Antifungal Susceptibility Testing of Dermatophytes

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Abstract

The cases of dermatophytoses have increased over the past few decades. In the last few years, a number of newer less toxic antifungal drugs have become available for clinical use. The increased use of antifungal, often for prolonged periods, has led to the recognition of the phenomenon of acquired antifungal resistance among previously susceptible strains or species and to the increased incidence of infections with less common species. Our study mainly focused on the in vitro susceptibility of clinical isolates of dermatophytes. The microbroth dilution method was performed according to CLSI standards. In the present study, antifungal susceptibility testing was done by microdilution method of dermatophytes against five antifungal agents namely, ketoconazole (imidazoles) fluconazole, itraconazole (triazoles), griseofulvin and terbinafine and their activity against significant number of strains, representing a wide spectrum of dermatophyte species is assessed. Dermatophytic strains: A total of 119 strains of dermatophytes belonging to 10 species were tested. They were *T. rubrum* ($n = 40$), *T. mentagrophytes* ($n = 19$), *T. violaceum* ($n = 15$), *M. gypseum* ($n = 12$), *E. flocossum* ($n = 9$), *M. audouinii* ($n = 8$), *T. schoenleinii* ($n = 5$), *M. canis* ($n = 5$), *T. tonsurans* ($n = 4$) and *T. verrucosum* ($n = 2$). The MIC ranges of all the 119 isolates of dermatophytes tested for antifungal susceptibility showed that terbinafine had the lowest MIC range 0.001–0.64 μg/ml followed by ketoconazole at a MIC range 0.01–3.84 μg/ml. The itraconazole showed a MIC range 0.082–20.45 μg/ml whereas the griseofulvin and fluconazole showed a highest MIC range 0.32–5.12 μg/ml. The MIC 50 of terbinafine was low at 0.02 μg/ml followed by ketaconazole 0.24 μg/ml. The MIC 50 of itraconazole and griseofulvin was 1.28 μg/ml .The highest MIC 50 with

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2.56 μg/ml was recorded for fluconazole. The MIC90 of terbinafine was low at 0.32 μg/ml followed by ketaconazole with 1.92 μg/ml. The MIC 90 itraconazole was 2.50 μg/ml and for griseofulvin it was 2.56 μg/ml. The highest MIC 90 of flucanozole was high at 10.24 μg/ml. In our study, we observed that terbinafine had the lowest MIC values compared to ketoconazole, itraconazole, griseofulvin and fluconazole.

10.1 Introduction

10.1.1 Historical Review

Historically, medical mycology, specifically relating to human disease, began with the discovery of the fungal etiology of favus and centered around three European physicians in the mid-nineteenth century: Robert Remak, Johann L. Schönlein, and David Gruby. According to Seeliger (1985), Remak (1842) first observed peculiar microscopic structures appearing as rods and buds in crusts from favic lesions. The real founder of dermatomycology was David Gruby on the basis of his discoveries during 1841–1844, his communications to the French Academy of Sciences, and his publications during this period. Independently, and unaware of the work of Remak and Schönlein, he described the causative agent of favus, both clinically and in microscopic details of the crusts, and established the contagious nature of the disease. He has described ectothrix invasion of the beard and scalp, naming the etiologic agent of the latter *Microsporum* (referring to the small spores around the hair shaft) *audouinii*, and described endothrix hair invasion by *Herpes* (*Trichophyton*) *tonsurans*. In addition to these observations on dermatophytes, he has also described the clinical and microscopic appearance of thrush in children.

Raymond Sabouraud (1970), one of the best known and most influential of the early medical mycologists, began his scientific studies of the dermatophytes around 1890, culminating in the publication of his classic volume, "Les Teignes", in 1910. Sabouraud's contributions included his studies on the taxonomy, morphology, and methods of culturing the dermatophytes and the therapy of the dermatophytoses. He classified the dermatophytes into four genera, *Achorion*, *Epidermophyton*, *Microsporum*, and *Trichophyton*, primarily on the basis of the clinical aspects of the disease, combined with cultural and microscopic observations. The medium that he has developed is in use till date for culturing fungi (although the ingredients are modified) and is named in his honor, Sabouraud glucose (dextrose) agar.

In 1934, Chester Emmons modernized the taxonomic scheme of Sabouraud and others and established the current classification of the dermatophytes on the basis of spore morphology and accessory organs. He eliminated the genus *Achorion* and recognized only the three genera *Microsporum*, *Trichophyton*, and *Epidermophyton* on the basis of mycological principles.

10.1.2 Medical Mycology

Medical mycology is the study of mycoses of man and their etiologic agents. Mycoses are the diseases caused by fungi. Among thousands of species of fungi that are known, less than 100 are pathogenic to man. In addition to these species which are generally recognized as pathogenic to man, it is firmly established that under unusual circumstances of abnormal susceptibility of patients or the traumatic implantation of the fungus, other fungi are capable of causing lesions and are known as opportunistic fungi. These circumstances may be:

(i) A debilitating condition of the host, as diabetes
(ii) A concurrent disease such as leukemia

(iii) Prolonged treatment with corticosteroids
(iv) Immunosuppressive drugs or an antibiotic treatment for long duration

Systemic and subcutaneous mycoses are caused by fungi which are essentially free-living dermatophytes in nature. The mycoses are not contagious and infection in man follows inhalation of spores or traumatic implantation of fungi. Certain fungi cause diseases and death in man, and these diseases vary from superficial skin infections to subcutaneous or generalized systemic deep mycoses. According to site of infection, the fungal diseases are classified into five types (Chander 2002):

10.1.2.1 Superficial Mycoses

The superficial (cutaneous) mycoses are usually confined to the outer layers of the skin, hair, and nails and do not invade living tissues. The fungi are called dermatophytes. Dermatophytes, or more properly keratinophilic fungi, produce extracellular enzymes (keratinases) which are capable of hydrolyzing keratin.

10.1.2.2 Subcutaneous Mycoses

These are chronic, localized infections of the skin and subcutaneous tissue following the traumatic implantation of the etiologic agent. The causative fungi are all soil saprophytes of regional epidemiology whose ability to adapt to the tissue environment and elicit disease is extremely variable.

10.1.2.3 Systemic Mycoses

These diseases involve the internal organs like the lungs and brain due to dissemination through blood. The causative fungi are usually dimorphic, which are endemic in some parts of America.

10.1.2.4 Opportunistic Mycoses

These diseases are caused by the nonpathogenic fungi or contaminants in persons whose immunological defense mechanisms are weakened by endogenous causes like cancer, leukemia, AIDS, or other exogenous causes like immunosuppressive therapy and aggressive use of drugs and corticosteroids.

10.1.2.5 Miscellaneous Mycoses

These are the diseases that include ones that could not be grouped under any of the above diseases.

10.2 Superficial Mycoses and Dermatophytes

The superficial mycoses are caused by dermatophytes which are included in *Deuteromycetes* depending upon the following features of *Deuteromycetes*:

1. Fungi have no demonstrable sexual reproductive cycle.
2. All fungi lacking sexual process are gathered in this group.
3. It includes many disease-producing fungi as dermatophytes.

Dermatophytes are fungi that can cause infections of the skin, hair, and nails due to their ability to utilize keratin. They colonize the keratin tissues and inflammation is caused by host response to metabolic by-products. These infections are known as ringworm or tinea, in association with the infected body part. Occasionally, the organisms do invade the subcutaneous tissues, resulting in kerion development. The organisms are transmitted by either direct contact with infected host (human or animal) or direct or indirect contact with infected exfoliated skin or hair in combs, hairbrushes, clothing, furniture, theater seats, caps, bed linens, towels, hotel rugs, and locker room floors.

Depending on the species, the organism may be viable in the environment for up to 15 months. There is an increased susceptibility to infection when there is a preexisting injury to the skin such as scars, burns, and wounds and during marching, high temperature, and humidity (Table 10.1).

The dermatophytes are included in three fungal genera, namely:

Table 10.1 Classification of dermatophytes

Anthropophilic	Zoophilic	Geophilic
Epidermophyton floccosum	*Microsporum canis* (cats, dogs, etc.)	*Microsporum gypseum*
Microsporum audouinii	*Microsporum equinum* (horses)	*Trichophyton ajelloi*
Microsporum ferrugineum	*Microsporum nanum* (pigs)	*Trichophyton terrestre*
Trichophyton concentricum	*Microsporum persicolor*(rodents)	
Trichophyton kanei	*Trichophyton equinum* (horses)	
Trichophyton megnini	*Trichophyton mentagrophytes*, granular (rodents, rabbits, hedgehogs, etc.)	
Trichophyton mentagrophytes (cottony and velvety)	*Trichophyton simii* (monkeys)	
Trichophyton raubitschekii	*Trichophyton verrucosum* (cattle)	
Trichophyton rubrum		
Trichophyton schoenleinii		
Trichophyton soudanense		
Trichophyton tonsurans		
Trichophyton violaceum		
Trichophyton yaoundei		

1. *Epidermophyton*: Produces only macroconidia, no microconidia. The macroconidia are abundant and born in clusters with smooth, thick walls and two to seven septa. This genus consists of two species, one of which is a pathogen.
2. *Microsporum*: Both microconidia and rough-walled macroconidia characterize *Microsporum* species. The macroconidia are abundant and spindle shaped or fusiform shaped with three to ten septa. There are 19 described species, but only nine are involved in human or animal infections.
3. *Trichophyton*: Macroconidia of *Trichophyton* species are smooth walled. They produce microconidia abundantly that are globose or pyriform and are born singly along the sides of hyphae or in the form of grape clusters. Macroconidia are rare and are elongated pencil-shaped structures. There are 22 species, most causing infections in humans or animals.

At the National Centre for Mycology, about 58 % of the dermatophyte species frequently isolated are *Trichophyton rubrum*, 27 % are *T. mentagrophytes*, 7 % are *T. verrucosum*, and 3 % are *T. tonsurans*. Infrequently isolated species (less than 1 %) are *Epidermophyton floccosum*, *Microsporum audouinii*, *M. canis*, *M. equinum*, *M. nanum*, *M. persicolor*, *Trichophyton equinum*, *T. kanei*, *T. raubitschekii*, and *T. violaceum*.

10.3 Dermatophytes

10.3.1 Distribution of Dermatophytes (Etiology and Ecology)

Epidermophyton floccosum is anthropophilic and worldwide in distribution. It infects the groin, body, epidemic athlete's foot, and occasionally nails but not hair. *M. audouinii* is also anthropophilic, worldwide in distribution, and rare except in Africa and Asia. It mostly infects the scalp and body, causing epidemic tinea capitis in prepubescent children, rarely spread by guinea pigs and dogs. *M. canis* is zoophilic and infects cats and dogs and less commonly monkeys, guinea pigs, horses, mice, cows, and rabbits. It is mostly worldwide in distribution, but less common in North America, the UK, and Scandinavia than the rest of the world. It infects the body in adults, scalp in children, and rarely nails. *M. equi*num is zoophilic, infects

horses, and is worldwide in distribution but rare in man. *M. ferrugineum* is anthropophilic and found in Asia, Africa, and Eastern Europe, but rare in the western hemisphere except Brazil. *M. gypseum* is geophilic and worldwide, but rare in North America and Europe and common in South America. It infects the feet, hand, body, scalp, and rarely nails and is usually acquired from soil but occasionally animals, including flies. *M. nanum* is zoophilic, present in pigs, and distributed worldwide but infects the scalp and body of man and shows ectothrix infection of hair. *M. persicolor* is zoophilic, with respect to bank vole and mice, but not found in soil. It is sporadic in Europe, especially the UK; there are reports of infection of the skin, not hair. *T. equinum* is zoophilic, seen in horses worldwide, and very rare in man. *T. kanei* is anthropophilic in the body and feet, while incidence in nails is very rare. *T. megnini* is anthropophilic and present in Spain, Portugal, and rarely Africa, while in the Mediterranean, it infects mostly the body, scalp, and beard and shows ectothrix infection. *T. mentagrophytes* is both anthropophilic and zoophilic – rodents and small and large mammals – worldwide, and found in soil and can infect the feet, body, nails, beard, scalp, hand, groin. The zoophilic ones are ectothrix; anthropophilic ones do not infect hair. *T. raubitschekii* is anthropophilic and seen in southern Asia and India. Mediterranean strain of which infects mostly the body and rarely the scalp. *T. rubrum* is anthropophilic, worldwide in distribution, seldom isolated from animals, and never found in soil. It infects the feet, nails, body, groin, and rarely scalp, is both endothrix and ectothrix, and is the most frequently isolated dermatophyte. *T. soudanense* is also anthropophilic; appears in Africa and occasionally North America, the UK, and Brazil; and infects the scalp and body primarily "shower sites." *T. terrestre* is geophilic, worldwide, and a nonpathogenic species. *T. tonsurans* is anthropophilic; is worldwide; infects the scalp, body primarily "shower sites," and occasionally nails; and is endothrix, and outbreaks are not rare. *T. verrucosum* is zoophilic, is worldwide seen in cattle and other domestic and wild animals, and infects the scalp, beard, body, and occasionally nails, and ectothrix hair infection is seen. *T. violaceum* is anthropophilic reported from areas seen in Near and Middle East, Eastern Europe, North Africa, occasionally Latin America, and the Mediterranean, imported to North America. Institutional outbreaks of *T. violaceum* have been reported in Western Europe. Infection is on the scalp, body primarily "shower sites", rarely feet, and nails. Incidence of endothrix infection is seen in most cases.

10.3.2 Laboratory Identification

Specimens are collected for laboratory identification of dermatophytes by scraping the skin from the margin of the lesion onto folded black paper. Hairs are plucked, not cut, from the edge of the lesion and are cut into short segments. Hairs that fluoresce under a Wood's lamp are chosen; if they do not fluoresce, choose broken or scaly ones. Nails are scraped or minced into small pieces. Nail scrapings are obtained from the nail bed or from infected areas after discarding outer layers. Each specimen is divided between at least two types of culture media. For direct examination, a small sample of the specimen is selected for direct microscopic examination and investigated for the presence of fungal elements. The specimen is mounted in a small amount of potassium hydroxide or calcofluor-white. The KOH slides are gently heated and allowed to clear for 30–60 min before examining on a light or phase contrast microscope. Calcofluor-white slides are examined on a fluorescent microscope. When present in the direct examination, dermatophytes appear as hyaline (nonpigmented), septated elements. Hyphae rounding up into arthroconidia are diagnostic of dermatophyte involvement. If arthroconidia are found absent, the elements could also be due to a non-dermatophyte agent of onychomycosis or a small segment of a contaminating organism. When hair is involved, the arthroconidia may be found on the periphery of the hair shaft (ectothrix) or within the shaft (endothrix). *Malassezia furfur* infections (tinea versicolor)

are diagnosed by the presence of spherical yeast cells with a single bud and a collar and short curved hyphal strands.

10.3.2.1 Culture Media

The following media are generally employed for culturing dermatophytes.

10.3.2.1.1 BCP

Bromocresol purple-milk solids-glucose agar is a differential media useful in the characterization of dermatophyte species. The growth pattern of restricted or profuse is determined by comparison to a tube of nutrient media such as SDA. Some species produce an alkaline reaction (change media to purple); others do not produce a pH change (leave the media a sky-blue color). Hydrolysis of the milk solids results in a zone of clearing around the colony.

10.3.2.1.2 PDA

Potato dextrose agar is used to observe pigment formation.

10.3.2.1.3 PYE

Phytone yeast extract agar is a nutritionally enriched media that supports luxurious growth of most fungi. It contains antibiotics to inhibit bacterial growth.

10.3.2.1.4 SDA

Sabouraud dextrose agar (Emmons modification) is a nonselective medium which supports the growth of most fungi. Species of the dermatophytes isolated during this study were identified on the basis of their growth characteristics.

10.3.2.2 Biochemical Tests

10.3.2.2.1 Slide Culturing

The slide culture is a method of examining the microscopic structures of a fungus. The organism is grown on a glass coverslip placed on a block of agar. When sufficient growth has occurred, the coverslip is placed on a drop of mounting media on a microscope slide and examined by phase contrast microscopy.

10.3.2.2.2 Scotch Tape Mount

The scotch tape mount is used for examining the microscopic structures of filamentous fungi. A piece of clear, transparent tape held with sterile forceps is touched on the surface of the colony. The tape is placed on a drop of mounting media on a slide, followed by another drop and a coverslip over it. The phase contrast microscope is used to examine the sample.

10.3.2.2.3 Hair Perforation

Many dermatophytes have the ability to degrade hair. The hair perforation test determines whether the organism simply erodes the hair shaft or produces an enzyme for penetration and invasion of the shaft resulting in perforating bodies or cones.

10.3.2.2.4 Rice Grain Test

Microsporum audouinii grows poorly on rice grains and produces a dark discoloration useful in differentiating it from atypical *M. canis* strains. The rice grains also enhance the production of macroconidia in some species.

10.3.2.2.5 Urease

Christensen's urea broth indicates the presence of the enzyme urease, which splits urea into ammonia, resulting in an alkaline environment. The phenol red indicator turns the media from a straw yellow to fuchsia at pH 8.4.

10.3.2.2.6 Vitamin Requirements

Certain species of dermatophytes have distinctive nutritional requirements that may be beneficial to differentiate from similar species. The agar base is vitamin free and various vitamins are added to the basal media. The growth on the vitamin-enriched media is compared to the vitamin-free media to determine enhancement, if any, of aerial mycelium.

10.3.3 Epidemiology

Dermatophytes are by far the most significant fungi because of their widespread involvement of population at large and their prevalence all

over the world. They are assuming greater significance both in developed and developing countries particularly due to the advent of immunosuppressive drugs and disease. Hot and humid climate in the tropical and subtropical countries like India makes dermatophytosis a very common superficial fungal skin infection.

The prevalence of dermatophytic infections are governed by environmental conditions, personal hygiene (Oyeka 1990), and individual susceptibility from place to place. The isolation of different dermatophytes also varies markedly from one ecological niche to another niche depending on their primary habitat (Aly 1994). Many saprophytic soil fungi are closely related to dermatophytes, sharing the ability to utilize the keratin as growth substrate, so it is believed that dermatophytes might have been evolved from these keratinophilic soil fungi. During the evolutionary process, they led to the development of epidemiological groups of anthropophilic, zoophilic, and geophilic species (Emmons et al. 1997). Some species of dermatophytes are endemic in certain parts of the world and have limited geographic distribution (Ajello 1968 on Epidemiologic profile of dermatophytosis in Stockholm, Sweden). Laboratory records comprising direct microscopy and culture results of 37,503 specimens from skin, hair, and nail scrapings collected from January 2005 through December 2009 were retrospectively analyzed in the mycology laboratory at Karolinska University Hospital. Onychomycosis had, over time, the highest overall prevalence of 14.1 %, followed by tinea pedis (4.4 %). *Trichophyton rubrum* was the predominant pathogen isolated from these cases (83.2 %), followed by *T. mentagrophytes* (7.4 %). In contrast, *T. violaceum* and *T. soudanense* accounted for 81.6 % of the isolates from patients with tinea capitis. Now *Trichophyton soudanense*, *T. gourvilii*, and *T. yaoundei* are geographically restricted to Central and West Africa (Singh and Beena 2003). *Microsporum ferrugineum* predominates in Japan and surroundings. *T. concentricum* is confined to the islands of South Pacific and in Central and South America; however, the increasing mobility of world population is disrupting several of these epidemiological patterns (Badillet 1991).

In recent times, *T. tonsurans* is replaced by *M. audouinii* as the principal causative agent of tinea capitis infections in the USA. This may be due to the mass migration of population from Mexico and other Latin American countries where the *T. tonsurans* was predominant. The most common etiological agents of dermatophytoses in the Western countries are *T. rubrum* and *M. canis*. *Microsporum distortum* is a rare case of tinea capitis in New Zealand and Australia. The prevalence of dermatophytoses varies in India. In 1900, Dr. Powell reported the first case of dermatophytoses from Assam, India. The commonest clinical types of dermatophytosis of man are tinea corporis (58.84 %), followed by tinea cruris (12.3 %), which concurs with reports from other parts of India (Kanwar et al. 2001). The incidence of tinea capitis was 6.92 %. Tinea capitis is less common in India than in other countries (Kaur 1970; Vasu 1966; Malik et al. 1978). This may be attributable to the use of hair oils (particularly mustard oil) which are customarily used by Indians and have been shown to have an inhibitory effect on dermatophytes in vitro (Hajini et al. 1970, Garg and Muller 1992). The reported incidence of tinea pedis varies from 26.4 % from Pune (Anand et al. 2001) to 0.4 % from Ahmedabad (Shah et al. 1975), and it is 11.53 % in our study.

The predominance of tinea pedis in Western countries could be because of the regular use of shoes and socks, predisposing to perspiration and maceration (Bhaskaran et al. 1977). *Trichophyton* species were more commonly isolated than *Epidermophyton* and *Microsporum*. *T. rubrum* is the main dermatophyte reported from India and other countries (Kanwar et al. 2001). Many other species of dermatophytes like *T. schoenleinii*, *T. tonsurans*, *T. verrucosum*, *T. ferrugineum*, *T. concentricum*, and *M. audouinii* are also isolated besides *T. rubrum*, *T. mentagrophytes*, *T. violaceum*, and *E. floccosum* (Varenker et al., 1991). *T. rubrum* has been found to be the main causative agent of tinea corporis, whereas tinea cruris is mainly caused by *E. floccosum* and tinea

capitis by *T. violaceum* (Kanwar et al. 2001). A higher incidence of dermatophytosis in males than in females has been reported both in India and abroad (Kanwar et al. 2001). Philpot suggested that males may be more vulnerable to infection due to higher exposures in the army, schools, and sporting activities and due to the type of shoes and socks they use (Philpot 1977). This is especially true for tinea cruris. Differences in the incidence of other clinical types were also observed in the present study, e.g., tinea corporis, tinea capitis, and tinea manuum are more common in males, while tinea pedis and tinea unguium are more common in females.

10.3.4 Immunology of Dermatophytes

Human infection is the result of a complex interplay of factors pertaining to the invading organism, the host, and the environment. This is best shown in human dermatophyte infections. Acute infections are usually short lived and easy to treat when the patient has good cell-mediated immunity, short-term antidermatophyte antibodies, and delayed hypersensitivity. In chronic infections, the infection is long term and resistant to therapy, and patients have poor in vitro assessed cell-mediated immunity and immediate hypersensitivity to fungal antigens. Antidermatophyte antibodies usually do not disappear quickly. The dermatophytes have the ability to invade keratinized tissue (skin, hair, and nails) but are usually restricted to the nonliving cornified layer of the epidermis because of their inability to penetrate viable tissue of an immunocompetent host. However, invasion does elicit as host response ranging from mild to severe. Acid proteinases, elastase, keratinases, and other proteinases reportedly act as virulence factors.

The development of cell-mediated immunity correlated with delayed hypersensitivity and an inflammatory response is associated with clinical cure, whereas the lack of or a defective cell-mediated immunity predisposes the host to chronic or recurrent dermatophyte infection. Chronic dermatophytosis is mostly caused by *Trichophyton rubrum*, and there is some evidence that mannan produced by this fungus suppresses or diminishes the inflammatory response. Dermatophyte colonization is characteristically limited to the dead keratinized tissue of the stratum corneum and results in either a mild or intense inflammatory reaction. Although the cornified layers of the skin lack a specific immune system to recognize this infection and rid itself of it, nevertheless, both humoral and cell-mediated reactions and specific and nonspecific host defense mechanisms respond and eventually eliminate the fungus, preventing invasion into the deeper viable tissue. This array of defense mechanisms thought to be active against dermatophytes consists of α2-macroglobulin keratinase inhibitor, unsaturated transferrin, epidermal desquamation, lymphocytes, macrophages, neutrophils, and mast cells. 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. Although the host develops a variety of antibodies to dermatophyte infection, i.e., immunoglobulin M (IgM), IgG, IgA, and IgE, they apparently do not help eliminate the infection since the highest level of antibodies is found in those patients with chronic infection. IgE, which mediates immediate hypersensitivity, appears to play no role in the defense process. Rather, the development of CMI which is correlated with DTH is usually associated with clinical cure and ridding the stratum corneum of the offending dermatophyte. In contrast, the lack of CMI or defective CMI prevents an effective response and predisposes the host to chronic or recurrent dermatophyte infections.

10.3.5 Treatment of Dermatophytosis

Topical antifungal preparations should be effective in treating small, uncomplicated tinea

infections located in areas other than the scalp. These include topical clotrimazole and iconazole (available over the counter) and terbinafine cream. The topical antifungal drugs such as ointments, lotions, powders, and sprays are used for tinea infections on the body. The creams are applied on affected parts twice or thrice daily for at least 1 month for better results. Powder and sprays are generally used for athlete's foot. Topical medications applied once or twice daily are the primary treatment indicated for tinea corporis/cruris and tinea pedis/manuum. In topical therapy, the improvement is more with allylamines than azoles.

Treatment of dermatophyte infection involves primarily oral and/or topical formulations of azoles or allylamines, particularly itraconazole and terbinafine. The use of oral antifungals may be practical where the tinea involvement is extensive or chronic or where application of a topical is not feasible. For tinea unguium (onychomycosis) and tinea capitis, oral therapies are the primary treatments provided. Recently, topical amorolfine and ciclopirox formulations have been approved for use in milder onychomycosis cases, and their role in the treatment of the different clinical forms of onychomycosis is currently being defined. Relapse of infection remains a problem, particularly with tinea pedis/unguium. Griseofulvin is the drug of choice for the treatment of tinea capitis till today, and azoles like imidazole and fluconazoles are used for the treatment of onychomycosis. But terbinafine is the effective antifungal drug for ringworm infections, which is less expensive, and the cure rate is more.

Appropriate follow-up duration and education of patients on proper foot hygiene are also important components in providing effective therapy. Sometimes, oral antifungal medication may be required if the condition is severe. Medications may include griseofulvin, itraconazole, terbinafine, and fluconazole. Tinea capitis (scalp), regardless of severity, is usually treated with oral antifungal medication, since topical antifungals do not penetrate hair follicles well. Corticosteroids may sometimes be used for the treatment of severely inflamed or potentially scarring lesions, such as scalp infections. Fungal infections involving the nails (onychomycosis) require oral treatment as well, because the dermatophyte is found deep in the nail. Tinea versicolor may be treated with selenium sulfide lotion or ketoconazole shampoo. Occasionally, the question arises as to whether a concurrent bacterial infection is complicating a fungal infection. This situation most commonly occurs when highly inflamed scalp lesions are draining purulent (pus) material. The lesions usually resolve with systemic (oral) antifungal and systemic corticosteroid therapy.

10.4 Antifungal Susceptibility Tests: (NCCLS Document M27-A, 1997; CLSI Standards M38-A, 2002)

In the last two decades, the incidence of infections caused by dermatophytes and other fungi has increased considerably (Weitzman and Summerbell 1995). 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 has become apparent (Ghannoum and Rice 1997). Establishment of a reference susceptibility testing method may allow the clinician to select the appropriate therapy for the treatment of infections caused by dermatophytic fungi. Recently, a standard method for antifungal susceptibility testing of yeasts has been established by the National Committee for Clinical Laboratory Standards (NCCLS M27-A document). This reference method for yeast is the first step in the establishment of a reliable, standardized, and clinically useful technique for the susceptibility testing of filamentous and dermatophytic fungi.

10.4.1 Culture Medium

Yeast nitrogen broth (YNB) supplemented with the following composition was used.

YNB base	6.7 g
Glucose	10.0 g
Distilled water pH 6.5	100 ml

This medium was filter sterilized and used as basal medium. It was diluted to 1:10 sterile (autoclaved) distilled water just before use.

10.4.2 Antifungal Agents: Antifungal Drugs

Antifungal drugs were donated as follows: ketoconazole by Janssen Pharmaceuticals, fluconazole by Hydex Chemicals Pvt. Ltd., terbinafine named "Terbicip" produced by Cipla Ltd., and griseofulvin (also known as *Grisovin*, a proprietary name of Glaxo Laboratories). Itraconazole was used in its commercial formulation (Sri Pharmacare, IndiaMART). All drugs were dissolved in 100 % dimethyl sulfoxide (Gibco) following the protocol of NCCLS and were prepared in stock solutions of 1000 μg/ml, and fluconazole was prepared in sterile distilled water and kept at -20^0 C until use. They were subsequently prepared as stock solution, and serial twofold dilutions were performed. Final concentrations ranged from 0.125 to 64 μg/mL for fluconazole; 0.03–16 μg/mL for ketoconazole, itraconazole, and terbinafine; and 0.03–8 μg/mL for griseofulvin.

10.4.3 Preparation of Inoculum

Testing was performed by a broth macrodilution method following the recommendation of the NCCLS M27-A (1997). In brief, stock inocula of dermatophytic stains were prepared from 7- to 14-day cultures grown on Sabouraud dextrose agar (SDA) with chloramphenicol. After the appearance of the sufficient growth, the fungal colonies were covered with 5 ml of sterile saline (0.9 %), and the suspensions were made by gently probing the surface with the tip of a sterile Pasteur pipette. The resulting suspended mixture was withdrawn and transformed to a sterile tube. Heavy particles of the suspension, when present, were allowed to settle for 15 min at room temperature, and the upper homogenous suspension was used for further testing. The suspensions were mixed with a vortex mixer for 15 s and adjusted with sterile normal saline to match the opacity of 0.5 McFarland standard.

10.4.4 Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. A $BaSO_4$ 0.5 McFarland standard may be prepared as follows: a 0.5-ml aliquot of 0.048 mol/L $BaCl_2$ (1.175 % w/v $BaCl_2 . 2H_2O$) is added to 99.5 ml of 0.18 mol/L H_2SO_4 (1 % v/v) with constant stirring to maintain a suspension.

1. The correct density of the turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.008–0.10 for the 0.5 McFarland standards.
2. The barium sulfate suspension should be transferred in 4–6-ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum.
3. These tubes should be tightly sealed and stored in the dark at room temperature.
4. The barium sulfate turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appear, the standard should be replaced. Latex particle suspensions should be mixed by inverting gently, not on a vortex mixer.
5. The barium sulfate standards should be replaced or their densities verified monthly.

The inoculum size was adjusted to between 1.0×10^6 and 5.0×10^6 spores/ml by

microscopic enumeration with a cell counting hemocytometer (Neubauer chamber). In some instance, where fungi do not readily produce conidia, a small portion of the mycelial growth was harvested and gently homogenized in 2 ml of sterile saline using Tenbroeck tissue grinder, and resulting suspensions were adjusted to opacity of 0.5 McFarland standard by adding sterile saline. Inoculum quantification was made by counting microconidia in a hemocytometer and by plating 0.01 ml of suspensions in SDA. The plates were incubated at 28 °C and were examined daily for the presence of fungal colonies before the test to check the viability of the fungus.

10.4.5 Method and Test Procedure

The NCCLS broth medium macrodilution method for yeasts which was modified for mold testing (NCCLS M27-A) was used for determination of the antifungal susceptibilities of dermatophytes. Twelve test tubes for each drug, i.e., fluconazole, itraconazole, ketoconazole, and griseofulvin, were arranged in a rack as per the requirement of different MIC ranges. An additional tube in the beginning was kept for griseofulvin and was later removed after antifungal dilutions were put up, such that the final range of the drug would be from 0.03 to 81.25 μg/ml. A set of 15 tubes were arranged for terbinafine MIC testing. All the tubes were arranged in ascending order with tube containing highest concentration on the left side. In addition, four control tubes were kept and labeled as C_1–C_4.

C_1	Sterility control (3 ml of YNB)
C_2	Positive control (2.7 ml of YNB + 0.3 ml of test inoculum)
C_3	Drug control (2.7 ml of YNB + 0.3 ML of drug stock solution)
C_4	Solvent control (2.7 ml of YNB + 0.3 ml of solvent used)

The stock solutions of antifungal agents were removed from the freezer and thawed to room temperature. The YNB was diluted 1 in 10 with distilled water under sterile conditions just before use. 2.7 ml of diluted YNB was dispensed in all the tubes. The stock solutions of antifungal drug tubes were vortex mixed for a few seconds to have a uniform suspension of the drug. 2.7 ml of the antifungal agent was pipetted and mixed in the first tube on the left, containing 2.7 ml of YNB. Serial twofold dilutions were made by pipetting 2.7 ml from the first tube to the second tube to the third tube and so on until the final tube (text figs. II–V). The final reservoir suspension was discarded. 0.3 ml of fungal inocula was added to the different drug dilutions and also into a positive (C_2) control tube for each test. The final dilution of the fungal inoculums was 1:10.

10.4.6 Incubation

Tubes in the rack were incubated at 35 °C in BOD incubator until growth appeared in the drug-free control tube. Incubation ranged 6–20 days. Control tubes were observed daily for the presence or absence of visible growth. When the growth was visible, each tube was vortexed for 10 s immediately prior to being scored, which allowed the detection of even a small amount of growth. The growth in each tube was compared with the growth of control tube (C2). Each tube was given a numerical score as follows:

O	Optically clear or the absence of growth
1	Slight turbidity compared to that of the fungus-free control tube (C_1)
2	Visible turbidity as compared to C_1
3	Clear-cut turbidity with or without formation of small hyphal fragments on the surface of the broth
4	Turbidity with the formation of a surface pellicle on the surface of the broth

The highest dilution of the drug, which inhibited the fungal growth, was taken as the MIC. MIC50 was calculated by taking the drug concentration, where 50 % of isolates are inhibited. Similarly, MIC90 was noted with drug concentration where 90 % of the isolates were inhibited.

10.4.7 Antifungal Susceptibility Test by CLSI Standards M38-A Method (Modified Method of NCCLS M27-A)

This is the modified method of NCCLS M27-A and was released as a document in 2002 as M38-A microdilution test. In this procedure, all the parameters are similar with macrodilution test, but instead of using tubes, the microtiter plate is used; hence, the size of inoculum also differs.

The samples from patients were collected in aseptic conditions from infected areas such as the skin, nail, and hair (Debono and Gordee 1994; Degreef 2008). Culturing of organisms from skin scraping was done on selective medium as Sabouraud dextrose agar for identification of dermatophytic species. For antifungal susceptibility testing, these species were used after identifying them on cultural, morphological, and biochemical characteristics (Favre et al. 2003). Five antifungal drugs were used for testing. The microbroth dilution method was performed according to CLSI standards M38-A (Fernández-Torres et al. 2002).

10.4.7.1 Culture Medium

Yeast nitrogen broth (YNB) supplemented with following composition was used:

YNB base 6.7 g
Glucose 10.0 g
Distilled water 100 ml and adjusting the pH at 6.5

This medium was filtered, sterilized, and used as basal medium (autoclaved). It was diluted to 1:10 with sterile distilled water just before use.

10.4.7.2 Antifungal Agents

Antifungal drugs used in this study were supplied from various firms, as follows: ketoconazole by Janssen Pharmaceuticals, fluconazole by Hydex Chemicals Pvt. Ltd., terbinafine named "Terbicip" produced by Cipla Ltd., and griseofulvin (also known as Grisovin, a proprietary name of Glaxo Laboratories). Itraconazole was used in its commercial formulation (Sri Pharmacare, IndiaMART). All drugs were dissolved in 100 % dimethyl sulfoxide (Gibco) following the protocol of CLSI and were prepared in stock solutions of 1000 μg/ml, and fluconazole was prepared in sterile distilled water and kept at −200 °C until used. They were subsequently prepared as stock solution and serial twofold dilutions were performed. Final concentrations ranged from 0.125 to 64 μg/mL for fluconazole; 0.03–16 μg/mL for ketoconazole, itraconazole, and terbinafine; and 0.03–8 μg/mL for griseofulvin.

10.4.7.3 Preparation of Inoculum

Testing was performed by a broth microdilution method following the recommendation of the CLSI M38-A. All the strains were obtained from the patient's samples of tinea infections. The species identification was based on morphological and biochemical characteristics and was used in inoculum preparation. In brief, stock inocula of dermatophytic stains were prepared from 7- to 14-day cultures grown on Sabouraud dextrose agar (SDA) with chloramphenicol. After the appearance of the sufficient growth, the fungal colonies were covered with 5 ml of sterile saline (0.9 %), and the suspensions were made by gently probing the surface with the tip of a sterile Pasteur pipette. The resulting suspended mixture was withdrawn and transformed to a sterile tube. Heavy particles of the suspension, when present, were allowed to settle for 15 min at room temperature, and the upper homogenous suspension was used for further testing. The suspensions were mixed with a vortex mixer for 15 s and adjusted with sterile normal saline to match the opacity of 0.5 McFarland standard.

10.4.7.4 Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for a susceptibility test, a BaSO4 turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. The inoculum size was

adjusted to between 1.0 × 106 and 5.0 × 106 spores/ml by microscopic enumeration with a cell counting hemocytometer (Neubauer chamber). In some instance, where fungi do not readily produce conidia, a small portion of the mycelial growth was harvested and gently homogenized in 2 ml of sterile saline using Tenbroeck tissue grinder, and resulting suspensions were adjusted to opacity of 0.5 McFarland standards by adding sterile saline. Inoculum quantification was made by counting microconidia in a hemocytometer and by plating 0.01 ml of suspensions in SDA. The plates were incubated at 28 °C and were examined daily for the presence of fungal colonies before the test to check the viability of the fungus.

10.4.7.5 Test Procedure

The tests were performed in polystyrene microtiter plates with flat bottom wells. By using a multichannel pipette, the aliquots of 100 µl of twofold drug dilutions were inoculated into the wells. Then, the microtiter plates were stored at −50 °C in a deep freezer until used. The microplate was inoculated with 100-µl fungal inoculum to maintain the dilutions with 0.5 × 104 to 5 × 104 spores ml-1. The plates were incubated at 28 °C for 7 days (Georgopapadakou and Tkacz 1995) for growth of the fungi. Growth and sterility control wells also maintained for each assay, and all the tests were performed in duplicate. The highest dilution of the drug, which inhibited the fungal growth, was taken as the MIC. MIC50 was calculated by taking the drug concentration, where 50 % of isolates are inhibited. Similarly, MIC90 was noted with drug concentration where 90 % of the isolates were inhibited. The MIC values were noted basing on the rate of growth inhibition.

10.4.7.6 Antifungal Susceptibility Investigations

The fungal infections are not completely cured with antifungal drugs. The treatment is less successful than that of bacterial infections because the fungal cells are eukaryotic and much more similar to human than the bacteria (Ghannoum and Rice 1999). Many drugs that inhibit or kill fungi are therefore quite toxic for humans also. Moreover, the fungal cells are equipped with a detoxifying system, which is able to modify many antibiotics, probably by hydroxylation (Gupta and Kohli 2003). Hence, the antibiotics used to treat the fungal infection will remain fungistatic for a period of time, and repeated usage of antibiotics is advised. The effective antifungal drugs may extract membrane sterols (da Silva Barros et al. 2007) or prevent their synthesis (Murray et al. 1999). Most antifungal compounds target the formation or the function of ergosterol, an important component of the fungal cell membrane (Nimura et al. 2001).

In the present study, a total of 119 strains of dermatophytes belonging to ten species were tested. All the strains were obtained from patient samples and were used in the tests. They were *T. rubrum* ($n = 40$), *T. mentagrophytes* ($n = 19$), *T. violaceum* ($n = 15$), *M. gypseum* ($n = 12$), *E. floccosum* ($n = 9$), *M. audouinii* ($n = 8$), *T. schoenleinii* ($n = 5$), *M. canis* ($n = 5$), *T. tonsurans* ($n = 4$), and *T. verrucosum* ($n = 2$).

10.4.7.7 Comparison of MICs of Five Antifungal Agents

The minimum inhibitory concentrations (MIC50, MIC90) of griseofulvin, ketoconazole, fluconazole, itraconazole, and terbinafine are compared, and the comparison of MIC values is used in determining the efficacy and the dosage of drug for the treatment of dermatophytosis.

10.5 Conclusion

In conclusion, it may be useful to undertake periodical screening programs to detect the antifungal susceptibility of newer antifungal agents. Our data on the antifungal susceptibility of dermatophyte isolates may contribute to a choice of antifungal treatment to ringworm infections. Terbinafine is considered as most potent drug followed by ketoconazole. But still the efficacy of ketoconazole drug was totally dependent upon the variation of causative dermatophytic strains

of particular tinea infections. We consider that our study on the antifungal susceptibility of dermatophytes can be beneficial for investigation of in vitro resistance of dermatophytic species and for management of cases clinically unresponsive to treatment.

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