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Original Research Article

Mycological profile of dermatophytes and their susceptibility pattern at tertiary care centre

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Abstract

Introduction: Dermatophytosis is the commonest contagious fungal infection, commonly known as ringworm infections, more prevalent in tropical & subtropical countries like India. Here heat & moisture helps in promoting its growth. In recent years, the Physician and Microbiologist take more interest in these infections due to various reasons like indiscriminate use of antibiotics, anticancer therapy and immunodeficient diseases like AIDS; as they help in varied clinical presentation of dermathophytic infections. It is observed that resistance to antifungal drugs have started to come up in dermatophytosis. In response to increased incidence of resistance to antifungal drugs, it is necessary to determine the antifungal susceptibility testing (AFST) of isolates to available drugs. In view of above, we have undertaken this study to develop a quick, easy & reliable method of AFST by Agar Based Disk Diffusion (ABDD) method for dermatophytes.

Aim and Objective: To study a quick, easy & reliable method of AFST by ABDD method for isolated dermatophytes.

Materials and Methods: To test AFST of dermatophytic isolates, following antifungal agents were included in the study. Fluconazole, Ketoconazole, Itraconazole, Clotrimazol discs were available commertially (HIMEDIA), and Griseofulvin and Terbinafine discs were prepared in laboratory from powders which were obtained from Siemens Company. Dermatophytic isolates were tested for antifungal susceptibility by ABDD method. Strains were reported as sensitive, intermediate and resistant and were reported to dermatologist for further management.

Results & Conclusion: In this study, we found 46 dermatophytic isolates from skin, nail and hair, in which most common pathogenic dermatophyte isolate was *T. mentagrophytes* (50%), followed by *T. rubrum* (43.47%), *M. gypseum* (4.3%) and *T. tonsurans* (2.1%). AFST was performed by ABDD method which we found quick and easy. All isolates were 100% sensitive Clotrimazole. All isolates of *M.gypseum* were sensitive to all drugs. All drugs were effective against *T.tonsurans* except Fluconazole. Approximately 95% strains were sensitive to Griseofulvin. Approximately 90% strains were sensitive to Terbinafine.

Keywords: Dermatophytosis, ABDD, AFST.

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1. Introduction

Dermatophytosis is the commonest contagious fungal infection, commonly known as ringworm infections.[1-5] It is not generally life threatening condition and it is the most common disease and disorder of mankind. These superficial skin infections are attributed to two sets of fungi, dermatophytes, and Malassezia.[6]

Dermatophytic fungal species belong to three genera:[2,4,7]

1. Trichophyton - Infects skin, hair & nail
2. Microsporum - Infects skin & hair
3. Epidermophyton - Infects skin & nail

Dermatophytic fungi produce proteases (keratinase) helps in digestion of keratin[8] & colonization & infection of stratum corneum of skin, hair & nail,[10,11] but do not penetrate in deeper anatomical sites.[12] They can invade the hair

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follicles, causing folliculitis or perifollicular abscesses.[8] These patients neglect such infections and seek medical attention mostly for cosmetic purposes.[12] When host's immunity is hampered the fungus may invade deeper layers of skin and multiply to develop inflammatory granuloma called as tinea profunda.[8]

Dermatophytic infections are more common in tropical & subtropical countries like India Where heat & moisture helps in promoting its growth.[2,7,13,14] Dermatophytosis constitutes about 10% all skin diseases.[11] Overcrowding, lack of personal hygiene and exposure to animals or cases are some of the risk factors which promote the dermatophytic infections.[2,8] Decreased cellular immune response due to various factors such as malignancy, administration of immunosuppressive drugs, endocrine disorders such as Cushing's can lead to invasive dermatophytic infections,[8,15] Risk factors for dermatophytosis also include people who uses communicable bath and who are involved in sports like wrestling.[3] In recent years, the Physician and Microbiologist take more interest in these infections due to various reasons like indiscriminate use of antibiotics, anticancer therapy and immunodeficient diseases like AIDS; as they help in varied clinical presentation of dermathophytic infections.[13.14]

The clinical presentation is mostly typical of Ringworm infection, and the diagnosis is made on clinical grounds; but it is often confused with other skin infections because of rampant application of skin ointments and creams, containing broad spectrum steroid, that leading to further misdiagnosis and mismanagement.[7,16] On examination the lesions have the outer ring of active progressing infection with central healing associated with itching, redness, scaling or fissuring of the skin. An abscess or cellulitis formation may occur due to aggressive infections.[7] Clinical diagnosis has to be supported by laboratory diagnosis hence culture and microscopic examination is required for the identification of etiological agent.[15]

It is observed that resistance to antifungal drugs have started to come up in dermatophytosis. There is need to perform AFST in at least chronic or recurrent cases of dermatophytic infections or in cases of dermatophytosis with treatment failure or relapse.[17] In response to increased incidence of resistance to antifungal drugs, it is necessary to determine the antifungal susceptibility testing.[12,18] Azole resistance in dermatophytic infections is reported as 19% worldwide. Also there is alarming trend of recalcitrant dermatophytic infections in India, which might be related to inadequate treatment or discontinuation of treatment, difficulty in elimination of source of infection and predisposing factors.[17]

Various methods are available for susceptibility testing like broth micro & macro dilution, agar dilution, E test; Sensititre, Colorimetric micro dilution & disk diffusion tests. CLSI has approved a reference broth dilution method for AFST (Antifungal Susceptibility Testing) of molds (CLSI M 38 A- 2008) & its later modifications (CLSI M 38 A2 – 2010) for dermatophytes as well.[17,19,20] Broth macro dilution and micro dilution reference methods are now available for susceptibility testing of both yeasts (NCCLS document M27) and molds (NCCLS document M38), but these methods are expensive, requiring specific media and equipment such as RPMI medium, MOPS buffer, and micro titer plates.[21] To have an antifungal susceptibility testing easily available to clinical microbiology laboratories, there is a need for alternative, simple, rapid, and cost-effective method. CLSI/NCCLS has approved disk diffusion testing for antibacterial agents and it also recommends and encourages the antifungal susceptibility testing by disk diffusion method for antifungal agents.[19] Dogra et al, reviewed many articles and stated that ABDD method is much simpler and easier to perform than broth dilution method, they also advised further research is needed before incorporating this technique to test AFST for dermatophytes in routine laboratory practice.[17] However there is scarce data on disk diffusion method for antifungal agents for dermatophytes.[22]

In view of above, to give scope for more studies we have undertaken this study to develop a quick, easy & reliable method of Antifungal Susceptibility Testing (AFST) by Agar Based Disk Diffusion (ABDD) method for dermatophytes.

1.1. Epidemiology of dermatophytes

Mycotic infections are worldwide[23,24] but dermatophytic infections are common in tropical and subtropical regions, in which heat and moisture plays a significant role in promoting growth of these fungi.[13,15,16,24,25] Dermatophytosis constitutes about 10% of all skin infections.[11] Prevalence of dermatophytosis is governed by environmental conditions, personal hygiene & habits[15] & individual's susceptibility. There is increase in prevalence and incidence of fungal infections in developing countries due to immunocompromised states such as corticosteroids, use of immunosuppressive drugs, anticancer drugs and HIV positivity.[26]

2. Aim and Objective

To study a quick, easy & reliable method of Antifungal Susceptibility Testing (AFST) by agar based disk diffusion (ABDD) method for isolated dermatophytes.

3. Material and Methods

3.1. Study populations

138 clinically suspected cases of dermatophytosis were studied, specimen like hair, nail and skin were collected.

3.2. Inclusion criteria

Clinically suspected dermatophytosis cases of all age groups & of both sexes were included.[3]

3.3. Exclusion criteria

Specimen from fungal diseases other than dermatophytosis and dermatophytosis cases with secondary bacterial infections were excluded.

3.4. Sample size calculation

Sample size was calculated by using below formula.

$$n = \frac{z^2 - p(1-p)}{d^2}$$

n = Sample size
p = Prevalence or incidence = 10% = 0.10
d = Allowable error =5% = 0.05
z = 1.96 for 95% C.I.

3.4. Study design

The present study was carried out in the department of Microbiology with cooperation of dermatology outpatient department, after getting Ethics Committee approval. The study was conducted from November 2016 to March 2018 and 46 isolates of dermatophytes were studied.

3.5. Antifungal susceptibility testing

The isolated dermatophyte species were processed for AFST by ABDD method.

Control strains like *Trichophyton rubrum* ATCC 28188 & *Trichophyton mentagrophytes* ATCC 9533.[19] ATCC strains were obtained from Himedia.

3.6. Antifungal agents

Following antifungal agents were included in the study.

1. Fluconazole – 25 µg,
2. Ketoconazole – 10 µg,
3. Itraconazole – 10 µg,
4. Clotrimazole – 10 µg

Above discs were available commertially (HIMEDIA), and

1. Griseofulvin – 10 µg and
2. Terbinafine – 2 µg

Above discs were prepared in laboratory from powders which were obtained from Siemens Company.

3.7. Standardization of AFST

Standardization of AFST for dermatophytes is very difficult; there are various critical parameters those need to be considered while performing the AFST. These are inoculums size, incubation temperature and duration, media which is used and time and percentage of growth inhibition for end point detection. We considered all above parameters taking references through various studies for performing the AFST.

3.8. Antifungal susceptibility testing

In Vitro susceptibility testing is helpful as it can help clinician to choose correct drug for the patient.[27] CLSI in 2002 approved a document M38-A for AFST of filamentous fungi, not included dermatophytes.[27,28] For dermatophytes CLSI approved the broth dilution method as per M38-A2 document.[26] It is of either broth macro dilution or broth micro dilution. Broth micro dilution is more preferred over macro dilution method.[19,27] For this test required things are proper media preparation, stock solution of antifungals, and final concentration of drug solutions, inoculum preparation, and MIC testing.[1] Disk diffusion test has limited application in antifungal drug susceptibility testing. The CLSI M51-A document was released in 2010 as a reference for antifungal disk diffusion susceptibility testing for non – dermatophytic filament fungi that cause invasive infections. Various testing methods for AFST are Etest, Neo-sensitabs, Colometric methods, spectrophotometric methods,[19] flowcytometry, vitek 2 yeast susceptibility test, bioluminescence assay, and ergosterol quantitation method.[1] Unlike antibacterial susceptibility testing, in spite of availability of reference method for dermatophytes, antifungal susceptibility testing is not that much developed, it is in its infancy.[21] Data on disk diffusion method for dermatophytes are scarce.[19] The agar based disk diffusion method for dermatophytes is becoming a focus of interest for many research workers as it is a simple, inexpensive and does not require specialized equipments. But it is not approved by CLSI.[23,29,30] The disk diffusion method correlated with reference dilution method and found to have a good correlation.[22,23]

3.9. Working solution

For preparing the discs, the pure powders were dissolved in DMSO (Dimethyl Sulfoxide) to give a concentration of 1 mg/ml & 200 µg/ml for Griseofulvin & Terbinafine respectively & then 10 µl from these dilutions were delivered to sterile empty discs. Sterile discs were also be impregnated with 10 µl of 1:100 dilution of DMSO to serve as control discs. Above all discs were applied to each inoculated & dried plates & were incubated at 28^0C for 5 days.[19,23,29]

3.9.1. Storage

Working solution was stored at -20^0C, while the prepared discs were stored at 2 to 8^0C.

3.10. Inoculums preparation

Culture colony from SDCCA subcultured on PDA (Potato Dextrose Agar) & it was allowed to incubate at 28^0C for 7 – 14 days to enhance sporulation.[19,22] The pure colony was scrapped and suspended in 3 to 4 ml of sterile saline. This colony was mixed properly and vortexed; heavy particles were allowed to settle down. Superficial homogenous suspension was adjusted to 3.0 McFarland standard by matching with standard tube of 3.0 McFarland.[12]

3.11. Inoculation of MHA plates

Plates of MHA (Muller Hinton Agar) were inoculated using a swab dipped in the inoculums suspension. The swab was rotated several times firmly against the inside wall of the tube which removed excess fluid from the swab. The plates were inoculated by evenly streaking the swab over the entire agar surface. This procedure was repeated for two more times by rotating plate approximately at 60^0 angles each time.[31] Finally the rim of agar was swabbed. The inoculated plates were then dried for 15 minutes at room temperature before applying the discs.[19] Then with the help of sterile forceps the antifungal disks were applied on the inoculated plates & kept at room temperature for five days.[19]

3.12. Measuring the Inhibition zone diameter

When growth was observed on plates, the size of zone of inhibition was measured for each antifungal agent as well as control disc after 5 days of incubation at room temperature. The control discs should not have any zone of inhibition around and in this study it was found the same, as there was no any antifungal agent coated in control discs. We were classified the strains into sensitive, intermediate sensitive and resistant by following the **Table 1**. The criteria of zone of inhibition for which to say it was sensitive, intermediate sensitive or resistant was estimated by examining two control strains 20 times for the listed antifungal agents. The zone of inhibition is measured for each strain at each time, mean and standard deviation for that was calculated and following chart was prepared. If the IZD was up to mean -1SD, it was regarded as sensitive, if it was between mean-1SD to mean-2SD, it was regarded as intermediate sensitive and if the IZD was less than mean -2SD, it was regarded as resistant.

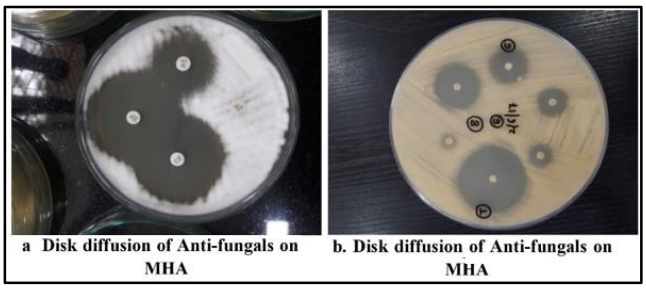

Figure 1: a & **b:** Disk diffusion of Anti-fungals on MHA plate.

Table 1: Sensitivity of the antifungal agent is decided by following the below chart

	Inhibition zone diameters			
	Mean ±SD	**Sensitive Mean – 1 SD**	**Intermediate Sensitive Mean – 1SD to – 2SD**	**Resistant Mean – 2SD**
Fluconazole	22.6±4.2	> 19	15 – 19	< 15
Itraconazole	27.70±3.7	> 24	21 – 24	< 21
Terbinafine	50.97±14.62	> 36	22 – 36	< 22
Ketoconazole	39.47±2.51	> 37	34 – 37	< 34
Griseofulvin	29.91±4.51	> 25	21 – 25	< 21
Clotrimazole	28.47±2.15	> 26	24 – 26	< 24

Strains were reported as sensitive, intermediate sensitive & resistant and were informed to Dermatologist for further management.

Table 2: Results of antifungal susceptibility testing of dermatophytes by ABDD (Agar Based Disk Diffusion) test: (total=46)

		***T.mentagrophyte* (23 isolates)**	***T.rubrum* (20 isolates)**	***M.gypseum* (2 isolates)**	***T.tonsurans* (1 isolate)**
Fluconazole	S	16 (69.56%)	13 (65%)	02 (100%)	00
	I	00	00	00	00
	R	07 (30.43%)	07 (35%)	00	01 (100%)
Itraconazole	S	20 (86.95%)	18 (90%)	02	01 (100%)
	I	03 (13.04%)	02 (10%)	00	00
	R	00	00	00	00
Terbinafine	S	21 (91.30%)	18 (90%)	02 (100%)	01 (100%)
	I	00	00	00	00
	R	02 (8.69%)	02 (10%)	00	00
Ketoconazole	S	18 (78.26%)	19 (95%)	02 (100%)	01 (100%)
	I	05 (21.73%)	01 (5%)	00	00
	R	00	00	00	00
Griseofulvin	S	22 (95.65%)	19 (95%)	02 (100%)	01 (100%)
	I	00	00	00	00
	R	01 (4.34%)	01 (5%)	00	00
Cotrimazole	S	23 (100%)	20 (100%)	02 (100%)	01 (100%)
	I	00	00	00	00
	R	00	00	00	00

4. Observations and Results

We found 46 dermatophytic isolates from skin, nail and hair, in which most common pathogenic dermatophyte isolate was *T. mentagrophytes* (50%), followed by *T. rubrum* (43.47%), *M. gypseum* (4.3%) and *T. tonsurans* (2.1%).

All 46 isolates of dermatophyte species and two ATCC control strains of *T. rubrum* and *T. mentagrophytes* were tested for AFST by ABDD.

Table 2 shows, out of 23 isolates of *T.mentagrophyte*, seven isolates were resistant to Fluconazole, two isolates were resistant to Terbinafine, one isolate was resistant to Griseofulvin; three isolates were intermediate sensitive to Itraconazole, five isolates were intermediate sensitive to Ketoconazole.

Out of 20 isolates of *T.rubrum*, seven isolates were resistant to Fluconazole, two isolates were resistant to Terbinafine and one isolate was resistant to Griseofulvin; two isolates were intermediate sensitive to Itraconazole and one isolate was intermediate sensitive to Ketoconazole.

All the two isolates of *M.gypseum* were sensitive all the drugs.

T. tonsurans was resistant to Fluconazole.

5. Discussion

Most common dermatophyte isolate in our study was *T. mentagrophytes* (50%), followed by *T. rubrum* (43.47%), *M. gypseum* (4.3%) and *T. tonsurans* (2.1%). Our finding coincides with the Findings of Soumya Nasimuddin et al[8] they also found T. mentagrophyte (38.75%) common isolate. But our this finding is not in accordance with Sabyasachi Banerjee et al (2015),[10] Kennedy Kumar et al (2004),[13] Dr. NIlekar et al (2015), [11] Hemangi Walke et al (2014),[24] Amodkumar Yadav et al (2013), Clarrisa J Lygdoh et al 92011-2012), Amita Pandey et al (2013),[16] Matnani G et al[12] (2007-2008), Gupta C M et al (2014),[25] and P.V. Doddamani et al (2013).[15] They all found *T. rubrum* was the most common species. Reason for more prevalence of *T. mentagrophyte* in our study may be due to geographical distribution or the difference in time period of the study.

In this study, out of 23 isolates of *T.mentagrophyte*, seven isolates are resistant to Fluconazole, two isolates are resistant to Terbinafine, one isolate is resistant to Griseofulvin; three isolates are intermediate sensitive to Itraconazole, five isolates are intermediate sensitive to Ketoconazole. Out of 20 isolates of *T.rubrum*, seven isolates are resistant to Fluconazole, two isolates are resistant to Terbinafine and one isolate is resistant to Griseofulvin; two isolates are intermediate sensitive to Itraconazole and one isolate is intermediate sensitive to Ketoconazole. All the two isolates of *M.gypseum* are sensitive all the drugs. *T. tonsurans* is resistant to Fluconazole. Our study shown clotrimazole was the most effective drug shown 100% sensitivity in all isolates, this finding was also revealed by Amodkumar Yadav et al. in our study we found Fluconazole is the least effective drug shown resistance in 15 isolates, similar findings were revealed by other workers like R.K. Agarwal et al,[21] Amodkumar Yadav et al,[3] Shalini Gupta et al, all had found that fluconazole was the least effective drug. Shalini Gupta et al correlated broth dilution method and disk diffusion method for AFST of dermatophyte and found good correlation between MIC and IZD of the drugs. R.K.Agarwal also found disk diffusion method was simple, reproducible, cheap and easily adaptable. Humera et al did disk diffusion testing for AFST for drugs like ITC, RAV, TRB, and VRC. They found that all strains showed measurable inhibition zones without microcolonies inside them. Against them voriconazole showed the widest IZD and Micafungin did not show any inhibition zone. Mona F et al done AFST of all isolates by ABDD method and found that most effective antifungal drugs were Clotrimazole and Miconazole. They found that ABDD was the simple, cost effective and promising method for AFST. Sudip Das et al,[20] studied dermatophytic AFST by broth dilution method, they found Itraconazole & Luliconazole were the most effective drugs in Trychophyton infections & Clotrimazole was the least effective drug. Dharmender Gupta et al, given conclusion that broth microdilution method is very cumbersome & labor intensive, so a simple method is needed for diagnostic purpose. Their study had shown good correlation & agreement between broth microdilution & disk diffusion method for AFST for dermatophytes. Murgesh Shamanur Basavarajappa et al stated that Luliconazole was the most effective drug against all dermatophytic infections, also with good susceptibility to Itraconazole, Ketoconazole & Terbinafine. But they had not tested against Clotrimazole.

6. Limitations

As there are no clinical break points defined as of now and it is urgently needed to establish epidemiological cut off values for dermatophytes. It is necessary to standardize the ABDD method by CLSI & more work, with large sample size & multi-centric studies are needed to reach a final conclusion. Limitations of this study are that, we have not compared the results of ABDD with broth microdilution method due to technical constraints and we were unable to include recent drug for AFST testing like Luliconazole and Tolnaftate, further studies can include these drugs. This may help clinicians to manage recalcitrant or resistant dermatophytosis

7. Conclusion

In the present study, *Trichophyton mentagrophytes* (50%) was the predominant isolate followed by *Trichophyton rubrum* (43.47%), *Microsporum gypseum* (4.34%) & *Trichophyton tonsurans* (2.1%) in the dermatophytes. Fluconazole showed the lowest activity and was resistant in

15 (32.60%) isolates. AFST was performed by agar based disk diffusion method which we found quick and easy.

1. All isolates were 100% sensitive Clotrimazole.
2. All isolates of *M.gypseum* were sensitive to all drugs.
3. All drugs were effective against *T.tonsurans* except Fluconazole.
4. Approximately 95% strains were sensitive to Griseofulvin.
5. Approximately 90% strains were sensitive to Terbinafine

8. Source of Funding

None.

9. Conflict of Interest

None.

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