Indian Journal of Medical Microbiology, (2015) 33(4): 533-537

Original Article

Determination of minimum inhibitory concentrations of itraconazole, terbinafine and ketoconazole against dermatophyte species by broth microdilution method

VK Bhatia, PC Sharma

Abstract

Purpose: Various antifungal agents both topical and systemic have been introduced into clinical practice for effectively treating dermatophytic conditions. Dermatophytosis is the infection of keratinised tissues caused by fungal species of genera *Trichophyton*, *Epidermophyton* and *Microsporum,* commonly known as dermatophytes affecting 20–25% of the world's population. The present study aims at determining the susceptibility patterns of dermatophyte species recovered from superficial mycoses of human patients in Himachal Pradesh to antifungal agents; itraconazole, terbinafine and ketoconazole. The study also aims at determining the minimum inhibitory concentrations (MICs) of these agents following the recommended protocol of Clinical and Laboratory Standards Institute (CLSI) (M38-A2). **Methodology:** A total of 53 isolates of dermatophytes (*T. mentagrophyte*-34 in no., *T. rubrum*-18 and *M. gypseum*-1) recovered from the superficial mycoses were examined. Broth microdilution method M38-A2 approved protocol of CLSI (2008) for filamentous fungi was followed for determining the susceptibility of dermatophyte species. **Results:** *T. mentagrophyte* isolates were found more susceptible to both itraconazole and ketoconazole as compared to terbinafine (MIC_{50}: 0.125 µg/ml for itraconazole, 0.0625 µg/ml for ketoconazole and 0.5 µg/ml for terbinafine). Three isolates of *T. mentagrophytes* (VBS-5, VBSo-3 and VBSo-73) and one isolate of *T. rubrum* (VBPo-9) had higher MIC values of itraconazole (1 µg/ml). Similarly, the higher MIC values of ketoconazole were observed in case of only three isolates of *T. mentagrophyte* (VBSo-30 = 2 µg/ml; VBSo-44, VBM-2 = 1 µg/ml). The comparative analysis of the three antifungal drugs based on *t*-test revealed that 'itraconazole and terbinafine' and 'terbinafine and ketoconazole' were found independent based on the $P < 0.005$ in case of *T. mentagrophyte* isolates. In case of *T. rubrum*, the similarity existed between MIC values of 'itraconazole and ketoconazole' and 'terbinafine and ketoconazole'. **Conclusion:** The MIC values observed in the present study based on standard protocol M38-A2 of CLSI 2008 might serve as reference for further studies covering large number of isolates from different geographic regions of the state. Such studies might reflect on the acquisition of drug resistance among isolates of dermatophyte species based on MIC values.

Key words: *Dermatophytes, itraconazole, ketoconazole, minimum inhibitory concentration, terbinafine*

Introduction

Dermatophytes are a specialised group of fungi, causing cutaneous infections of human and other vertebrates that are among the most prevalent cutaneous infections globally. These infections are commonly known as ring worm infections that are caused by species of genera *Trichophyton*, *Epidermophyton*, and *Microsporum*. Although, these infections are not life-threatening, they cause physical discomfort to the affected persons. An increasing frequency of dermatophytosis has been observed during last two decades especially in immunocompromised patients such as AIDS, diabetes mellitus, cancer and organ transplantation patients, etc. Dermatophytes are also associated with secondary bacterial infections leading to systemic skin infections.[1,2]

Some antifungal agents are commonly used to treat determatophytosis; among these, griseofulvin was the only approved systemic antifungal agent, initially. However, at present new agents both topical (clotrimazole [imidazoles], naftifine [allylamines], ciclopirox olamine [pyridine]) and systemic (Itraconazole and fluconazole [triazoles], ketoconazole [imidazoles], terbinafine [allylamines]) have been introduced into clinical practice during last 5–10 years for effectively treating dermatophytic conditions. Besides the availability of wide range of antimicrobial agents, the failure of treatment possibly due to resistance to the agent by dermatophyte implicated in mycoses has been reported by other workers.[3] Although the exact role of drug resistance in treatment failure is not clearly understood, all species of dermatophytes do not have the same pattern of susceptibility

*Corresponding author (email: <dr.sharmapc@gmail.com>)
Department of Microbiology (VKB, PCS), School of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan, Himachal Pradesh, India
Received: 19-09-2014
Accepted: 30-04-2015

Access this article online	
Quick Response Code:	Website: www.ijmm.org
	DOI: 10.4103/0255-0857.167341

to different antifungal agents. *In vitro*, antifungal susceptibility testing could therefore, prove helpful in the better management of the dermatophytosis because effective antifungal agents for the optimisation of antifungal therapy can be selected by this method by determining minimum inhibitory concentrations (MICs) of these agents. Broth macro- and micro-dilution methods, agar dilution and disc diffusion methods are routinely used for this purpose.[4,5] For determining MICs, Clinical and Laboratory Standards Institute (CLSI) approved protocol M38-A2 for filamentous fungi including dermatophytes has been recommended in its guidelines of 2008.[6]

The present study presents the susceptibility patterns of dermatophyte species recovered from superficial mycoses of human patients to itraconazole, terbinafine, and ketoconazole.

Methodology

Ethical statement

The research project SUIEC/12/04 was approved by the Institute Ethics Committee through its letter no. SUBMS/IEC/12/45, dated 19 March, 2012.

Dermatophyte species studied

We have previously isolated dermatophyte species recovered from superficial mycoses from human patients at Solan and Shimla regions of Himachal Pradesh.[1] In the present study of 53 of these isolates (*T. mentagrophyte*-34, *T. rubrum*-18 and *Microsporum gypseum*-1) have been tested for the susceptibility to itraconazole, terbinafine, and ketoconazole. The isolates were maintained in sterile distilled water and cultured on potato dextrose agar (PDA) medium at 30°C for 5–7 days before subjecting them to susceptibility testing. *Candida parapsilosis* strain ATCC-22019 and *Candida krusei* strain ATCC-6258 were included as reference strains in the test.

Antifungal agents

Three antifungal drugs itraconazole (Metro Chem API Pvt. CTD Erragadda, Hydrabad, India), ketoconazole (Aarti drugs Ltd., Thanne, Maharashtra, India) and terbinafine (Shreeji Pharma International, Sarabhi, Vadodara, Gujarat, India) in powdered form were used in the study.

Determination of antifungal susceptibility testing

Broth microdilution method

Broth microdilution method M38-A2 approved protocol of CLSI (2008) for filamentous fungi was followed for determining the susceptibility of dermatophyte species.

Drug dilutions

Stock dilutions of itraconazole, ketoconazole and terbinafine were prepared in dimethyl sulfoxide (HiMedia) according to the standard protocol. The two-fold dilutions of the stock solution were further prepared in RPMI 1640 medium with L-glutamine and without sodium bicarbonate (HiMedia). These dilutions were used in the test at a pH of 7.0 ± 0.1 with 3-(N-morpholino) propanesulfonic buffer (HiMedia) along with 1N NaOH. The concentrations of different dilutions of the antifungal drugs ranged from 0.0078 µg/ml to 128 µg/ml.

Preparation of inoculums of dermatophyte species

Cultures of dermatophyte species (7–8 days old) grown on PDA slants at 30°C were used to prepare inoculums. The fungal growth was covered with 5 ml of sterile normal saline and suspensions prepared by scraping the growth from the surface of the slants with a sterile swab that contained conidia and hyphal fragments. The heavy particles were allowed to settle down for 10–15 min. The upper clear suspension was transferred to fresh tube, and its optical density was set equal to 0.5 McFarland standards. The final cell density was set between 2×10^3 and 6×10^3 colony forming units per ml. which was used in the assay.

Test procedure

Flat-bottomed, 96 well microtitre plates (Costar-3596) having 8 rows and 12 columns were used to perform the susceptibility test. Eight test organisms in a volume of 100 µl each was placed in the wells of 8 rows of the plates (one test organism in each row). The dilutions (100 µl) of the drugs were added in the each well of ten columns of the plate from left to right. The concentration of the drug was highest in the first column and decreases from left to right. The contents were incubated at 35°C for 4–5 days. The 11th and 12th columns contained inoculated positive controls and un-inoculated negative control respectively.

Quality control reference strains

Candida parapsilosis strain ATCC-22019 and *C. krusei* strain ATCC-6258 were used as quality control reference strains as approved by the CLSI and their susceptibilities to itraconazole, terbinafine and ketoconzole were also tested. The plate containing these strains was incubated at 28°C for 48 h, as recommended by CLSI.

Determination of minimum inhibitory concentration values

The MIC value of a drug is defined as the lowest antifungal concentration at which no growth is visible in the wells when detected visually (80–100% inhibition) [Figure 1]. These values for each drug were recorded.

Data analysis

The mean values, MIC range, MIC_{50} and MIC_{90} values were determined for all antifungal agents, used in the assay, as per the standard protocol. The statistical

analysis was done by *t*-test using IBM-SPSS 20 (IBM-International Business Machine. SPSS-Statistical Product and Service Solutions version 20. SPSS Graphical Tools for use with *IBMSPSS Statistics* and other SPSS products.) software in order to find the independence of the variables or whether they had similarity in their MIC values with $P < 0.005$.

Results

The MIC ranges, Minimum concentration that inhibited 50% of the isolates (MIC_{50}) and 90% of the isolates (MIC_{90}) and the mean MIC values of the each antifungal drug are shown in Table 1. In order to illustrate the determination of MIC values, a representative case demonstrating MIC of terbinafine against dermatophyte isolates is presented through Figure 1.

The MIC range, MIC_{50}, MIC_{90} and mean values of itraconazole against *T. mentagrophyte* were 0.0156–1 μg/ml, 0.125 μg/ml, 0.50 μg/ml and 0.2486 μg/ml respectively while the values were 0.0156–1 μg/ml, 0.625 μg/ml, 0.50 μg/ml and 0.1918 μg/ml in case of *T. rubrum* isolates. Higher MIC values of antifungal agents have been observed against few dermatophyte species. Three isolates of *T. mentagrophytes* (VBS-5, VBSo-3 and VBSo-73) and one isolate of *T. rubrum* (VBPo-9) had higher MIC values of itraconazole (1 μg/ml).

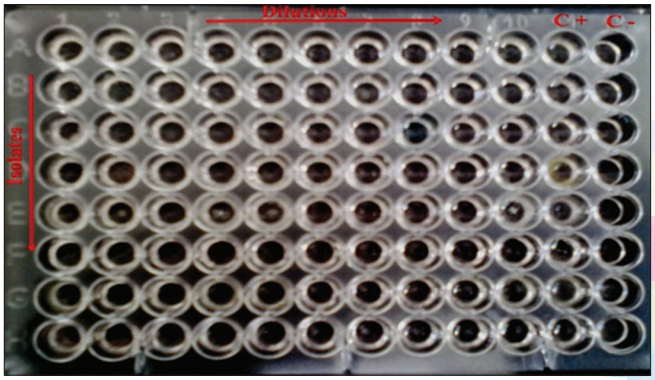

Figure 1: Determination of minimum inhibitory concentrations (MICs) of terbinafine by micro-broth dilution method against different dermatophyte isolates VBSo-44, VBSo-30, VBS-17, VBS-18 (MIC = 1 μg/ml) VBP-24 (MIC = 0.25 μg/ml), VBP-37 (MIC = 1 μg/ml), VBPo-13 (0.125 μg/ml), VBPo-15 (MIC = 0.5 μg/ml) (A to H rows respectively). The wells 1–10 containing the isolates were treated with two fold dilutions of terbinafine starting from a concentration of 4–0.0156 μg/ml. Eleventh column does not contain terbinafine (abbreviated as C+) and 12th column is blank (contains medium only, abbreviated as C−)

The MIC range, MIC_{50}, MIC_{90} and mean values of terbinafine against *T. mentagrophyte* were 0.0625–4 μg/ml, 0.50 μg/ml, 2.0 μg/ml and 0.9319 μg/ml respectively while these values were 0.0313–1 μg/ml, 0.50 μg/ml, 0.2 μg/ml and 0.7378 μg/ml against *T. rubrum* isolates. As shown in Table 1, higher MIC values of terbinafine were also observed in eight *T. mentagrophyte* isolates (VBS-1 = 4 μg/ml; VBS-3, VBSo-5, VBSo-17, VBSo-18, VBSo-39, VBSo-50, M-2 = 2 μg/ml) and two *T. rubrum* isolates (VBSo-22 = 2 μg/ml, VBPo-9 = 4 μg/ml).

The MIC range, MIC_{50}, MIC_{90} and mean values of ketoconazole against *T. mentagrophyte* were 0.0156–2 μg/ml, 0.0625 μg/ml, 0.50 μg/ml and 0.2642 μg/ml while these values were 0.0156–0.5 μg/ml, 0.125 μg/ml, 0.50 μg/ml and 0.1954 μg/ml against *T. rubrum* isolates. Similarly, the higher MIC values of ketoconazole were observed only in the three isolates of *T. mentagrophyte* (VBSo-30 = 2 μg/ml; VBSo-44, VBM-2 = 1 μg/ml).

Among the 34 *T. mentagrophyte* isolates examined in this study, 22.52% (8/34) exhibited MIC values of 0.0625 μg/ml of itraconazole, 20.5% (7/34) isolates had 1 and 2 μg/ml each of terbinafine and 29.41% (10/34) isolates had MIC values of 0.125 μg/ml of ketoconazole. In case of *T. rubrum*, 33.33% (6/18) isolates exhibited MIC values of 0.0625 μg/ml of itraconazole, 22.22% (4/18) isolates, each of 0.125, 0.5,1 μg/ml of terbinafine and 33.33% (6/18) isolates had MICs of 0.125 μg/ml of ketoconazole.

Only one isolate of *M. gypseum* (VBM-32) was tested for its susceptibility against antifungal drugs. The MIC values of this isolates were 0.125 μg/ml, 2.0 μg/ml

Table 1: Determination of MIC values of antifungal drugs against dermatophyte species (broth microdilution method)

Dermatophyte species	MIC values	Concentration (in μg/ml)		
		Itraconazole	Terbinafine	Ketoconazole
Trichophyton rubrum (18)*	MIC range	0.0156-1	0.0313-4	0.0156-0.5
	MIC_{50}	0.0625	0.50	0.125
	MIC_{90}	0.50	2.0	0.50
	Mean MIC value	0.1918	0.7378	0.1954
Trichophyton mentagrophyte (34)*	MIC range	0.0156-1	0.0625-4	0.0156-2
	MIC_{50}	0.125	0.50	0.0625
	MIC_{90}	0.50	2.0	0.50
	Mean MIC value	0.2486	0.9319	0.2642
Microsporum gypseum (1)*	MIC	0.125	2.0	0.0625

*Number of isolates tested. MIC: Minimum inhibitory concentration, MIC_{50} and MIC_{90}: MIC inhibiting 50% and 90% of isolates

and 0.0625 μg/ml against itraconazole, terbinafine and ketoconazole respectively.

Discussion

Dermatophytosis is the most common superficial mycoses in humans and domestic animals.[7] A number of antifungal agents have been introduced for treating this condition and more are underway.[8] Different dermatophyte strains have different antifungal susceptibility patterns. Strains of dermatophyte resistant to particular antifungal agent have been reported.[9] The introduction of wide range of new antifungal agents and the recovery of clinical isolates exhibiting resistance to antifungal agents such as amphotericin B, azole group etc., makes testing of the susceptibility of dermatophytes to these agents more important particularly for surveillance of resistant strains, in epidemiological studies. It plays an important role in detecting resistant strains that might help clinicians for better management of the disease caused by them by selecting appropriate therapeutic options for checking further spread.

Prior to CLSI guidelines of 2008, there was no definitive system to determine the susceptibility of dermatophytes to different antifungal agents. Due to the lack of suitable and effective methods of determining the *in vitro* antifungal susceptibility and the MICs of the antifungal drugs against dermatophytosis, it is not possible to ensure effective treatment. A number of techniques have been used for this purpose, e.g., disk diffusion method, broth macro and microdilution method, colorimetric microdilution method, E-test etc.[10-13] Some researchers followed the protocol M38-A of CLSI 2002 for determining the susceptibility of dermatophytes that was intended for filamentous fungi.[14] Later, the document was modified to M38-A2 by CLSI in 2008. This document also includes the protocol for dermatophytes which has been followed by us for determining the MIC values of itraconazole, terbinafine and ketoconazole against different dermatophyte species. The unavailability of such reference method previously was due to the difficulty in the standardisation of some parameters such as temperature, incubation time, selection of growth medium etc., for different species of dermatophytes.[15] In the present study, we incubated *T. rubrum, T. mentagrophyte* and *M. gypseum* at 35°C as mentioned in the M38-A2 protocol. Some researchers have obtained better growth of dermatophyte species at 28°C.[16-18] For determining the MICs of itraconazole, terbinafine and ketoconazole, the cultures of *T. mentagrophyte* and *M. gypseum* were incubated for 4 days and *T. rubrum* for 5 days as good growth was observed after incubation of specified period. Good inhibitory activity of all the three antifungal agents against *T. mentagrophyte*, *T. rubrum* and *M. gypseum* was demonstrated in the present study [Table 1]. Itraconazole and ketoconazole had the lower mean MIC values as compared to terbinafine. This suggests more effectiveness of both the drugs as compared to terbinafine. Low MIC values for these antifungal agents have also been reported by others.[9,19]

Trichophyton mentagrophyte isolates were found more susceptible to both itraconazole and ketoconazole as compared to terbinafine since lower MIC_{50} values of these drugs against *T. mentagrophyte* (itraconazole-0.125 μg/ml and ketoconazole-0.0625 μg/ml) were observed whereas this value was recorded at 0.5 μg/ml for terbinafine. The values are comparable to those reported by others in respect of *T. rubrum* and *T. mentagrophyte*.[20,21] As no significant difference of MIC_{50} values among *T. rubrum* and *T. mentagrophyte* isolates was observed by us, both the species exhibited similar susceptibility to terbinafine also. The MIC_{50} and MIC_{90} values of this drug were recorded at 0.5 μg/ml and 2 μg/ml respectively. These values are higher than those reported by other workers.[14,22] These results may be linked to the observations of Gupta *et al*., 1998 and Roberts in 1997 who reported that the oral drug formulations of terbinafine and itraconazole were required in more extensive and severe fungal infections.[23,24] Ketoconazole was found most effective against a single isolate of *M. gypseum* tested in the study as compared to other two antifungal agents as reflected by the MIC values. In order to obtain a better picture of susceptibility pattern of *M. gypseum*, a large number of isolates of this species need to be analysed before making definitive conclusion.

Comparative analysis of the effectiveness of the three antifungal drugs based on *t*-test revealed that 'itraconazole and terbinafine' and 'terbinafine and ketoconazole' were found independent based on the $P < 0.005$ in case of *T. mentagrophyte* isolates. However, the similarity existed between MIC values of 'itraconazole and ketoconazole' against *T. mentagrophyte* as the $P > 0.005$ was recorded in this case. In case of *T. rubrum*, the MIC values of 'itraconazole and terbinafine' were found independent on the $P < 0.005$. However, the similarity existed between MIC values of 'itraconazole and ketoconazole' and 'terbinafine and ketoconazole'. The MIC_{50} and MIC_{90} observed in the present study based on standard protocol M38-A2 of CLSI 2008 might serve as reference for further studies covering large number of isolates from different geographic regions of the state. Also, such studies might reflect on the acquisition of drug resistance among isolates of dermatophyte species based on MIC values.

Acknowledgments

The authors express their gratitude to Professor P.K. Khosla, Vice Chancellor of Shoolini University for providing necessary facilities to carry out this piece of work. Sincere thanks to Professor and Head Dr. Anil Kanga, Department of Microbiology at Indira Gandhi Medical College, Shimla and Dr. Vinay Patyal, Skin Specialist, Regional Hospital, Solan, Himachal Pradesh for providing skin samples and technical guidance.

References

1. Bhatia VK, Sharma PC. Epidemiological studies on dermatophytosis in human patients in Himachal Pradesh, India. Springerplus 2014;3:134.
2. Gong JQ, Liu XQ, Xu HB, Zeng XS, Chen W, Li XF. Deep dermatophytosis caused by *Trichophyton rubrum*: Report of two cases. Mycoses 2007;50:102-8.
3. Vandeputte P, Ferrari S, Coste AT. Antifungal resistance and new strategies to control fungal infections. Int J Microbiol 2012;2012:713687.
4. Bueno JG, Martinez C, Zapata B, Sanclemente G, Gallego M, Mesa AC. *In vitro* activity of fluconazole, itraconazole, voriconazole and terbinafine against fungi causing onychomycosis. Clin Exp Dermatol 2010;35:658-63.
5. Nweze EI, Ogbonna CC, Okafor JI. *In vitro* susceptibility testing of dermatophytes isolated from pediatric cases in Nigeria against five antifungals. Rev Inst Med Trop Sao Paulo 2007;49:293-5.
6. CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard – Document M38-A2. 2nd ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
7. Chinelli PA, Sofiatti Ade A, Nunes RS, Martins JE. Dermatophyte agents in the city of São Paulo, from 1992 to 2002. Rev Inst Med Trop Sao Paulo 2003;45:259-63.
8. Chadeganipour M, Nilipour S, Havaei A. *In vitro* evaluation of griseofulvin against clinical isolates of dermatophytes from Isfahan. Mycoses 2004;47:503-7.
9. Fernández-Torres B, Carrillo AJ, Martín E, Del Palacio A, Moore MK, Valverde A, *et al. In vitro* activities of 10 antifungal drugs against 508 dermatophyte strains. Antimicrob Agents Chemother 2001;45:2524-8.
10. Karaca N, Koç AN. *In vitro* susceptibility testing of dermatophytes: Comparison of disk diffusion and reference broth dilution methods. Diagn Microbiol Infect Dis 2004;48:259-64.
11. Perea S, Fothergill AW, Sutton DA, Rinaldi MG. Comparison of *in vitro* activities of voriconazole and five established antifungal agents against different species of dermatophytes using a broth macrodilution method. J Clin Microbiol 2001;39:385-8.
12. Santos DA, Hamdan JS. Evaluation of broth microdilution antifungal susceptibility testing conditions for *Trichophyton rubrum*. J Clin Microbiol 2005;43:1917-20.
13. Fernández-Torres B, Carrillo-Muñoz A, Ortoneda M, Pujol I, Pastor FJ, Guarro J. Interlaboratory evaluation of the Etest® for antifungal susceptibility testing of dermatophytes. Med Mycol 2003;41:125-30.
14. Mota CR, Miranda KC, Lemos Jde A, Costa CR, Hasimoto e Souza LK, Passos XS, *et al.* Comparison of *in vitro* activity of five antifungal agents against dermatophytes, using the agar dilution and broth microdilution methods. Rev Soc Bras Med Trop 2009;42:250-4.
15. Jessup CJ, Warner J, Isham N, Hasan I, Ghannoum MA. Antifungal susceptibility testing of dermatophytes: Establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. J Clin Microbiol 2000;38:341-4.
16. Pujol I, Capilla J, Fernández-Torres B, Ortoneda M, Guarro J. Use of the sensititre colorimetric microdilution panel for antifungal susceptibility testing of dermatophytes. J Clin Microbiol 2002;40:2618-21.
17. da Silva Barros ME, de Assis Santos D, Hamdan JS. Evaluation of susceptibility of *Trichophyton mentagrophytes* and *Trichophyton rubrum* clinical isolates to antifungal drugs using a modified CLSI microdilution method (M38-A). J Med Microbiol 2007;56:514-8.
18. Araújo CR, Miranda KC, Fernandes Ode F, Soares AJ, Silva Mdo R. *In vitro* susceptibility testing of dermatophytes isolated in Goiania, Brazil, against five antifungal agents by broth microdilution method. Rev Inst Med Trop Sao Paulo 2009;51:9-12.
19. Ghannoum MA, Chaturvedi V, Espinel-Ingroff A, Pfaller MA, Rinaldi MG, Lee-Yang W, *et al.* Intra- and inter-laboratory study of a method for testing the antifungal susceptibilities of dermatophytes. J Clin Microbiol 2004;42:2977-9.
20. Adimi P, Hashemi SJ, Mahmoudi M, Mirhendi H, Shidfar MR, Emmami M, *et al. In-vitro* activity of 10 antifungal agents against 320 dermatophyte strains using microdilution method in Tehran. Iran J Pharm Res 2013;12:537-45.
21. Barros ME, Santos Dde A, Hamdan JS. *In vitro* methods for antifungal susceptibility testing of *Trichophyton* spp. Mycol Res 2006;110:1355-60.
22. Favre B, Hofbauer B, Hildering KS, Ryder NS. Comparison of *in vitro* activities of 17 antifungal drugs against a panel of 20 dermatophytes by using a microdilution assay. J Clin Microbiol 2003;41:4817-9.
23. Gupta AK, Einarson TR, Summerbell RC, Shear NH. An overview of topical antifungal therapy in dermatomycoses. A North American perspective. Drugs 1998;55:645-74.
24. Roberts DT. Oral terbinafine (Lamisil) in the treatment of fungal infections of the skin and nails. Dermatology 1997;194 Suppl 1:37-9.

How to cite this article: Bhatia VK, Sharma PC. Determination of minimum inhibitory concentrations of itraconazole, terbinafine and ketoconazole against dermatophyte species by broth microdilution method. Indian J Med Microbiol 2015;33:533-7.

Source of Support: Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan. **Conflict of Interest:** None declared.