Journal of Pharma Research
Available online through
www.jprinfo.com

Research Article
ISSN: 2319-5622

Antifungal Susceptibility Testing of Dermatophytes from all Tinea cases, by Broth Micro Dilution Method

Jha B. K.*, Murthy S. M., Mathur M., Gupta B. P.
Department of Microbiology, Center for PhD studies, Yubraja College, Mysore, India.

Received on: 01-04-2013; Revised and Accepted on: 09-04-2013

ABSTRACT

Dermatophytosis is a common fungal disease difficult to so we plan to evaluate the antifungal activities of five antifungal drugs like Terbinafine (0.03-0.5), Itraconazole (0.03-0.25), Ketoconazole (0.03-4), Fluconazole (4-64) and Griseofulvin (0.24-1) µg/ml were used to evaluate MIC against (n=132) dermatophytes isolates. The isolates belongs to three genera and eight species as T.mentagrophytes 52 (39.4%), T.rubrum 30(22.7%), T.violacium 18(13.6%), T.verrucosum 11(8.3%), E.floccosum 10(7.6%), M.canis 6(4.5%), T.tonsurans 03(2.3%) and T.schollenii 2(1.5%). MIC showing high against the Fluconazole and Ketoconazole so it signifies that slowly it is going towards the resistance according to drug exposed so these fungi. This study shows that Terbinafine and Itraconazole drugs are drug of choice when there is no response against Ketoconazole, Fluconazole as we know due to more human toxicity Griseofulvin is now a day's not commonly used.

Keywords: *Dermatophytosis, Microdilution test, Antifungal agents, Terbinafine.*

INTRODUCTION

The susceptibility testing of bacteria, actinomycetes is now well accepted, but still researches are going on to make more technologically well advanced [1, 2]. Whereas, antifungal susceptibility testing remains less well developed and less utilized than antibacterial testing. The incidence of dermatophytosis cause by *Trichophyton*, *Epidermophyton* and *Microsporum* species [3, 4] has increase day by day especially among immunocompromised patients [5]. Relapse reported for some dermatophytes species and primary resistance of *Trichophyton rubrum* strains to terbinafine [6-9]. So the need for determination of their in Vitro susceptibility test against antifungal agents is essential.

In vitro antifungal susceptibility tests could help to optimize the therapy and to select an effective antifungal agent for dermatophytosis [10]. A standard method for susceptibility testing of dermatophytes is only MIC using either broth macro dilution or broth microdilution tests have been obtained in several tests reports [11-14]. The main purpose of this work was to establish in vitro antifungal susceptibility of fluconazole, itraconazole, ketoconazole, terbinafine and griseofulvin against clinical dermatophytes isolated in KRL Hospital, Mysore, India using broth microdilution method.

MATERIALS AND METHODS

Dermatophytes:

A total of 132 isolated and conformed by PCR strains were taken, which includes *Trichophyton mentagrophytes* (n=52), *T.rubrum* (n=30), *T.violacium*, *T. flocossum* (n=10), *M.canis*, *T.tonsurans* (n=03) and *T.scholenii* (n=02) tested for antifungal susceptibility. All microorganisms were clinical isolates obtained from nail, skin and hair specimens taken for research purpose from KRL Hospital Mysore, India from 2010-Janaury to 2011-December. All these fungi were maintained on Potato Dextrose Agar (PDA) without antibiotics at 28°C for seven to 14 days for pure colony *Candida parapsilosis* ATCC-22019 was also cultured and maintained as reference strains obtained from CMC-Vellore.

Antifungal susceptibility tests:

The broth microdilution assay for antifungal susceptibility tests for dermatophytes was performed according to the CLSI guidelines M38-A for filamentous fungi.

***Corresponding author:**
Jha B. K.
Department of Microbiology, Center for PhD studies,
Yubraja College, Mysore, India.
**E-Mail: baiju010@gmail.com*

Broth Microdilution:

The drugs were obtained from their respective manufacturers: fluconazole (Cipla Pharmaceuticals Limited, India), ketoconazole, itraconazole (Cadila Pharmaceuticals, India), terbinafine (HiMedia, India) and griseofulvin (HiMedia, India). Fluconazole was dissolved in distilled water while the other drugs were dissolved in 100% dimethyl sulfoxide (Cipla Pharmaceuticals Limited, India). The RPMI-1640 supplemented with L-glutamine was used to prepare stock solution according to the Stock solutions were stored at -70°C until used. Serial dilutions started with different range of antifungal agents like Terbinafine (0.03-0.5), Itraconazole (0.03-0.25), Ketoconazole (0.03-4), Fluconazole (4-64) and Griseofulvin (0.24-1) µg/ml were used [15-17].

Test procedure:

The dermatophytes were grown on Potato Dextrose agar at 28°C. Then slowly with wire loop scrap the surface of the colony and make suspension in distilled water with Conidia and hyphae. Optical density adjusted at 530 nm to obtain final inoculums size 5×10^4 cells/ml. If requires opacity adjusted with 500 ml RPMI 1640 supplemented with L- glutamine (broth).

Sterile microdilution plates 96-U-shaped used for the study. Rows 1-10 contains the series of drug dilution in 100µl volumes starting with the concentration of 32µg/ml. 100µl of inoculum suspension were added to each well. The eleventh control with conidia, 100µl of inoculums and 100µl of drugs free medium were added. These plates were covered with cello tape, incubated at 28°C and examined after 48 hours to 72 hours incubation for conidia as control and dermatophytes as test organisms respectively.

Test reading:

End point of the tests value were performed by visual no growth in the medium, i.e every 24hrs until growth in the central well drug free medium. Azole agent and griseofulvin: The lowest concentrations of the drug produce 80% of growth inhibition. Terbinafine: The lowest concentration of the drug showing 100% growth inhibition.

RESULTS

MIC of antifungal agents for 132 isolates was determined after four days for *Trichophyton mentagrophytes* and five days for *Trichophyton rubrum*, *Microsporum canis* and *Epidermophyton floccosum* species when incubated at 28°C. All these species of dermatophytes isolated identified and conformed by PCR as dermatophytes were selected for antifungal sensitivity tests. It was now known that either these 132 isolates were sensitive or resistance but high MIC value indicates that it is also slowly acquired adaptation towards the drug. Twenty three isolates (14.4%) were showing high MIC valve

(*T.mentagrophyte*-8, *T.rubrum*-5 and *T.verrucosum*-7) of fluconazole and *M.canis* -3 had MIC_{50} of 16µg/ml. Second most frequent by used drug next to fluconazole is ketoconazole, which had MIC_{50} of 0.125µg/ml of the most of the isolated. Griseofulvin, itraconazole and terbinafine drugs are all most showing similar result in between MIC value of 0.03-0.06µg/ml.

Table-1 gives all ranges of concentration inhibiting 50% of isolates and 90% of isolated and geometric mean of 5 drugs against 132 isolates where MIC_{50} of MIC_{90} was not determined because of small number of samples i.e. <10 samples as seen in (**Table-1, 2, 3 and 4**). The MIC ranges of fluconazole, itraconazole and ketoconazole for *C. parapsilosis* ATCC-22019 were within the value standardized by CLSI document M38-A.

Table No. 1: MIC of the Terbinafine against 132 isolates of conformed dermatophytes in µg/ml

Isolates	Range	MIC50	MIC90	GMIC
***T.mentegrophytes* (n=52)**	(0.03-0.5)	0.06	0.25	**0.07**
***T.rubrum* (n=30)**	(0.03-0.5)	0.06	0.25	**0.06**
***T.violacium* (n=18)**	(0.03-0.5)	<0.03	0.03	**0.03**
***T.verrucosum* (n=11)**	(0.03-0.5)	<0.03	0.06	**0.03**
***E.flocosum* (n=10)**	(0.03-0.5)	0.06	-	**0.05**
***M.canis* (n=6)**	(0.03-0.5)	0.06	-	**0.05**
***T.tonsurans* (n=3)**	(0.03-0.5)	0.0	-	**0.03**
***T.schoelneii* (n=2)**	(0.03-0.5)	0.0	-	**0.03**

Table No. 2: MIC of the Itraconazole against 132 isolates of conformed dermatophytes in µg/ml

Isolates	Range	MIC50	MIC90	GMIC
***T.mentegrophytes* (n=52)**	(0.03-4)	0.125	0.25	**0.10**
***T.rubrum* (n=30)**	(0.03-4)	0.125	0.5	**0.12**
***T.violacium* (n=18)**	(0.03-4)	<0.03	0.03	**0.03**
***T.verrucosum* (n=11)**	(0.03-4)	0.125	0.25	**0.11**
***E.flocosum* (n=10)**	(0.03-4)	0.125	-	**0.07**
***M.canis* (n=6)**	(0.03-4)	0.125	-	**0.07**
***T.tonsurans* (n=3)**	(0.03-4)	0.0	-	**0.04**
***T.schoelneii* (n=2)**	(0.03-4)	0.0	-	**0.03**

Table No. 3: MIC of the Ketoconazole against 132 isolates of conformed dermatophytes in µg/ml

Isolates	Range	MIC50	MIC90	GMIC
***T.mentegrophytes* (n=52)**	(0.03-4)	0.125	0.25	**0.10**
***T.rubrum* (n=30)**	(0.03-4)	0.06	2	**0.11**
***T.violacium* (n=18)**	(0.03-4)	0.125	0.25	**0.11**
***T.verrucosum* (n=11)**	(0.03-4)	0.25	4	**0.24**
***E.flocosum* (n=10)**	(0.03-4)	0.06	-	**0.04**
***M.canis* (n=6)**	(0.03-4)	0.125	-	**0.08**
***T.tonsurans* (n=3)**	(0.03-4)	0.0	-	**0.04**
***T.schoelneii* (n=2)**	(0.03-4)	0.0	-	**0.03**

Table No. 4: MIC of the Fluconazole against 132 isolates of conformed dermatophytes in µg/ml

Isolates	Range	MIC50	MIC90	GMIC
***T.mentegrophytes* (n=52)**	(4-64)	32	64	**18.20**
***T.rubrum* (n=30)**	(4-64)	16	32	**11.69**
***T.violacium* (n=18)**	(4-64)	1	2	**4.0**
***T.verrucosum* (n=11)**	(4-64)	4	8	**5.27**
***E.flocosum* (n=10)**	(4-64)	32	-	**17.15**
***M.canis* (n=6)**	(4-64)	16	-	**11.31**
***T.tonsurans* (n=3)**	(4-64)	0.0	-	**4.0**
***T.schoelneii* (n=2)**	(4-64)	0.0	-	**4.0**

Table No. 5: MIC of the Gresiofulvin against 132 isolates of conformed dermatophytes in µg/ml

Isolates	Range	MIC50	MIC90	GMIC
***T.mentegrophytes* (n=52)**	(0.25-1)	0.5	0.5	**0.26**
***T.rubrum* (n=30)**	(0.25-1)	0.5	0.5	**0.34**
***T.violacium* (n=18)**	(0.25-1)	0.5	1	**0.32**
***T.verrucosum* (n=11)**	(0.25-1)	>1	>1	**0.47**
***E.flocosum* (n=10)**	(0.25-1)	0.5	-	**0.21**
***M.canis* (n=6)**	(0.25-1)	0.25	-	**0.14**
***T.tonsurans* (n=3)**	(0.25-1)	0.0	-	**0.07**
***T.schoelneii* (n=2)**	(0.25-1)	0.0	-	**0.06**

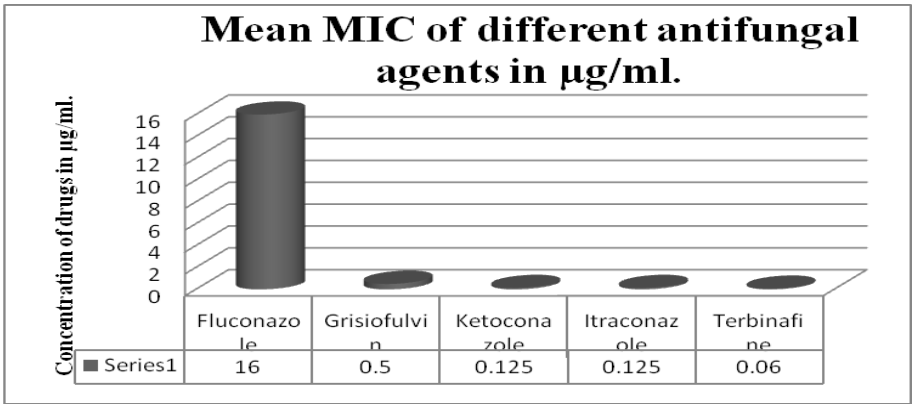

Fig. 1: Mean MIC of fluconazole, grisiofulvin, ketoconazole, itraconazole, terbinafine by microtube antifungal susceptibility test

DISCUSSION

As antifungal tests is not so common in this Asian countries like India and Nepal, but it is very much essential to know the resistance patterns among microbes i.e not only in bacteria but also in fungus, which help to assess interventional efforts and empirical therapy to the patient. This MIC data are also essential to obtain distribution profiles of MIC values for fungal correlations of MICs with clinical response Nir-Paz et al [18] advised the method of testing susceptibility of antifungal agents against yeast and also additional effort to adapt NCCLS broth microdilution method for molds.

As given in fig 1 MIC done by incubation at 28°C for 7 days gives only microconidia in buffered RPMI 1640 allows adequate growth for the study. Differences in MIC values cannot be attributed to the incubation temperature (28 or 35°C) as Perea et al. [19] shown that anyone temperature can influence MIC. We incubated 7days for *T.rubrum* and *T.mentagrophytes* as Gupta and Kohli [20] these fungal growths were luxurious and taken only for 5 days for growth. Terbinafine was most potent agent tested in our study but slow and steady increasing MIC of Terbinafine in T.mentagrophytes is also a point of view [21, 22]. Fluconazole is the drug that had high MIC value in *Trichophyton, Microsporum* and *Epidermophyton* which is 16-32µg/ml, similar report was found in latest research done by Santos et al. in 2006 [23]. Next drug which is more frequently used ketoconazole is also showing MIC increasing 0.125µg/ml as compared to other researchers work [24-26].

Monitoring antimicrobial resistance is useful because apart from tracking and detection of resistance trends by microorganisms, it also gives clues to emerging threats of new resistance. Where as in other researcher terbinafine also showing mild type of resistance but in our study most frequently used drugs by patients were ketoconazole and fluconazole weekly for months, even if it was not getting treated then few patients were taking one tab for Flustat for 5 days direct from drug house without any Doctors prescription. That may be the one of the region for drug resistance among few isolates. This type of drug resistance mostly we have observed in zoonotic infection.

Lastly we conclude that Terbinafine as most active and has excellent in vitro potency and broad spectrum activity against all the tested species. This can be used to treat a majority of dermatophytic infections and also in those infection causing dermatophytes with high MIC values with azoles can easily treated by this drugs.

CONCLUSION

We have demonstrated that terbinafine and itraconazole should preserve for drug resistance infection treatment use. As we have seen that terbinafine has least MIC and next itraconazole, whereas fluconazole and ketoconazole has high MIC. MIC need to correlate with clinical form of disease for break point development against the dermatophytes.

ACKNOWLEDGEMENT:

We thank Dr. S. Mahadeva Murthy (Prof. and HOD) for providing funding from Dept. of Microbiology for the test, Dr khilasa for statistical analysis and Dr Surya for typing the manuscript.

REFERENCES:

1. Barry AL, Pfaller MA, Brown SD, *et al.* Quality control limits of broth microdilution susceptibility tests of ten antifungal agents. J. clin. Microbiol., **2000**; 33: 3457-9.
2. Pfaller MA. Antifungal susceptibility testing. Progress and future developments. Braz. J. Infect. Dis., **2000**; 4(2): 55-60.
3. Elewski BE and Charif MA. Prevalence of onychomycosis in patients attending a dermatology clinic in northeastern Ohio for other conditions. Arch. Dermatol., **1997**; 133: 1172-3.
4. Georg LK and Camp LB. Routine nutritional tests for the identification of dermatophytes. J. Bacteriol., **1956**; 74: 113-21.
5. Odds FC, Motyl M, Andrade R, *et al.* Interlaboratory comparison of results of susceptibility testing with caspofungin against *Candida* and *Aspergillus* species. J. Clin. Microbiol., **2004**; 42: 3475-82.
6. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, 2nd ed. Approved standard. Clinical and Laboratory Standards Institute, Wayne, PA. CLSI document **2008**; M38-A2.
7. Carrillo-Munõz AJ, Quindós G, Ruesga M, *et al*. *In vitro* susceptibility testing of filamentous fungi with Sensititre Yeast One TM. Mycoses., **2006**; 49: 293-7.
8. Favre B, Hofbauer B, Hildering KS and Ryder NS. Comparison of *in vitro* activities of 17 antifungal drugs against a panel of 20 dermatophytes by using microdilution assay. J. clin. Microbiol., **2003**; 41: 4817-9.
9. Espinel-Ingroff A. Clinical utility of in vitro antifungal susceptibility testing. Rev. Esp. Quimioter., **2003**; 13(2): 161-6.
10. Rex JH, Pfaller MA, Galgiani JN, Bartlett MS, Espinel-Ingroff A, Ghannoum MA, et al. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of *in vitro-in vivo* correlation data for fluconazole, itraconazole and *Candida* infections. Clin. Infect. Dis., **1997**; 24: 235-47.
11. Macura AB. In vitro susceptibility of dermatophytes to antifungal drugs: comparison of two methods. Int. J. Dermatol., **1993**; 32:533-6.
12. Barros MES, Santos DA and 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. Clin. Microbiol., **2007**; 56: 514-8.
13. Ghannoum MA, Chatuverdi V, Espinel-Ingroff A, Pfaller MA, Rinaldi MG, Lee-Yang W and Warnock DW. Intra- and interlaboratory study of a method for testing the antifungal susceptibilities of dermatophytes. J. Clin. Microbiol., **2004**; 42: 2977-9.
14. Mukherjee PK, Leidich SD, Isham N, Leitner I, Ryder IN and Ghannoum MA. Clinical *Trichophyton rubrum* strain exhibiting primary resistance to terbinafine. Antimicrob Agents Chemother., **2003**; 47:82-6.
15. Nweze EI and Okafor JI. Prevalence of dermatophytic fungal infections in children: a recent study in Anambra State, Nigeria. Mycopathologia., **2005**; 160: 239-43.
16. Jesenska Z. Comparative study of the growth inhibition of vegetative hyphae of some dermatophytes and keratinophilic fungi on glucose-peptoneagar with griseofulvin. Dermatol Monatsschr., **1979**; 165: 292-9.
17. Fernández-Torres B, Vázquez-Veiga H, Liovo X, Pereiro Jr and Guarro J. *In vitro* susceptibility to itraconazole, clotrimazole, ketoconazole and terbinafine of 100 isolates of *Trichophyton rubrum*. Chemotherapy, **2000**; 46: 390-4.
18. Nir-Paz RH, Elinav GE, Pierard D, Walker A, Maly M, Shapiro RC, Barton A and Polacheck I. 2003. Deep infection by *Trichophyton*

rubrum in an immunocompromised patient. J. Clin. Microbiol., **2003**; 41: 5298-301.
19. Perea S, Fothergill AW, Sutton DA and Rinaldi MG. (2001): 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(1): 385-8.
20. Gupta AK and Kohli Y. In vitro susceptibility testing of ciclopirox, terbinafine, ketoconazole and itraconazole against dermatophytes and nondermatophytes and in vitro evaluation of combination antifungal activity. Br. J. Dermatol., **2003**; 149: 296-305.
21. Ghannoum MA, Wraith LA, Cai B, Nyirady J and Isham N. Susceptibility of dermatophytes isolates obtained from a large worldwide terbinafine tinea capitis clinical trial. Brit. J. Derm., **2008**; 159: 711-3.
22. Ryder NS and Favre B. Antifungal activity and mechanism of action of terbinafine. Rev. Contemp. Pharmacother., **1997**; 8:275-87.
23. Santos DA, Barros MES and Hamdan JS. Establishing a method of inoculum preparation for susceptibility testing of Trichophyton rubrum and Trichophyton mentagrophytes. J. Clin. Microbiol., **2006**; 44: 98-101.
24. Singh J, Zaman M and Gupta AK. Evaluation of microdilution and disk diffusion methods for antifungal susceptibility testing of dermatophytes. Med. Mycol., **2007**; 45: 595-602.
25. Mukherjee PK, Leidich SD, Isham N *et al*. Clinical *Trychophyton rubrum* strain exhibiting primary resistance to terbinafine. Antimicrob. Agents Chemother., **2003**; 47: 82-6.
26. Weitzman I and Summerbell RC. The dermatophytes. Clin. Microbiol. Rev., **1995**; 8: 240-59.

Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nil