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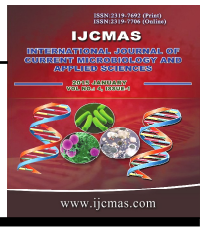

Original Research Article

Antifungal Susceptibility Testing of Dermatophytes by Agar Based Disk Diffusion Method

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A B S T R A C T

Keywords

Agar Based Disk Diffusion **(**ABDD), Dermatophytes, Antifungal sensitivity

Incidence of dermatophytosis is on increase especially in immunocompromised individuals. Resistance to antifungals have started coming up in dermatophytes. Methods are available for testing antifungal activity against dermatophytes but no simple reference method is available. We adopted agar based disk diffusion (ABDD) method to test the sensitivity of common dermatophytes against two azoles – Fluconazole, Itraconazole, Griseofulvin and Terbinafine. Six strains were found to be resistant to Fluconazole and 5 to terbinafine by the above method. The ABDD method appears to be simple, cost effective & promising.

Introduction

The incidence of dermatophytosis is increasing in recent times especially in geriatric & paediatric population (Ghannoum *et al.*, 2000) & in immuno compromised (Berg *et al.*, 2007; Nir-Paz *et al.*, 2003). Although *Trichophyton rubrum* among other dermatophytes is a major causative agent for superficial dermatophytosis (Johnson *et al.*, 2000), it is known to cause deep infections as well in immuno compromised patients (Nir-Paz *et al.*, 2003).

There are many antifungal agents that are used to treat dermatophytosis. However, not all species of dermatophytes have the same susceptibility pattern and relative or absolute resistance may occur (Fernandez *et al.*, 2002).

Evaluation of in vitro susceptibility testing had been hampered due to lack of reliable in vitro techniques for testing of antifungal agents against dermatophytes.

Various methods such as broth micro & macro dilutio, agar dilution, E test, sensititre, colorimetric micro dilution & disk diffusion have been available (Karaca *et al.*, 2004, Niewerth *et al.*, 1998; Pujol *et al.*, 2002). Clinical and Laboratory Standard Institute has approved a reference micro

dilution method for antifungal susceptibility testing of molds (CLSI, M-38 A, 2008) and its later modification (CLSI, M-38 A2, 2010) for dermatophytes as well. However, these dilution tests are difficult to be performed in routine laboratory.

A disk diffusion method to test yeasts has recently been standardised (NCCLS, 2004). The agar based disk diffusion (ABDD) susceptibility method for dermatophytes is quick, easy and a good option (Matar *et al.*, 2003). However data on disk diffusion methods for dermatophytes are scarce (Esteban *et al.*, 2005). The present study was therefore undertaken to determine in vitro activity of two azole derivatives (Fluconazole & Itraconazole), Griseofulvin & Terbinafine that are most commonly used to treat dermatophyte infection by ABDD against commonly isolated species of dermatophytes.

Materials and Methods

Fifty five clinical isolates of dermatophytes were tested along with *Trichophyton rubrum* ATCC 28188 and *Trichophyton mentagrophytes* ATCC 9533 as control. The ABDD was performed as described by Nweze *et al.* (2010). Dermatophytes were subcultured on Potato Dextrose Agar (PDA) & incubated at 28°C for 7 days to enhance sporulation.

The growth was harvested in sterile saline & the conidial and hyphal suspension was adjusted to $1x10^6$/ml using a haemocytometer. Plates of Mueller Hinton Agar (MHA) were inoculated using a swab dipped in the inoculums suspension. The inoculated plates were then dried before applying the disks.

Fluconazole (25μg) & Itraconazole (10 μg) disks were available commercially (HIMEDIA), Griseofulvin (10 μg) & Terbinafine (2μg/disk) were prepared in lab by dissolving the pure powders in DMSO & then diluting it to give a final concentration of 1mg/ml & 200 μg/ml for Griseofulvin & Terbinafine respectively & then delivering 10 μl onto each sterile disk.

Sterile disks were also impregnated with 10 μl of 1:100 dilution of DMSO to serve as control. The above 5 disks were applied to each inoculated & dried plate & then incubated at 28°C for up to 5 days .When growth took place, the size of zones of inhibition were measured for each antifungal agent (Pakshir *et al.*,2009).

Results and Discussion

A total of 55 strains of dermatophytes were tested for antifungal susceptibility by ABDD method. Isolates belonged to 2 genera and 6 species of dermatophytes as shown in table 1.

The zones of inhibition were seen in all the strains except 5 strains of *T. rubrum* without micro colonies within them (Fig. 1).

The zone of inhibition varied from 10-32 mm for Fluconazole, 17-36 mm for Itraconazole, nil-44 mm for Terbinafine and 21-49 mm for Griseofulvin with mean ± SD of 22.6 ± 4.2, 27.3 ± 6.2, 32.1 ± 6.1 and 35.9 ±4.9 respectively. No zone of inhibition was seen with disk containing DMSO against any of the species tested. The results of zone of inhibition for all the drugs for each group of fungi are summarised in table 2.

Successful treatment of fungal infections depends on the ability of a given antimycotic agent to eradicate the fungus from the tissue (Santos *et al.*, 2001).Though some in vitro antifungal susceptibility tests are now available (Fernández-Torres *et al.*,

2001; Karaca *et al.,* 2004; Santos *et al.,* 2001) including CLSI document regarding filamentous fungi (CLSI, 2008, 2010), no simple reference method has been standardised for testing the drug susceptibility of dermatophytes.

We tested 55 strains of dermatophytes against 4 commonly used antifungal agents viz. Fluconazole, Itraconazole, Terbinafine & Griseofulvin by disk diffusion method & the strength of each disk being 25μg, 10 μg, 2 μg &10 μg respectively.

The zone sizes varied from10–32 mm, 17–36 mm, 0–44 mm & 21–49 mm for Fluconazole, Itraconazole, Terbinafine & Griseofulvin with an average of 22.6 ± 4.2, 27.3 ± 6.2, 32.1 ± 6.1 & 35.9 ± 4.9 respectively.

No zone of inhibition was seen in 5 strains of *T. rubrum* against terbinafine. Perhaps these strains were intrinsically resistant to terbinafine. However, all these 5 strains were fully sensitive to other antifungal agents tested indicating that cross resistance to azoles & griseofulvin does not exists. Strains of *T. rubrum* showing primary resistance to terbinafine have also been reported by other workers also (Nweze *et al.,* 2010; Mukherjee *et al.,* 2002).

Disk strength & inhibition zone diameters (IZDs) are two very important variables. Variable IZDs have been reported by various workers employing different disk strength of antifungal agents. Pakshir *et al.* (2009) used terbinafine disks of 30 μg & griseofulvin of 25 μg & reported that IZD of more than 20 mm & 10 mm should be regarded as sensitive. On the other hand most of the workers have used a disk strength of 10 μg for griseofulvin & 1–2 μg for terbinafine (Venugopal *et al.,* 1995; Nweze *et al.,*2010) & have found much wider IZD usually >35 mm.

Diogo *et al.* (2010) have found IZD of >40 mm even with 0.125 μg disk of terbinafine. We also found IZD ranging from 30 to 44mm with 2 μg disk of terbinafine except in 5 strains of *T. rubrum* where IZD was nil & perhaps these strains were intrinsically resistant to terbinafine. Griseofulvin 10 μg/disk showed IZD ranging from 21-49 mm. Similarly Itraconazole with disk strength of 10 μg gave IZD of 17-36 mm. However, fluconazole with a disk strength of 25 μg gave IZD ranging from 10 mm to 32 mm. An IZD of <19 mm to fluconazole was found in some strains of, *T. mentagrophytes, T. rubrum, T. tonsurans & M. audonnii.* Fluconazole has been found to be least effective against dermatophytes by others also (Galuppi *et al.,* 2010; Barros *et al.,* 2007).

Another important variable that can affect the IZD is the type of inoculum preparation; many workers including CLSI guidelines recommend the use of microconidia (Nweze *et al.,* 2010; Barros *et al.,* 2007). It is known that microconidia of the tested species present higher susceptibility to antifungal drugs than hyphal preparations (Santos *et al.,* 2001) and it may be the reason for getting low MIC or very large IZD by several of them. However, we have used a mixture of hyphae &conidia & perhaps that is the reason for getting moderate IZD in the present study.

Inoculum size and incubation temperature may also affect the results of antifungal sensitivity testing. We have used an inoculum size of $1x10^6$ CFU/ml. However, Fernández-Torres *et al.* (2001) and Santos *et al.* (2001) have demonstrated that inoculum size does not affect the result.

Table.1 Number of dermatophytes tested

Fungi	No. of strains
Trichophyton mentagrophytes	23
T.rubrum	24
T.tonsurans	02
Microsporum audonii	01
M.gypseum	04
M.ferrugineum	01

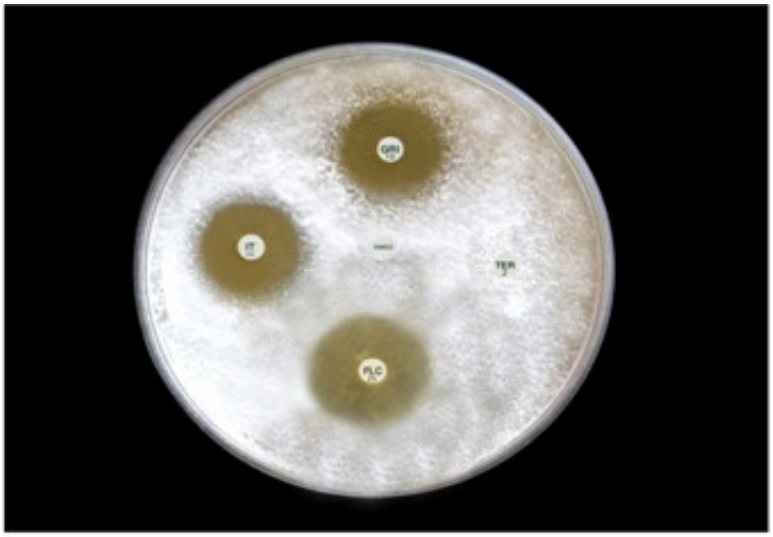
Table.2 IZDs obtained with different dermatophytes

Fungi	No.of strains	Drugs	Range	Arithmetic Mean
T.mentagrophytes	23	ITC FLU TRB GRI	20-35 13-30 30-41 30-49	29.3 25 33.6 37.6
T.rubrum	24	ITC FLU TRB GRI	17-33 10-32 Nil-44 32-43	24.7 20.9 28.5 35.5
T.tonsurans	02	ITC FLU TRB GRI	31-36 16-23 40-49 21-38	33.5 19.5 44.5 29.5
M.audonnii	01	ITC FLU TRB GRI	25 19 37 31	25 19 37 31
M.gypseum	04	ITC FLU TRB GRI	23-36 20-28 38-44 35-40	28.3 22 40.5 36.5
M.ferrugineum	01	ITC FLU TRB GRI	19 20 40 37	19 20 40 37

Table.3 Cut off values for IZDs for each of the four drugs

Drugs	Inhibition Zone Diameters			
	Mean ± SD	Sensitive Mean ± 1 SD	Intermediate Sensitive Mean-1 SD to Mean-2 SD	Resistant <Mean -2 SD
FLC	22.6±4.2	>19	14-19	<14
ITR	27.3±6.2	>21	15-21	<15
TER	32.1±6.1	>26	20-26	<20
GRI	35.9±4.9	>31	26-31	<26

Fig.1 ABDD of *Trichophyton rubrum* showing resistance to terbinafine



We have used an incubation temperature of 28°C but some workers have used a temperature of 35°C as it eliminates the need of second incubator (Norris *et al.*, 1999). Santos *et al.* (2001) have concluded that temperature alone (28°C or 35°C) does not significantly affect the results.

We have tried to classify the strains into sensitive, intermediate sensitive and resistant depending on mean and standard deviation of IZD for a particular antifungal. If the IZD was up to mean -1 SD, it was regarded as sensitive, if it was between mean -1 SD to mean-2 SD, it was regarded as intermediate sensitive and if the IZD was less than mean -2 SD, it was regarded as resistant.

Following the above criterion 6 strains were resistant to Fluconazole and 5 to Terbinafine and 5, 4 and 3 strains were found intermediate sensitive to FLC, ITR and GRI respectively.

The agar based disk diffusion (ABDD) susceptibility testing methods for dermatophytes is simple, inexpensive & does not require specialised equipment & can be adapted for routine assessment of dermatophyte resistance to antifungal agents. Further studies are necessary to properly standardize antimycotic sensitivity testing by disk diffusion method to make it useful & necessary procedure for selection of appropriate drug in a routine mycology laboratory.

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