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Microbiology Section

Comparison of Broth Microdilution Method vs Disk Diffusion Method for Antifungal Susceptibility Testing in Dermatophytosis: A Cross-sectional Study

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ABSTRACT

Introduction: Dermatophytosis is frequently associated with relapses following the interruption of antifungal therapy. The incidence of fungal infections, including resistant infections, has increased during the last few years, may be due to inadequate use of drugs or increased incidence of immunodeficiency states.

Aim: To compare disk diffusion and broth microdilution methods and to determine in vitro activity of antifungal agents which are most commonly used to treat the dermatophytic infection.

Material and Methods: This was a cross-sectional study which was conducted from November 2016 to April 2018 on 50 dermatophytic strains isolated from skin, hair and nail specimen collected from Dermatology Outpatient Department (OPD) of Shri Ram Murti Smarak Institute of Medical Sciences, Bareilly, Uttar Pradesh, India. All samples were cultured on Sabouraud Dextrose Agar (SDA) and Sabouraud's Cycloheximide, Chloramphenicol Agar (SCCA) medium and incubated at 25°C upto 21 days. Then antifungal susceptibility was done using disk diffusion and broth microdilution method. Result was analysed Statistical software namely Statistical Package for the Social Sciences (SPSS) 20.0 and graph pad were used to analyse the data.

Results: Data analysis of drug susceptibility tests involved a standard 2×2 contingency table. The isolates belong to two genera and five species namely *Trichophyton menatagrophytes* (44%), *Trichophyton rubrum* (32%), *Trichophyton violaceum* (18%), *Trichophyton verrucosum* (4%) and *Epidermophyton floccosum* (2%). The kappa value for fluconazole, itraconazole, terbinafine and griseofulvin were 0.419, 0.464, 0.444 and 0.451 respectively for disk diffusion and broth microdilution methods.

Conclusion: A good agreement was observed between disk diffusion and broth microdilution method.

Keywords: Dermatophytes, Microscopy, Subculture

INTRODUCTION

Dermatophytosis is a very common problem infecting about 20-25% of the world's population and the incidence is increasing on a steady basis [1]. Dermatophytes comprise of filamentous pathogenic fungi including three important genera of *Epidermophyton*, *Microsporum*, and *Trichophyton* which may lead to superficial infections in both humans and animals [2]. The incidence of fungal infections, including resistant infections, has increased during the last few years, and may be due to inadequate or irregular use of drugs or increased incidence of immunodeficiency states [3-5]. Antifungal-drug resistance is usually quantified using the Minimum Inhibitory Concentration (MIC), in which growth in the presence of a range of drug concentrations is measured over a defined time period according to a standard protocol [6]. Although various groups of antifungal drugs are available for dermatophytosis, cases of treatment failure have been noted worldwide [7,8]. Different species of dermatophytes may have different pattern of susceptibility to different antifungal agents [9,10]. Antifungal Susceptibility Testing (AFST) is performed to provide information to allow clinicians to select appropriate antifungal agents useful for treating a specific fungal infection and preventing resistant dermatophytes. There are several antifungal susceptibility methods like broth dilution method, agar disk diffusion method, E-test, colourimetric methods, flowcytometry, etc., that have been developed. Multicentre studies to develop a standardised antifungal susceptibility assay were initiated by the Clinical and Laboratory Standards Institute (CLSI) in 1983 [11]. Standard guidelines (M38-A2) for broth microdilution method have been proposed by the CLSI for in vitro AFST of dermatophytes and moulds [12].

As the fungal infections are rising and due to misuse of the antifungal agents as these are over the counter drugs, the resistance for common antifungal agents is rising and patients are not getting response for the treatment. Above fact prompted us to take this study and also such study was never conducted in Rohilkhand region of Uttar Pradesh which caters the patients from Rokilkhand area, adjacent Uttrakhand and Nepal. Hence, it's a novel study for this region to give insight to the dermatotologists. The present study was therefore undertaken to evaluate and compare broth microdilution method and disk diffusion method for AFST for antifungal agents which are most commonly used to treat the dermatophytic infection.

MATERIALS AND METHODS

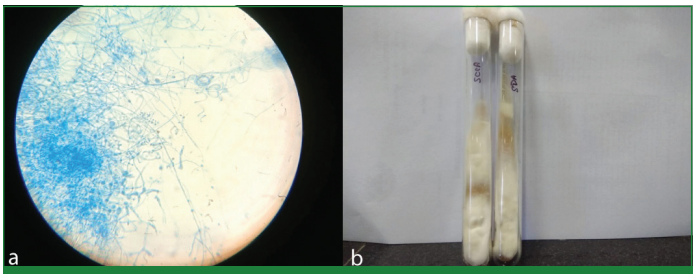
This was a cross-sectional study, conducted on the patients who attended the Dermatology OPD of Shri Ram Murti Smarak Institute of Medical Sciences, Bareilly, Uttar Pradesh, India, from November 2016 to April 2018. All the patients who were the suspected cases of fungal infection of skin, nail and hair were included. Suspected cases included the patients presenting with the symptoms like enlarging raised red rings of ringworms on skin or thicken, discoloured or crumbled nail or red scaly itchy patches or bald patches of scalp were sent to the laboratory. The study was started after obtaining approval from College Ethical Committee (IEC REF NUMBER- SRMS/2016-1767-D).

Inclusion criteria: Patients who were clinically diagnosed cases of fungal infection and Potassium hydroxide (KOH) positive and were willing for further investigations were included in the study.

Exclusion criteria: Patients not willing for investigation, and isolated fungi was other than dermatophytes on culture growth were excluded from the study.

Sample collection: Skin was decontaminated with 70% alcohol to remove surface bacterial contaminants, then skin scrapping was taken from the erythematous, peripheral, actively growing margins of the lesions. For nail scrapping and clipping hands or foot were washed with soap and water with emphasis on the nail. After drying, the nails were further decontaminated with 70% alcohol. For hair sample, after cleaning the selected area with spirit, dull lustreless hair and stubs of hairs were chosen and plucked by sterile forceps. All the scraping material was placed into a sterile container. Samples were collected in two parts, for culture sensitivity and KOH examination. Only KOH positive cases were included in the study.

Isolation of dermatophytes: The samples were cultured under sterile conditions on the SDA (Himedia, India) and SDA containing cyclohexamide (0.05%) and chloramphenicol (0.004%) (Himedia, India). The samples were incubated at 25°C upto 21 days. The culture was further examined for gross and microscopic features. The colonies on the slants of the SDA and SDA containing cyclohexamide (0.05%) and chloramphenicol (SCCA) tubes were examined for their morphology, texture and pigmentation (front and reverse) etc., upto 21 days. When growth came earlier fungus was identified on the same day. The confirmation was done by microscopic examination of the stained preparations by Lactophenol Cotton Blue (LPCB). The growth on the slants of the tubes were examined for their morphology, texture and pigmentation (front and reverse) etc., [Table/Fig-1a].

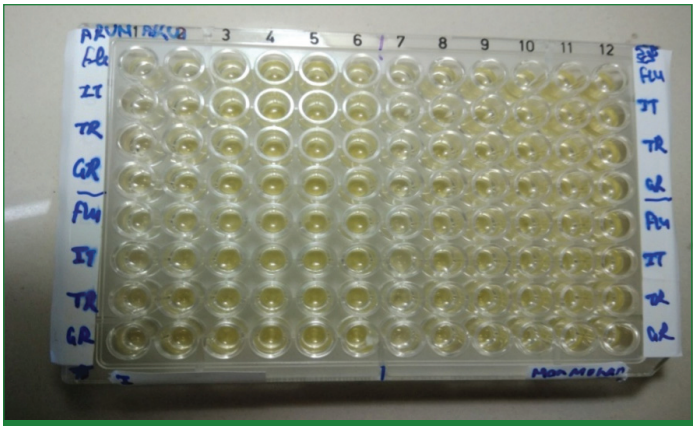


[Table/Fig-1]: Growth of *T.mentagrophyte* a) LPCB microscopic colonies of *T.mentagrophyte* in 40X; b) Slant showing growth of *T.mentagrophyte*.

Identification by microscopy and subculture: Fungus confirmation was done by microscopic examination of the stained preparations by LPCB (Himedia, India) and observed under 40X lens of light microscope [Table/Fig-1a]. The isolated dermatophytes were then sub cultured on Potato Dextrose Agar (PDA) and oatmeal agar (for *T. rubrum*) (Hi Media, India) at 28°C upto 7-15 days.

Broth Microdilution Method

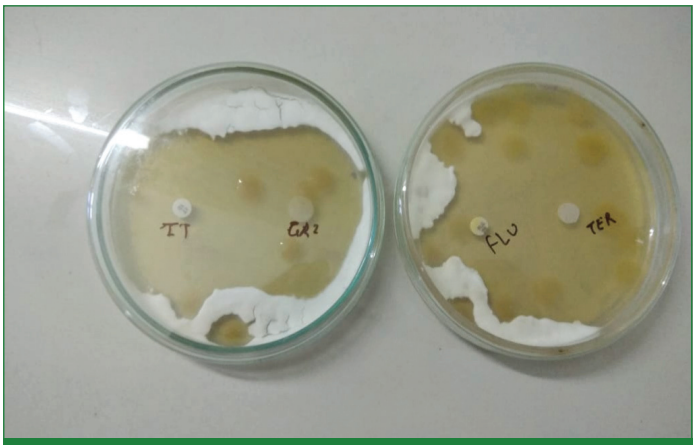
The CLSI M38-A2 guidelines were followed [6]. The test was performed in microtiter plates with Roswell Park Memorial Institute Medium (RPMI)-1640 without bicarbonate and buffered to pH 7.0 with (N-morpholino) propane sulfonic acid (MOPS). Crude powder of antifungal drugs including fluconazole, itraconazole, terbinafine and griseofulvin (Synergene active ingredients) were prepared by dissolving the powder in their specific solvent. Fluconazole was dissolved in distilled water and itraconazole, terbinafine, and griseofulvin dissolved in Dimethyl Sulfoxide (DMSO) (HI MEDIA, India). The clear suspension of inoculum having conidia was transferred to fresh tube, and its optical density was set equal to 0.5 McFarland standards. All the tests were performed in sterile, flat-bottomed, 96-well micro plates. Fungal conidial suspension was made in RPMI-1640 and its 100 µl suspension was dispensed in the wells of microtitre plate. To note the effect of the drugs 100 µL of diluted drug was added sequentially to every well of microtiter plate. Two wells were utilised for quality control and sterility control of the test. The microtiter plates were covered to check the desiccation and then incubated at 28°C and first reporting was done after seven days of incubation with the help of an inverted mirror. Concentration of fluconazole used was 2.0-64.0 µg/mL, griseofulvin was 0.125-4.0 µg/mL itraconazole was 0.62-2.0 µg/mL and for terbinafine was 0.15-0.5 µg/mL [Table/Fig-2].



[Table/Fig-2]: Antifungal Susceptibility Testing (AFST) by Broth microdilution method.

Disk Diffusion Method [13]

Commercially available disks 9 mm diameter preloaded with fluconazole 25 µg and itranconazole 10 µg were used (HI MEDIA, India). Disks containing griseofulvin 10 µg and terbinafine 2 µg were not commercially available and were prepared in the laboratory. For these two antifungal, the drugs terbinafine and griseofulvin (synergene active ingredients) were obtained in powder form. A stock solution of each drug was prepared using DMSO as follow: terbinafine 0.1 mg/mL and griseofulvin 1.25 mg/mL. Blank paper disks of Whatmann filter paper (Number 1, 6 mm and 9 mm diameter were loaded with 20 µL of the prepared stock solution to obtain the desired drug concentration per disks 2 µg and 10 µg for terbinafine and griseofulvin, respectively. Two plates of Muller hinton agar were used for sensitivity testing. A 0.5 Mcfarland turbidity dilution of every isolated strain was made and inoculated on Muller hinton agar plate by lawn culture method. On one plate the disks of terbinafine and Itraconazole were applied while on other plate the disks of fluconazole and griseofulvin were applied. Plates were inverted and incubated at 28°C upto seven days [Table/Fig-3]. Inhibition Zone Diameters (IZD) were measured in millimetres. The standard zone diameter of every antifungal agent is shown in the [Table/Fig-4].



[Table/Fig-3]: Antifungal Susceptibility Testing (AFST) by disk diffusion method (for all the four drugs).

Drugs	Potency	Zone wise interpretation (mm)		
		Sensitive	Intermediate sensitive	Resistant
Fluconazole	25 µg	≥21	15-22	≤14
Itraconazole	10 µg	≥19	11-18	≤10
Terbinafine	2 µg	≥20	12-19	≤11
Griseofulvin	10 µg	≥10	0	No Zone

[Table/Fig-4]: Criteria for interpretation of antifungal drugs.

STATISTICAL ANAYLSIS

Statistical software namely SPSS 20.0 and GraphPad were used to analyse the data. Data analysis of drug susceptibility tests involved a standard 2×2 contingency table. The rate of agreement of isolation

between two techniques was determined by the kappa statistic which was calculated using Graph pad software. Kappa value was interpreted as: <0.40 low agreements, 0.41-0.6 moderate agreement, 0.61-0.80 substantial agreement, >0.80 perfect agreement.

RESULTS

Total 63 samples were positive for KOH examination i.e. showed the presence of fungal element. All these 63 samples was cultured on the SDA and SDA containing cyclohexamide (0.05%) and chloramphenicol (SCCA) for a maximum of 21 days. Out of these 63 samples. four samples showed no growth after 21 days, one sample showed growth of bacteria while eight samples showed growth of non dermatophytic fungi, so these 13 cases were excluded from the study. Only 50 samples which showed the growth of dermatophytic fungi were processed further in the study. Out of these 50 cases, 40 (80%) were males and 10 (20%) were females. Most common age group affected was 15-30 years. The isolates belonged to 2 genera and 5 species which are as follows: *Trichophyton mentagrophyte Trichophyton rubrum*, *Trichophyton violaceum*, *Trichophyton verrucosum* and *Epidermophyton flocossum*. *T.mentagrophyte* was most frequently isolated dermatophyte in present study [Table/Fig-5].

Species isolated	Number of cases (N=50)	Percentage (%)
T.mentagrophyte	22	44
T.rubrum	16	32
T.violaceum	9	18
T.verrucosum	2	4
Epidermophyton flocossum	1	2

[Table/Fig-5]: Number of dermatophytes isolated.

In broth microdilution method [Table/Fig-6] the MIC was taken as the lowest concentration of antifungal agent that substantially inhibit growth of the organism as detected visually. The result of broth dilution sensitivity is shown in [Table/Fig-7]. The interpretation of sensitivity pattern by Antibiotic Disk Diffusion (ABDD) is shown in [Table/Fig-8].

Species		FLU	ITR	TER	GRI
T. mentagrophyte	GM	19.32	0.213	0.122	0.427
	MIC50	32	0.25	0.12	0.5
	MIC90	64	0.5	0.125	0.95
	Range	2-64	0.062-0.5	0.015-0.25	0.25-1
T. rubrum	GM	24.67	0.25	0.125	0.369
	MIC50	32	0.125	0.125	0.25
	MIC90	64	0.5	0.125	0.75
	Range	4-64	0.062-1	0.015-0.25	0.125-4
T. violaceum	GM	24.78	0.198	0.052	0.5
	MIC50	16	0.25	0.06	0.5
	MIC90	38.4	0.3	0.25	0.5
	Range	4-64	0.062-0.5	0.015-0.25	0.25-0.5
T. verrucosum	GM	32	0.353	0.125	0.353
	MIC50	40	0.375	0.125	0.375
	MIC90	59.2	0.475	0.125	0.475
	Range	16-64	0.25-0.5	0.062-0.125	0.25-0.5
E. flocossum	MIC	2	0.5	0.5	2
	Range	2	0.5	0.5	2
Total	GM	20.25	0.205	0.072	0.411
	MIC50	32	0.25	0.122	0.5
	MIC90	64	0.5	0.137	1
	Range	2-64	0.062-2	0.015-0.5	0.125-4

[Table/Fig-6]: In vitro activities of four antifungal drugs against dermatophytes by broth microdilution method.
FLU: Fluconazole; ITR: Itraconazole; TER: Terbinafine; GRI: Griseofulvin

On comparison of Disk Diffusion and Broth Microdilutuion method an attempt was made to correlate MICs obtained in broth micro dilution method with zone of inhibitions obtained in disk diffusion method [Table/Fig-9].

Dermatophytes	Drugs			
	FLU	ITRA	TER	GRI
T.mentagrophyte (n=22)	16 (72.7%)	16 (72.7%)	21 (95.4%)	19 (86.4%)
T.rubrum (n=16)	11 (68.7%)	10 (62.5%)	15 (93.7%)	10 (62.5%)
T.violaceum (n=9)	5 (55.6%)	6 (66.7%)	7 (77.8%)	9 (100%)
T.verrucosum (n=2)	1 (50%)	1 (50%)	2 (100%)	2 (100%)
Epi. flocossum (n=1)	0	1 (100%)	1 (100%)	0

[Table/Fig-7]: Result of broth microdilution sensitivity.
FLU: Fluconazole; ITR: Itraconazole; TER: Terbinafine; GRI: Griseofulvin

Dermatophytes	Drugs			
	FLU	ITRA	TER	GRI
T.mentagrophyte (n=22)	7 (31.8%)	13 (59%)	18 (81.8%)	19 (86.4%)
T.rubrum (n=16)	5 (31.3%)	8 (50%)	12 (75%)	13 (81.25%)
T.violaceum (n=9)	4 (44.4%)	6 (66.7%)	4 (44.4%)	9 (100%)
T.verrucosum (n=2)	0	2 (100%)	2 (100%)	2 (100%)
Epidermophyton flocossum (n=1)	1 (100%)	1 (100%)	0	0

[Table/Fig-8]: Result of disk diffusion sensitivity.
FLU: Fluconazole; ITR: Itraconazole; TER: Terbinafine; GRI: Griseofulvin

	Broth microdilution	Disk diffusion		Total	Kappa	p-value
		S	R			
FLU	S	17	16	33	0.419	0.0006
	R	0	17	17		
	Total	17	33	50		
ITR	S	28	10	38	0.464	0.0001
	R	2	10	12		
	Total	30	20	50		
TER	S	36	9	45	0.444	0.0001
	R	0	5	5		
	Total	36	14	50		
GRI	S	39	3	42	0.451	0.0001
	R	4	4	8		
	Total	43	7	50		

[Table/Fig-9]: Agreement between broth microdilution and disk diffusion.
FLU: Fluconazole; ITR: Itraconazole; TER: Terbinafine; GRI: Griseofulvin

For fluconazole, out of 50 strains, 17 (34%) strains were found sensitive by both disk diffusion and broth microdilution method, 16 (32%) strains were found to be sensitive by broth microdilution method but resistant by disk diffusion method, no strain was found resistant by broth microdilution method and sensitive by disk diffusion method, and 17 (34%) strains were found resistant by both disk diffusion and broth microdilution method. For itraconazole, out of 50 strains, 28 (56%) strains were found sensitive by both disk diffusion and broth microdilution method, 10 (20%) strains were found to be sensitive by broth microdilution method but resistant by disk diffusion method, 2 (4%) strains were found resistant by broth microdilution and sensitive by disk diffusion method, and 10 (20%) strains were found resistant by both disk diffusion and broth microdilution method. For terbinafine, out of 50 strains, 36 (72%) strains were found sensitive for both disk diffusion and broth microdilution method and 9 (18%) strains were found to be sensitive by broth microdilution method but resistant by disk diffusion, no strain was found resistant by broth microdilution method and sensitive by disk diffusion method, and 5 (10%) strains were found resistant by disk diffusion and broth microdilution method.

For griseofulvin, out of 50 strains, 39 (78%) strains were found sensitive by both disk diffusion and broth microdilution, 3 (6%)

strains were found to be sensitive by broth microdilution method but resistant by disk diffusion, 4 (8%) strains was found sensitive by disk diffusion and resistant by broth microdilution, 4 (8%) strains were found resistant by both disk diffusion and broth microdilution method. In this study, statistical agreement between disk diffusion and broth microdilution method was represented by kappa statistic. The agreement between two methods was found to be 0.419, 0.464, 0.444 and 0.451 respectively. While the p-value calculated using Graph pad, were 0.0006, 0.0001, 0.0001 and 0.0001 which showed high significance.

DISCUSSION

Dermatophytosis is a major health problem in tropical and subtropical countries, yet remains unresolved. Therefore, it is essential that good laboratory methods should be available for the rapid and precise identification of the dermatophytes involved in order to apply appropriate treatment and prevention measures. In the present study, a higher incidence of dermatophytosis was seen in males than in females i.e., 4:1, which was supported by Agarwal E et al., [14]. Male predominance is due to increased outdoor physical activities and increased sweating. Most of the infections were seen in the younger age group between 15-30 years of age due to working culture which predisposes them to the hot and humid climatic conditions. In addition, personal hygiene and the nature of the job also act as an additive factor in the occurrence of dermatophytosis in young adults. Similar findings were observed by Peerapur BV et al., reported higher incidence in 21-30 years age group [15].

In present study, dermatophytes isolated belong to 2 genera (*Trichophyton* and *Epidermophyton*) and 5 species. No *Microsporum* species were isolated. *Trichophyton* species were the most common species isolated. Among *Trichophyton, T.mentagrophytes* was the most common species isolated in 44% patients followed by *Trichophyton rubrum* in 32%, *T. violaceum* in 18%, and *T.verrucosum* in 4%. *Epidermophyton flocossum* was isolated in 2% patients. Similar findings were observed by Pakshir K et al., and Gadangi I, in their study also showed the higher incidence of *Trichophyton rubrum* [16,17]. The reverse trend in present study was due to the fact that *T.rubrum* is usually linked to chronic dermatophytoses. In present study, most cases were reported within six months of onset of disease. Besides, extended use of antifungal therapy to treat patients might have also reduced the occurrence of this species in the region.

In this study, four antifungal agents were used including fluconazole, itraconazole, terbinafine, and griseofulvin which were tested against 50 dermatophytic strains of clinical origin using disk diffusion and broth microdilution methods.

On analysing the results by disk diffusion method, in *Trichophyton* maximum number of cases were found sensitive to griseofulvin i.e., 87.7%, followed by terbinafine 73.5%, still followed by itraconazole i.e., 60% and least for fluconazole i.e., 34%, which were consistent with the study done by Zaki MS et al., found griseofulvin and itraconazole have good activity and fluconazole displayed no visible inhibition zone [18]. Pakshir K et al., found griseofulvin was sensitive in 92.5% cases [16]. As far as broth microdilution was concerned, MIC50, MIC90 and geometric mean values were determined for all antifungal drugs. When susceptibility profile was viewed, fluconzole showed higher MIC50 value 32 µg/mL and terbinafine showed the lowest MIC50 value 0.122 µg/mL. Similar findings correlates with the Gupta S et al., has also reported higher MIC50 value for fluconazole and lowest MIC50 value for terbinafine [19].

There was moderately significant relation observed between the AFST of fluconazole, itraconazole, terbinafine and griseofulvin by disk diffusion and broth microdilution method. In this study, the relation between both methods for fluconazole was 0.419 (kappa), for itraconazole was 0.464, for terbinafine was 0.444 and for griseofulvin was 0.451 and p-value for fluconazole, itraconazole, terbinafine and griseofulvin were 0.0006, 0.0001, 0.0001 and 0.0001, respectively. The study conducted by Alim E Al et al., showed highly significant agreements with kappa for fluconazole, itraconazole, and terbinafine were 0.878, 1.00 and 0.947, respectively, p-value was 0.0001, 0.0001 and 0.0001, respectively [20]. Contrary to these, in a study of evaluation of broth microdilution and disk diffusion methods for AFST of dermatophytes made by both Singh J et al., and Mendez CC et al.,, reported contrary results with poor or no correlation between broth microdilution and disk diffusion [21,22], they justified their results by the use of Dermasel agar medium which is probably not be the ideal medium for AFST. Torres B et al., stated that the factors that may affect the results of broth microdilution and disk diffusion are type and size of inoculums, composition of the media, temperature and duration of incubation and disc strength [23]. Establishing the relationship between the inoculums size, optimum conditions, medium for conidial formation incubation time duration and end point determination is of utmost importance. A standard reference method for AFST of dermatophytic infection is still lacking.

Limitation(s)

Though the good association of broth microdilution and disk diffusion was found on the study, the broth microdilution is cumbersome and costly and also it cannot be conducted on everyday basis on every patient. The disk diffusion test do not have the above limitations but to standardise this method for AFST further comparative study on more number of samples is needed to reach to any conclusion.

CONCLUSION(S)

As broth microdilution method is very cumbersome and labour intensive, so a simple method is always needed. The disc diffusion method is very simple and easy to conduct, so it can be the best substitute of the broth microdilution method. As present study has shown good correlation and agreement between two methods, so for daily purpose disk diffusion susceptibility testing can be used for determining the fungal sensitivity. Though its standardisation by CLSI and more work is needed for generation of a final conclusion.

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