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A study on the antifungal susceptibility pattern of dermatophytes isolated in a tertiary care hospital

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Abstract: Recent years, due to increased usage of antifungal treatment worldwide, there is an increased chance of rising resistance among antifungal drugs too. Dermatophytic infections causes' superficial mycosis and it affects skin, hair and nail. These infections are more common and antifungal drugs are used everywhere to treat those common infections. To conduct a study by determining the antifungal susceptibility pattern in dermatophytic isolates from patients attending dermatology OPD in a tertiary care hospital. A total of 217 samples like hair, nail and skin scrapings were obtained and isolation of dermatophytes was done. Antifungal susceptibility testing for dermatophytes was performed by micro broth dilution method. Antifungal drugs tested were Griseofulvin, Fluconazole, Itraconazole and Ketoconazole. Minimum inhibitory concentration for each drug for fungal isolates was tested and results studied. Fluconazole showed a higher MIC values in the range of 1-8µg/ml. Itraconazole showed the lowest MIC values by micro broth dilution method. Since there is limitation of standard guidelines and protocol, meticulous research must be conducted on effect of antifungals and derive at universally implementable guidelines.

Key words: Dermatophytes; antifungal drugs; susceptibility

Introduction

Over the past few years, there is an increased usage of antifungal drugs in the treatment, which has led to increased chance of antifungal resistance also.(1) Due to lack of standard protocol in testing and implementing antifungal management, resistance rates are increasing nowadays. Resistance has resulted in increased rates of morbidity and mortality, especially in critically care patients and also in community.(2) Dermatophytic infections are more prevalent in tropical countries, which utilizes keratin and causes skin, hair and nail infections.(3) Based on the site of infection, dermatophytosis is classified clinically as Tinea cruris, Tinea capitis, Tinea unguuim, Tinea pedis, Tinea barbae. Even though dermatophytic infections are limited to skin, hair and nail, antifungal resistance due to irrational treatment can lead to spread of resistance to other colonizing species and thus may lead to invasive infections.(4) Testing methods to detect antifungal susceptibility among fungal isolates has been developed by Clinical Standards Laboratory Institute (CLSI); yet universal protocol for testing is still in development process.(5) Moreover, cost of testing the susceptibility using antifungal drugs is challenging to utilize the testing methods in all levels of health care and diagnostic laboratories. CLSI M-38-A describes the standard parameters for testing Minimum Inhibitory Concentration (MIC) of established agents against filamentous fungi. This has been modified with incubating temperature (28 versus 35°C) and duration of incubation (4 to 10 days versus 21 to 72 hours) for testing dermatophytes M-38-P.(6,7) *In vitro* susceptibility testing of fungi is dependent upon many factors such as inoculum size, composition of the medium, pH, incubation duration, and temperature and MIC end point determination. (8,9) Several studies have attempted to correlate the MIC results with outcome. However, there is only little evidence available with *in-vitro* outcomes. (10,11) The retrospective nature of the studies, the documented variability of the non-standardized in vitro methods and the difficulty in defining mycoses and their responses to therapy are responsible for this status. Due to above reasons, antifungal susceptibility testing is receiving increased attention and many studies are being conducted to assess the true burden of resistance. Hence, we planned to conduct a study by determining the antifungal susceptibility pattern in dermatophytic isolates from patients attending dermatology OPD in a tertiary care hospital.

Materials and Methods

This cross-sectional study conducted over a period of one year from samples obtained from patients attending dermatology clinic was carried out in Department of Microbiology in a tertiary care teaching hospital. A total of 217 samples like hair, nail and skin scrapings were obtained and isolation of dermatophytes was done as per standard protocol; then determination of their

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antifungal susceptibility was done based on previous studies and CLSI guidelines.

Antifungal susceptibility testing for dermatophytes:
Antifungal susceptibility testing for dermatophytes was performed by micro broth dilution method.

Medium:
Medium used was RPMI 1640 with glutamine, without bicarbonate in MOPS (3N-Morpholino propane sulphonic acid), buffer sterilized by membrane filtration.

Stock solution:
For each drug, 5ml stock solution was prepared. For water soluble drugs (eg. Fluconazole): Two-fold dilutions of a water-soluble antifungal agent is used, they were prepared volumetrically in broth. For water insoluble drugs diluent used was DMSO. To prepare for a broth micro dilution test series containing a water-insoluble drug that can be dissolved in DMSO, for which the highest desired test concentration is 16µg/ml, first 4.8 mg of the drug is weighed (assuming 100% potency) of antifungal powder and dissolved in 3.0 ml of DMSO. This will provide a stock solution at 1,600 µg/ml. Next further dilutions of this stocks solution in DMSO was prepared. The solutions in DMSO was further diluted 1:50 in the test medium and a further two-fold dilution was done when inoculated.

Drug Dilution:
To prepare 5 ml volumes of antifungal agent, 4.9 ml volumes of RPMI 1640 medium was pipetted into each of 10 sterile test tubes. Then, a single pipette was used and added 0.1 ml of DMSO alone to one 4.9 ml lot of medium (control medium), then 0.1 ml of lowest (3.13 microgram /ml) drug concentration added in DMSO, then 0.1 ml of the 6.25 µg/ml, concentration and then continued in sequence up the concentration series, each time adding 0.1 ml volumes to 4.9 ml medium. These volumes were adjusted according to the total number of test required. The working solution obtained was in 1:2 dilutions.

Inoculum Preparation:
Isolates obtained by fungal culture on Sabouraud`s Dextrose agar (SDA) with cycloheximide incubated at 25°C of skin scrapings, nail clippings and hair samples were taken for performing susceptibility. 7-15 days old cultures grown on SDA at 25°C were used. Mature colonies were covered with 10ml of sterile saline (0.85%). Growth was scraped by sterile Pasteur pipette. Heavy particles were allowed to settle for 15-20 minutes at room temperature. Supernatant was then mixed with a vortex for 15 seconds. Turbidity of supernatant was adjusted by spectrophotometry to 530nm 65-70% absorbance. Each suspension was then diluted 1:50 in RPMI 1640.

Inoculation:
Each well was inoculated on the day of test with 0.1 ml of 2x inoculum suspension. This step will dilute the drug concentration, inoculums densities, and solvent used to the final desired test concentration. The growth control well contains 0.1ml of the corresponding diluted inoculums suspension and 0.1ml of the drug diluents without anti-fungal agents. Test was performed in sterile microtitre plates. Aliquots of 100µl of drug dilutions inoculated in 1-10 microtitre wells. Concentration of Fluconazole was from 0.01-64 µg/ml and concentration of other drugs 0.0039-16 µg/ml). Added 100 µl of inoculums into each well from 1 to 12. Growth control was tube 12 with inoculums and without antifungal drug. All micro dilution trays were incubated at 28°C without agitation.

Interpretation:
The MIC was taken as the lowest concentration of antifungal agent that substantially inhibit growth of the organism as detected visually. For the conventional micro dilution procedure, the growth in each MIC well is compared with that of the growth control with the aid of reading mirror. Each micro titer well was then given a numerical score as follows: 4- No reduction in growth; 3-Slight reduction in growth or approximately 80% of growth control (drug free medium); 2-Prominent reduction in growth or approximately 50% of growth control; 1- Slight growth or approximately 25% of growth control; 0-Optically clear or absence of growth (NCCLS/CLSI M -38A)(12). End point for MIC for Itraconazole is score 0; for Fluconazole and Ketoconazole it was score 2 or less.

Results

Among 217 specimens processed, 36.8% showed culture positivity. Various species were isolated and their distributions were *Trichophyton rubrum* 63 (78.75%), *Trichophyton mentagrophytes* 7 (8.75%), *Trichophyton verrucosum* 6 (7.5%), *Epidermophyton floccosum* 2 (2.5%), *Trichophyton tonsurans* 1(1.25%) and *Microsporum gypseum* 1 (1.25%). (Figure 1) Minimum inhibitory concentration for each drug for fungal isolates was tested and results shown as follows.

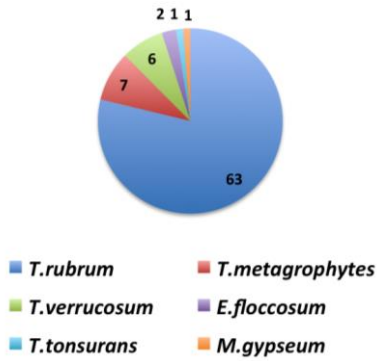

Figure 1: Distribution of dermatophytes among study population

Table 1: MIC for drug Fluconazole

Species	Drug concentrations in µg/ml											
	0.03	**0.06**	**0.012**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**MIC 50**	**MIC 90**
T.rubrum n=63	-	-	-	-	-	42	18	3	-	-	1	4
T.mentagrophytes n=7	-	-	-	-	-	-	5	1	1	-	2	8
T.verrucosum n=6	-	-	-	-	-	-	4	2	-	-	2	4
T.tonsurans n=1	-	-	-	-	-	-	1	-	-	-	2	2
E.floccosum n=2	-	-	-	-	-	-	-	2	-	-	4	4
M.gypseum n=1	-	-	-	-	-	-	-	1	-	-	1	1

Table 2: MIC for drug Itraconazole

Species	Drug concentrations in µg/ml										
	0.0075	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**MIC 50**	**MIC 90**
T.rubrum n=63	-	-	5	28	23	7	-	-	-	0.12	0.25
T.mentagrophytes n=7	-	3	2	-	2	-	-	-	-	0.03	0.12
T.verrucosum n=6	-	-	1	3	-	2	-	-	-	0.06	0.25
T.tonsurans n=1	-	-	-	1	-	-	-	-	-	0.06	0.06
E.floccosum n=2	-	-	1	1	-	-	-	-	-	0.03	0.06
M.gypseum n=1	-	-	-	1	-	-	-	-	-	0.06	0.06

Table 3: MIC for drug Ketoconazole

Species	Drug concentrations in µg/ml											
	0.03	**0.06**	**0.012**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**MIC 50**	**MIC 90**
T.rubrum n=63	-	4	39	14	6	-	-	-	-	-	0.12	0.5
T.mentagrophytes n=7	1	1	2	3	-	-	-	-	-	-	0.12	0.25
T.verrucosum n=6	-	2	1	-	3	-	-	-	-	-	0.06	0.5
T.tonsurans n=1	-	1	-	-	-	-	-	-	-	-	0.06	0.06
E.floccosum n=2	-	1	-	-	1	-	-	-	-	-	0.06	0.5
M.gypseum n=1	-	-	1	-	-	-	-	-	-	-	0.12	0.12

Table 4: MIC for drug Griseofulvin

Species	Drug concentrations in µg/ml											
	0.03	**0.06**	**0.012**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**MIC 50**	**MIC 90**
T.rubrum n=63	-	8	11	42	2	-	-	-	-	-	0.25	0.5
T.mentagrophytes n=7	1	2	1	3	-	-	-	-	-	-	0.12	0.25
T.verrucosum n=6	-	1	2	3	-	-	-	-	-	-	0.12	0.25
T.tonsurans n=	-	-	-	1	-	-	-	-	-	-	0.25	0.25
E.floccosum n=2	-	1	-	1	-	-	-	-	-	-	0.06	0.25
M.gypseum n=1	-	1	-	-	-	-	-	-	-	-	0.06	0.06

Table 5: Comparison of present study results with another study

	Antifungal Drugs	Present Study			M.A.Ghannoum *et al.*, Study (12)		
		MIC range µg/ml	**MIC 50µg/ml**	**MIC 90µg/ml**	**MIC range µg/ml**	**MIC 50µg/ml**	**MIC 90µg/ml**
All Dermatophytes	Griseofulvin	0.03-0.5	0.25	0.5	0.12-64	0.12	0.5
	Ketoconazole	0.03-0.5	0.06	0.5	0.12-16	2	16
	Fluconazole	1-8	2	4	0.001-0.05	0.015	0.125
	Itraconazole	0.015-0.25	0.06	0.12	0.001-0.5	0.008	0.03

Discussion

Dermatophytic infections are a major health problem worldwide, especially in tropical countries like India.(13,14) Yet, proper methodology for testing and interpreting antifungal susceptibility is lacking in all centers due to unavailability of universal protocol for antifungal drugs. In the present study, which aimed at determining antifungal susceptibility of dermatophytes isolated from skin, hair and nail specimens with MIC range set according to drugs and MIC 50 and MIC 90 was estimated and compared. When susceptibility profile was viewed, Fluconazole showed a higher MIC values in the range of 1-8µg/ml. Itraconazole showed the lowest MIC values by micro broth dilution method and found to be the most potent drug. These findings correlate well with other studies conducted by C. J. Jessup *et al.,* and M. A. Ghannoum *et al.,* (12,15). The MIC results obtained in present study is compared and listed in Table 5. MIC values of M. A. Ghannoum *et al.*, shows that there is an increased MIC value for Griseofulvin and Fluconazole. Previously, Griseofulvin was the only drug used for treating dermatophytic infections. Clinical usage of antifungal drugs is mainly based on dosage of drugs, side effects and easy availability. Universal usage of Fluconazole is due to its low cost and dosage and its widespread availability in all level of health care centers, which in turn has turned up to, increased resistance profile for that drug. Now antifungal susceptibility profile shows promising results that newer drugs like Itraconazole are also effective.

Many of the studies were aimed at establishing the relationship between the inoculums size, optimum condition, optimum medium for conidial formation, incubation time duration and end point determination. (9,12,15) A standard reference method for antifungal susceptibility testing of dermatophytic infection is still lacking. There are studies which compared different methods, different medium for performing

susceptibility. (16,17) For the present study, micro dilution method was chosen because of its convenience, reproducibility and greater ease of performance. Due to increased awareness among clinicians about newer antifungal drugs and their promising clinical results, widespread usage has become increased. Hence it is advisory for coordinated work between testing laboratories and clinical physicians to combat arising resistance.

Conclusion

Although usage of antifungal drugs shows promising results in clinical improvements of fungal infections, determination of its susceptibility and formulation of a protocol for testing and guidelines for antifungal treatment is in need now. It is advisory to conduct research at many centers on antifungal testing to implement a universal guideline for testing and treatment of fungal infections. This aids in combating the ongoing and evolving resistance in future.

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