Original Article

Antifungal Susceptibility Profile Of Dermatophytes By Stanadard Broth Microdilution Method

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Abstract

The in vitro activities of 5 antifungal drugs against a set of 100 Dermatophytes which are consisting of 4 different species were determined using broth microdilution method. Out of 100 samples Trichophyton mentagrophytes accounts highest number (41%) followed by Trichophyton rubrum (35%), Trichophyton tonsurans (22%), Microsporum canis (2%). Miconazole nitrate was the most potent drug while Itraconazole and Ketoconazole were the other potent drugs resulted in this study. The emerging resistance of dermatophytes makes necessary to test antifungal susceptibility to treat dermatophyte infection.

Key words: Dermatophyte, Antifungals, Tinea, superficial fungal infection, Resistance, Trichophyton, Microsporum canis

INTRODUCTION

Dermatophytes are the group of fungi which are capable of digesting and obtaining the nutrients from the primary component of skin, hair, and nail i.e., keratin by unique enzymatic (keratinase) activity. The disease caused by dermatophytes is known as dermatophytosis, ringworm or tinea. The dermatophytes usually colonize outermost layer of the skin and its derivatives. The skin infection which is caused by non-dermatophytic fungi and cutaneous manifestations of systemic fungi is known as dermatomycosis.

Superficial fungal infection which or dermatomycoses have been showing the enormous increase both in incidence and prevalence in recent past. Dermatophytes spreads easily and rapidly especially in lower economic classes of population.[1] Infection usually restricted to the cutaneous portion, due to the inability of fungi to penetrate the deeper tissues. The organisms colonize the keratin tissue and inflammation is caused by host response to metabolic by-products. Mainly these infections affect our superficial layers of the skin, nails and hair without invading tissue and are often caused by dermatophyte molds belonging to *Trichophyton, Microsporum, Epidermophyton*, Pityriasis versicolor, candida and non dermatophytic molds like *Aspergillus, Penicillium, Fusarium, Rhizopus* etc [(2)]

The prevalence of fungal infections is increasing in both the developing and developed countries due to underlying predisposing factors such as immunocompromised patients, anticancer drugs, diabetes, immunosuppressive agents, use of corticosteroids, HIV positivity etc.[1] The diagnosis is done with the help of history, physical examination, occupation, microscopy, culture, serodiagnosis and molecular testing.

The emergence of antifungal resistant strains is due to their widespread usage over years, self-medication, overuse and misuse of antifungal medication and a weak or non-existent antifungal policy and poor infection control.[2] In vivo antifungal susceptibility test method of fungi depended on various agents like quantity of the inoculum, contents of broth medium, incubating period, temperature and MIC end point calculation. The reported viability of the non-standard in vivo methods and difficulties in understanding fungal infection and infection activity are main reason for increased attention on antifungal susceptibility testing on and many studies are being conducted to approach the true burden of resistance.

Answers to the problems in making standardizing the existing methods have been already demonstrated in many studies. The first document [M38-A] related to the antifungal susceptibility is published in the year 2002 by CLSI [Clinical and Laboratory Standards] previously called NCCLS [National Committee for Clinical Laboratory Standards] is dealing in the Standardizing of susceptibility protocols has not included the dermatophytes Now a days most of the laboratories have opted to use broth micro dilution method as it is more practicable, less cumbersome with more consistent MIC results.

The present research might contribute in controlling antifungal resistance and standardizing the protocol for antifungal susceptibility testing for dermatophytes. Therefore, present study determines the antifungal susceptibility profile of

dermatophytes against 5 antifungal agents i.e., Amphotericin B, Ketoconazole, Miconazole nitrate, Fluconazole and Itraconazole Which are used as antifungal agents against dermatophytes using microbroth dilution technique, conducted in the department of Microbiology JSS Hospital Mysuru. This study would help to make a policy to treat dermatophyte infections by evaluating the standardized method and MIC breakpoint of antifungal drugs.

MATERIALS AND METHODS

A total of 233 clinical samples (skin scrapings, nail clippings and hair) which are suspected dermatophyte infection were included in the study. All the collected samples are subjected to KOH mount (10% for skin, 20% for hair, and 40% for nail) and also culture inoculation done on saborauds cycloheximide and chloramphenicol agar and incubated at 25-27°C temperature for 4-5 weeks. The grown dermatophytes than identified by macroscopic and microscopic observation of cultures and some standard testing methods.

Preparation of inoculum:

The clinical isolates which are grown dermatophytes sub-cultured on Potato dextrose agar and incubated for 5 days at 27°C and grown colonies are covered with the 1ml of normal sterile saline and suspension made gently by probing the surface with sterile cotton swabs then dip that cotton swab into sterile 4ml of sterile saline. allow the suspensions stand for 5-10 mins, count the conidia or spores with the usage of hemocytometer. The inoculum size standardized for 0.5×10^4 to 5×10^4 CFU/ml. The inoculum further diluted with RPMI medium in 1:50 ratio to obtain desired concentration of inoculum.

Antifungal drugs

The antifungal powders weighed on calibrated analytical balance machine. All the antifungal agents weighed by the following formula

$$W(mg)= \frac{\text{Target vol.} \times \text{desired Conc.}}{\text{Potency of drug}}$$

The antifungal agents dissolved with respective with their solubility[1]

Stock solution preparation

The stock solution prepared for different concentration according to the CLSI guidelines M-28A document (Fluconazole-0.125 – 64µg/ml Ketoconazole-0.0313 - 16µg/ml, Miconazole- 0.0313 - 32µg/ml Itraconazole- 0.0313 - 16µg/ml, Amphotericin B- 0.0313 - 16µg/ml)

Test procedure:

U-bottomed microdilution plates (96 wells) were set up in accordance with the CLSI reference method. Each microdilution well containing 100µl of the twofold drug concentration was inoculated with 100µl of the diluted inoculum suspension. For each test plate, two drug-free controls were included, one with the medium alone (sterile control) and the other with 100µl of medium plus 100µl of inoculum suspension (growth control). The microdilution plates were incubated at 35°C and were read visually after 4 days of incubation.

Reading and interpretation of MICs

Endpoint determination readings were performed visually based on comparison of the growth in wells containing the drug with that of the growth control. The MIC is the lowest concentration of an azole agents the MIC was defined as the lowest concentration showing prominent growth inhibition (a drop in growth corresponding to approximately 80% of the growth control. Here we noted 50% and 90% of inhibition.

RESULTS

The present study entitled "ANTIFUNGAL SUSCEPTIBILITY PATTERN OF DERMATOPHYTES IN BROTH MICRODILUTION METHOD" was carried out in Department of Microbiology, J.S.S Hospital from 2019 to 2020. In this present study, clinical specimens which include skin scrapings, nail clipping and hair samples are collected which culture positive. KOH positive samples are also subjected to culture which are suspected for dermatophytes and positive cultures are taken as isolates. Total 233 samples are collected out of that male contributed 134 samples. Whereas female contributed 99 samples. Out of that 100 dermatophytes cultures are isolated. In these 100 samples male contributed 45 for skin scrapings, 14 for nail clipping, 9 for hair specimen whereas females contributed 18 for skin scrapings 10 for nail clipping and 4 for hair in total 68% samples collected from male and 32% samples collected from female. The selected 100 isolates samples KOH mount shown 84 % KOH positive and culture positive and 16% was KOH negative and culture positive.

The most of the clinical samples of suspected dermatophyte infection belongs to the clinical type Tinea corporis (62%) followed by Tinea pedis (13%), Tinea ungium(9%), Tinea capitis (7%), Tinea cruris (5%). The most frequently isolated dermatophyte organism in the 100 test samples is *Trichophyton mentagrophyte* which accounts for 41% of total samples, followed by *Trichophyton rubrum* (35%), *Trichophyton tonsurans* (22%) and *Microsporum canis* (2%).

Table 1 Showing Susceptibility Patterns Of The Isolates

Dermatophyte organism	MIC Values	Fluconazole	Itraconazole	Micanazole	Ketoconazole	Amphotericin-B
T. rubrum (27)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50	64 µg/ml	0.0625 µg/ml	0.031 µg/ml	0.25 µg/ml	8 µg/ml
	MIC90	125 µg/ml	0.125 µg/ml	0.0625 µg/ml	0.5 µg/ml	16 µg/ml
T. mentagrophyte (37)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50	64 µg/ml	0.0625 µg/ml	0.031µg/ml	0.25 µg/ml	8 µg/ml
	MIC90	125 µg/ml	0.125 µg/ml	0.0625 µg/ml	0.5 µg/ml	16 µg/ml
T. tonsurans (21)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50	64 µg/ml	0.0625 µg/ml	0.031 µg/ml	0.25 µg/ml	8 µg/ml
	MIC90	125 µg/ml	0.125 µg/ml	0.0625 µg/ml	0.5 µg/ml	16 µg/ml
M. canis (2)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50	64 µg/ml	0.31 µg/ml	< 0.031 µg/ml	0.25 µg/ml	4 µg/ml
	MIC90	< 64µg/ml	0.0625 µg/ml	0.031 µg/ml	0.062 µg/ml	8 µg/ml

Table 2 Showing Emerging Resistance Of *Trichophyton Rubrum* Isolates

Dermatophyte organism	MIC Values	Fluconazole	Itraconazole	Micanazole	Ketoconazole	Amphotericin-B
T. rubrum (1)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50 (µg/ml)	64	0.125	0.031	0.25	32
	MIC90 (µg/ml)	>64	0.25	0.0625	0.5	>32
	GM* (µg/ml)	>64	0.187	0.046	0.375	>32
T. rubrum (2)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50(µg/ml)	16	0.0625	0.031	0.25	8
	MIC90(µg/ml)	32	0.125	0.0625	0.5	16
	GM* (µg/ml)	24	0.093	0.046	0.375	12
T. rubrum (3)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50 (µg/ml)	16	0.0625	0.031	0.25	4
	MIC90 (µg/ml)	32	0.125	0.0625	0.5	8
	GM* (µg/ml)	24	0.093	0.046	0.375	6
T. rubrum (4)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50 (µg/ml)	8	0.125	0.031	0.5	2
	MIC90 (µg/ml)	16	0.25	0.0625	1	4
	GM*(µg/ml)	12	0.187	0.046	0.75	3
T. rubrum (5)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50 (µg/ml)	8	0.125	0.031	0.25	8
	MIC90 (µg/ml)	16	0.25	0.0625	0.5	16
	GM* (µg/ml)	12	0.187	0.046	0.375	12
T. rubrum (6)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50 (µg/ml)	32	0.0625	0.031	0.5	2
	MIC90 (µg/ml)	64	0.25	0.0625	1	4
	GM* (µg/ml)	48	0.093	0.046	0.075	3
T. rubrum (7)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50 (µg/ml)	8	0.125	0.031	0.25	2
	MIC90 (µg/ml)	16	0.25	0.0625	0.5	4
	GM* (µg/ml)	12	0.187	0.046	0.375	3

Table 3 Showing Emerging Resistance Of *Trichophyton Tonsurans* Isolates

Dermatophyte organism	MIC Values	Fluconazole	Itraconazole	Micanazole	Ketoconazole	Amphotericin-B
T. tonsurans (1)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50 (µg/ml)	16	0.0625	0.031	0.5	8
	MIC90 (µg/ml)	32	0.125	0.0625	1	16
	GM* (µg/ml)	24	0.093	0.046	0.75	12
T. tonsurans (2)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50 (µg/ml)	16	0.125	0.031	0.25	8
	MIC90 (µg/ml)	32	0.25	0.0625	0.5	16
	GM* (µg/ml)	24	0.187	0.046	0.375 l	12

Table 4 Showing Emerging Resistance Of *Trichophyton Mentagrophytes* Isolates

Dermatophyte organism	MIC Values	Fluconazole	Itraconazole	Micanazole	Ketoconazole	Amphotericin-B
T. mentagrophyte (1)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50(µg/ml)	32	0.125	0.031	0.25	2
	MIC90(µg/ml)	64	0.25	0.0625	0.5	4
	GM*(µg/ml)	48	0.187	0.046	0.375	3
T. mentagrophyte (2)	**MIC range**	**64µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50(µg/ml)	16	0.0625	0.031	0.25	4 l
	MIC90(µg/ml)	32	0.125	0.0625	0.5	8
	GM*(µg/ml)	24	0.093	0.046	0.375	12
T. mentagrophyte (3)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50(µg/ml)	16	0.125	0.031	0.25	2
	MIC90(µg/ml)	32	0.25	0.0625	0.5	4
	GM*(µg/ml)	24	0.187	0.046	0.375 l	3
T. mentagrophyte (4)	**MIC range**	**64-512µg/ml**	**0.031-16µg/ml**	**0.015-32µg/ml**	**0.031-16µg/ml**	**2-16 µg/ml**
	MIC50(µg/ml)	8	0.0625	0.031	0.25	2
	MIC90(µg/ml)	16	0.125	0.0625	0.5 l	4
	GM*(µg/ml)	12	0.093	0.046	0.375	3

DISCUSSION

Superficial fungal infections are the commonly encountered fungal diseases prevalent in the most parts of the world. The dermatophytes play the major role in superficial cutaneous fungal infections due to their widespread and their involvement in population at a large range and prevalence. The most frequent clinical type observed in the present study is Tinea corporis followed by Tinea pedis. Similar results seen in studies made by Mota CR et, al (2009)[3].

In present study, out of 100 samples most common isolated organism is Trichophyton mentagrophyte (41%) followed by Trichophyton rubrum (35%), Trichophyton tonsurans (22%) and Microsporum canis(2%) similar findings i.e., most frequently isolated organism is T. mentagrophytes followed by T. rubrum seen in studies C. J. JESSUP et. al. (2000)[5], D.A.Santos et, al.(2005)[6], Ravika K. Budhiraja et. al (2018)[2], Sowmya N et, al. (2015)[4], were tested against Fluconazole, Amphotericin-B, Ketoconazole, Itraconazole and Micanazole nitrate.

The number of authors proposed variety of incubation timing ref. D. A. Santos et, al.(2005)[6], Emerson Roberto SIQUEIRA (2008)[7,] Ravika K. Budhiraja et. al (2018)[2,] but in present study all the dermatophytes shown the growth at 4 days of incubation.

In present study Fluconazole testing done for the 0.5-64 µg/ml MIC range and results shown the highest GM value. The normal GM value seen among the T. rubrum , T. mentagrophyte and T. tonsurans is 12 µg/ml and in Microsporum canis GM value was 6 µg/ml (mentioned in **Table no.- 01**). The fluconazole resistance is well seen and documented in many studies ref Satyendra Kumar Singh et, al. (2018)[8], Ravika K. Budhiraja et. al (2018)[2], Santos et, al.(2005)[6] similarly the 3 Trichophyton rubrum, 2 T. tonsurans and 3 T. mentagrophytes shows the emerging resistance and 1 Trichophyton rubrum complete resistance to tested MIC range of fluconazole (>64µg/ml) (mentioned in **Table no. 2,3 and 4**).

The Amphotericin-B tested in the MIC range between 2-32 µg/ml. The results shown GM value of 6 µg/ml for T. rubrum, T. mentagrophyte and T. tonsurans and 3 µg/ml for M. canis (mentioned in **Table no.- 01**). Out of all samples 1 Trichophyton rubrum shown resistance for MIC range 2-32 µg/ml, 3 T. rubrum, 1 T. mentagrophytes and 2 T. tonsurans shown the emerging resistance to this drug (mentioned in **Table no. 2,3 and 4**). But any how this drug is not commonly used against the dermatophyte infection. Similar to Study made by Sowmya.N et, al. (2011) [9]

The itraconazole MIC are tested in between the range 0.0313-16 µg/ml and it shown the GM value of 0.093 µg/ml for T. rubrum, T. mentagrophytes, T.tonsurans and M.canis (mentioned in **Table no.- 01**). Anyhow 4 isolates of T. rubrum and 2 t. mentagrophytes and 1 T. tonsurans were shown the emergence of resistance to the itraconazole drug (mentioned in **Table no. 2,3 and 4**). Ref Santos et, al. (2005)[6,10]

The ketoconazole drug tested in between the MIC ranges of 0.0313-16 µg/ml and the result shown the GM value of 0.375 µg/ml for T. rubrum, T. mentagrophytes and T. tonsurans and M. canis shows the GM value of 0.312 µg/ml (mentioned in **Table no.- 01**). similarly, M. Suganthi et.al, (2018)[11], Ahmed Medhat Hanafy (2012)[12] Out of all isolates 2 isolates of T. rubrum and 1 *T mentagrophytes* and 1 *T tonsurans* shown the emerging resistance (mentioned in **Table no. 2,3 and 4**).

Other than the MIC results for Miconazole drug shown in Ahmed Medhat Hanafy (2012)[12] In this present study the Miconazole nitrate drug tested between the MIC ranges of 0.0313-16 µg/ml and the result shown the GM value of 0.046 µg/ml for T. rubrum, T. mentagrophytes and T. tonsurans and M. canis shows the GM value <0.031 µg/ml. There were no resistant isolates found against the Miconazole nitrate drug among the tested isolates. (Mentioned in **Table no.1,2,3 and 4**)

Overall, the fluconazole shows the highest GM value in Trichophyton species and M. canis i.e., 12 µg/ml and 6 µg/ml respectively. Followed by Amphotericin-B i.e, 6 µg/ml and 3 µg/ml for T. species and M.canis. Ketoconazole shown the GM value of 0.37µg/ml and 0.312 µg/ml and finally the Miconazole nitrate shown the GM value of 0.046 µg/ml in T.species and <0.0313 µg/ml in M.canis and it found to be an effective drug.

CONCLUSION

In present study most of the dermatophytosis cases were from the age group 21-30 (31%). Males were more commonly affected than females. The most commonly seen clinical type is Tinea corporis followed by Tinea pedis and unguium respectively. In conclusion the broth microdilution is the best antifungal susceptibility testing method for dermatophytes and it is less cumbersome and reproducible MIC. [6]

The most efficient drug was miconazole followed by ketoconazole, itraconazole and fluconazole respectively. The fluconazole is not much effective drug among the 5 drugs which is tested in present study and its emerging resistance making the drug useless to treat the patients. The fluconazole is required in higher concentration to treat. Whereas, the miconazole requires the lesser concentration i.e., 0.0313 µg/ml and shown the lower GM value 0.046 µg/ml. The 1 dermatophyte isolate shown complete resistance to Amphotericin-B and fluconazole for tested MIC range. The isolates also shown emerging resistance against drugs amphotericin-B, Itraconazole, Ketoconazole. With the increasing resistance of dermatophytes to antifungal agents, in resistant cases or treatment failure cases antifungal susceptibility test will have to be done. In the course of time antifungal susceptibility test become mandatory just like the antibiotic susceptibility testing. As the present method microbroth dilution is cumbersome automated antifungal susceptibility testing may become imperative.

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