intermediate conc (µg/ml)	3200	1600	800	400	200	100	50	25	12.5	6.25
Add drugs from tubes of Row 1 (ml)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
RPMI (ml)	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45

Conc 1:5	64	32	16	8	4	2	1	0.5	0.25	0.125
Add drugs from Row 2 to microtitreplate(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Add inoculum (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final conc 1:100 (µg/ml)	32	16	8	4	2	1	0.5	0.25	0.125	0.06

Terbinafine:

Desired conc = 0.512 to 0.001 µg/ml

Potency = 1000 µg/mg

Volume of DMSO = 1ml

Weight of the drug needed (mg) = $\frac{1\text{ml} \times 51.2\ \mu\text{g/ml}}{1000\mu\text{g/mg}}$

= 0.0512 mg in 1 ml of DMSO .For the convenience of measurement, we multiply the weight of the drug by 1000.0.0512 mg X 1000 = 51.2 mg. Hence dissolve 51.2 mg of drug in 1 ml of DMSO – Master stock. (TUBE 1).

10 µl of this Master stock is diluted with 90 µl of DMSO will give concentration as 0.512 mg in 100 µl of DMSO – Junior stock I. (TUBE 2). Again take 10 µl from this Junior Stock I to 990 µl of DMSO will give the final concentration as 0.0512 mg in 1 ml of DMSO. (TUBE 3).

Tubes	Tube 1	Tube 2	Tube3	Tube4	Tube5	Tube6	Tube7	Tube8	Tube9	Tube10
Source	Tube 0 = 1	Tube 1	Tube 1	Tube 1	Tube4	Tube4	Tube4	Tube7	Tube7	Tube7
Drug amount(ml)	-	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
RPMI solvent (ml)	-	0.25	0.75	1.75	0.25	0.75	1.75	0.25	0.75	1.75
Intermediate conc (µg/ml)	51.2	25.6	12.8	6.4	3.2	1.6	0.8	0.4	0.2	0.1
Add drugs from tubes of Row1 ml	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
RPMI (ml)	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Conc 1:5	1.024	0.512	0.256	0.128	0.064	0.032	0.016	0.008	0.004	0.002
Add drugs from Row 2 to microtitreplate(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Add inoculum (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final conc 1:100 (µg/ml)	0.152	0.256	0.128	0.064	0.032	0.016	0.008	0.004	0.002	0.001

Griseofulvin:

Desired conc = 64 to 0.125 µg/ml

Potency = 1000 µg/mg

Volume of DMSO = 1ml

Weight of the drug needed (mg) = $\frac{1\text{ml} \times 6400\mu\text{g/ml}}{1000\mu\text{g/mg}}$

= 6.4 mg in 1 ml of DMSO = 6.4.For the convenience of measurement, we multiply the weight of the drug by 10.6.4 mg X 10 = 64 mg. Hence dissolve 64 mg of drug in 1 ml of DMSO – Master stock. 100 µl of this Master stock is diluted with 900 µl of DMSO will give the final concentration as 6400 µg in 1 ml of DMSO – Junior stock.

Tubes	Tube 1	Tube 2	Tube3	Tube4	Tube5	Tube6	Tube7	Tube8	Tube9	Tube10
Source	Tube 0 = 1	Tube 1	Tube 1	Tube 1	Tube4	Tube4	Tube4	Tube7	Tube7	Tube7
Drug amount(ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
RPMI solvent (ml)	3.5	0.5	1.5	0.5	0.75	1.75	0.5	0.75	1.75	0.5
Intermediate conc (µg/ml)	640	320	160	80	40	20	10	5	2.5	1.25

Add drugs from tubes of Row 1 (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
RPMI (ml)	2	2	2	2	2	2	2	2	2	2
Conc 1:5	128	64	32	16	8	4	2	1	0.5	0.25
Add drugs from Row 2 to microtitreplate(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Add inoculum (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final conc 1:100 (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125

Fluconazole:

Desired conc = 64 to 0.125 µg/ml

Potency = 1000 µg/mg

Volume of water = 1ml

Weight of the drug needed (mg) = $\frac{1\text{ml} \times 6400\mu\text{g/ml}}{1000\mu\text{g/mg}}$

= 6.4 mg in 1 ml of water = 6.4.

For the convenience of measurement, we multiply the weight of the drug by 10.6.4 mg X 10 = 64 mg. Hence dissolve 64 mg of drug in 1 ml of water – Master stock.100 µl of this Master stock is diluted with 900 µl of water will give the final concentration as 6400 µg in 1 ml of water – Junior stock.

Tubes	Tube 1	Tube 2	Tube3	Tube4	Tube5	Tube6	Tube7	Tube8	Tube9	Tube10
Source	Tube 0 = 1	Tube 1	Tube 1	Tube 1	Tube4	Tube4	Tube4	Tube7	Tube7	Tube7
Drug amount(ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
RPMI solvent (ml)	3.5	0.5	1.5	0.5	0.75	1.75	0.5	0.75	1.75	0.5
Intermediate conc (µg/ml)	640	320	160	80	40	20	10	5	2.5	1.25
Add drugs from tubes of Row 1 (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
RPMI (ml)	2	2	2	2	2	2	2	2	2	2
Conc 1:5	128	64	32	16	8	4	2	1	0.5	0.25
Add drugs from Row 2 to microtitreplate(ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Add inoculum (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final conc 1:100 (µg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125

Annexure

PSG Institute of Medical Science and Research, Coimbatore

INFORMED CONSENT

I, Dr.Sowmya.N, Post graduate student, Department of Microbiology, of the PSG Institute of Medical Sciences & Research (PSG IMS&R), are carrying out a study titled "Isolation, Identification and In Vitro antifungal susceptibility of Dermatophytes from Clinical Samples" under the guidance of Dr.B.Appalaraju, Prof and HOD, Department of Microbiology, PSG IMS & R.

The objectives of this study are:

> Isolation, Identification, characterization and performing In vitro antifungal susceptibility of dermatophytes from three hundred samples from patients with ring worm infections attending the Dermatology out-patient department.

This goal of the study is: Isolate, Characterization and to study the prevalence of dermatophytes from clinical samples in Coimbatore and to find out the MIC values of antifungal drugs.

Sample size: Three hundred.

Samples to be collected: Infected skin / nail / hair

Benefits from this study, if any: Effectiveness of drugs given to the patients.

How the results will be used: The results of the study help to determine the emergence of resistant isolates.

We request you to kindly cooperate with us in this study. We propose collect background information and other relevant details related to this study.

If you are uncomfortable in answering any of our questions during the course of the interview / sample collection, you have the right to withdraw from the interview / study at anytime. You will NOT be paid any remuneration for the time you spend with us for this interview / study. The information provided by you will be kept in strict confidence. Under no circumstances shall we reveal the identity of the respondent or their families to anyone. The information that we collect shall be used for approved research purposes only.

Consent: The above information regarding the study, has been read by me/ read to me, and has been explained to me by the investigators from the PSG IMS&R. Having understood the same, I hereby give my consent to them to interview me. I affixing my signature / left thumb impression to indicate my consent and willingness to cooperate in this study.

Respondent Name: _____. Date:

Signature / Left thumb impression of the Respondent: Signature of the witness

ஒப்புதல் படிவம்

பி.எஸ்.ஜி. மருத்துவக் கல்லூரியின் நுண்ணுயிரியல் துறையினை சேர்ந்த மருத்துவர் சௌமியா, மருத்துவர் அப்பலராஜு அவர்களின் வழிநடத்துதலில், தோல் நோயினை உண்டாக்கும் காளான்கள் பற்றிய ஆராய்ச்சியினை மேற்கொள்ள போகின்றோம்.

நோக்கம்:

தோல் நோயினை உண்டாக்கும் காளான்கள் பற்றியும்,அதன் சிகிச்சைக்கு பயன்படுத்தப்படும் மருந்துகளின் சரியான பயன்பாட்டு அளவினையும் அறிவதே இந்த ஆராய்ச்சியின் நோக்கமாகும். இதற்காக 300 நோயாளிகளிடம் இருந்து மாதிரிகள் எடுக்கப்படும்.இதற்கான தங்களின் முழு ஒத்துழைப்பும் தருமாறு கேட்டு கொள்கிறேன.இந்த ஆராய்ச்சியில் தங்களின் பாதிக்கப்பட்ட தோல் / நகம் / முடிகள் மட்டுமே எடுக்கப்படும்.இந்த ஆராய்ச்சியில் ரத்த பரிசோதனையோ மருந்து உட்கொள்ளுதலோ இருக்காது என்று தெரிவித்து கொள்கின்றோம்.

ஆராய்ச்சியின் பயன்கள்:

தோல் நோயினை உண்டாக்கும் காளான்கள் எது என்பதனையும் ,அதனை எதிர்க்கும் மருந்துகளின் சரியான பயன்பாட்டு அளவும் சிகிச்சைக்கு பெரிதும் பயன்படும்.

இந்த ஆராய்ச்சியில் இருந்து எந்த நேரமும் விலகி கொள்ள தங்களுக்கு உரிமை உள்ளது.இதனால் தங்களின் சிகிச்சை முறையில் எந்த விதமான மாறுதலும் இருக்காது. இந்த ஆராய்ச்சிக்காக தாங்கள் எங்களுடன் செலவிடும் நேரத்திற்கு எந்தவித ஊதியமும் வழங்க பட மாட்டது. தங்களிடம் இருந்து பரப்படும் தகவல்கள் அனைத்தும் ரகசியமாக வைக்கப்படும்.

ஒப்புதல்:

மேற்கூறிய ஆராய்ச்சியின் பயன்கள் மற்றும் பாதகங்கள் எனக்கு விளக்கப்பட்டது.நானாக என் முழு சம்மதத்துடன் இந்த ஆராய்ச்சியில் பங்கு பெற ஒப்புதல் அளிக்கின்றேன்.என்னிடம் இருந்து பெறப்பட்ட விபரங்கள் இந்த ஆய்விற்கு பயன்படுத்த சம்மதம் தருகின்றேன்.இந்த ஆய்வு பற்றிய விபரங்கள் அறிவியல் சார்ந்த பத்திரிகைகள் மற்றும் கருத்தரங்குகளில் இடம் பெரும் போது எனது அடையாளம் இடம் பெறாது என்று உருதி அளிக்கப்பட்டுள்ளதை நன்கு அறிவேன்.

பெயர் :

தேதி / நேரம் :

கையொப்பம் :

சாட்சி கையொப்பம் :

PROFORMA

Consent Obtained -

Data Collection from the Volunteer

1. Name -

2. Age / Sex - Male ☐ Female ☐

3. Residential Address -

4. Occupation -

5. Contact Number -

6. Clinical history –

H/o Diabetes Yes ☐ No ☐

H/o Atopic Dermatitis **Yes** ☐ **No** ☐

H/o similar illness in the family – **Yes** ☐ **No** ☐

H/o contact with animals **Yes** ☐ **No** ☐

If yes, to which animal **Dog** ☐ **Cat** ☐ **Fowl** ☐

Horse ☐ **Sheep** ☐ **Cow** ☐

7. Clinical presentation:

Tinea Capitis ☐

Tinea Corporis ☐

Tinea Cruris ☐

Tinea pedis ☐

Tinea manum ☐

Tinea unguinum ☐

8.Signature of the investigator:

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : psgethics2005@yahoo.co.in

PROPOSAL NUMBER	:	**09/154**
PROJECT TITLE	:	**Isolation, Identification and in vitro antifungal Susceptibility of Dermatophytes from clinical sample.**
NAME OF THE INVESTIGATOR/S	:	**Dr N Sowmya**
NAME OF THE GUIDE	:	**Dr B Appalaraju**
WAIVER OF CONSENT	:	**No**
REVIEW TYPE	:	**Exempt**
DATE OF THE MEETING	:	**N/A**
DECISION	:	**Approved**
APPROVAL DATE	:	**27.08.2009**
VALIDITY OF THE APPROVAL	:	**One year**
CONTINUING PANEL REVIEW	:	**Not Needed**

Dr S Bhuvaneshwari
Secretary
Institutional Human Ethics Committee

SECRETARY
PSG IMS & R
COIMBATORE-641 004
INSTITUTIONAL HUMAN ETHICS COMMITTEE