MINIMUM INHIBITORY CONCENTRATION ESTIMATION AGAINST MULTIDRUG RESISTANCE DERMATOPHYTES SPECIES USING BROTH MICRODILUTION METHOD

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Abstract:

Antifungal drug resistance and a lack of clinical response in 20% of cases of dermatophytosis demand valuation of resistant dermatophytes using a standardized simple and reproducible in vitro analyze, to permit a clinician to select the suitable antifungal agent based on the susceptibility of the isolate to the antifungal agent. Skin, hair and nail samples were collected from patients already under antifungal treatment cases of dermatophytosis. A total of 118 isolates were tested for minimum inhibitory concentration (MIC) against four antifungal drugs in the study. Itraconazole, ketoconazole, terbinafine and fluconazole, was the antifungal drugs tested using the broth microdilution method. MIC50 and MIC90 values were recorded. A total of 118 dermatophytes isolates were tested. Dermatophytic isolates in this study were T. mentagrophyte-80, T. rubrum-31, T. interdigitale-05 and M. gypseum-02.MIC90 value for terbinafine and fluconazole was significantly higher for all isolated dermatophytes. MIC of 68.64 % isolates for terbinafine and 35.59% isolates for fluconazole were lower than the cut-off value, which indicates that not all treatment failure cases are due to drug resistance.

Keywords: Dermatophytes, Minimum inhibitory concentration (MIC), Antifungal drugs, Broth microdilution method

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1. Introduction

Dermatophytosis is a superficial fungal infection that affects millions of people worldwide with an estimated lifetime risk of 10-20 %. The pathogens responsible for superficial fungal infections are dermatophytes, yeasts and moulds. Dermatophytes are the most commonly encountered causative agent of superficial fungal infections; most important are tinea infections which are classified according to the body site affected (Sahin et al, 2004). Dermatophytosis is an infection of the skin, hair and nails caused by dermatophytes, a group of related filamentous fungi also known as ringworm fungi. These can be classified into three groups' anthropophilic, zoophilic and geophilic depending on their natural habitat and host preferences (Kwon-Chung et al, 1992).Dermatophytes have been classified into three genera of Trichophyton, Microsporum and Epidermophyton(Rippon, 1998). As the transmittance of the dermatophytoses merely requires contact and low personal hygiene, its occurrence in a community may become persistent. Keeping in view the fact that dermatophytoses and other fungal infections are readily caught by immunocompromised individuals they are increasing critically at a sharp rate(Burkhartet al, 2003).The establishment of a reference antifungal susceptibility testing method may allow the clinician to select the appropriate therapy for the treatment of infections caused by dermatophytes fungal (Sowmya et al,2015) (Graseret al, 2007).Although the exact role of drug resistance in treatment failure is not clearly understood, all species of dermatophytes do not have the same pattern of susceptibility to different antifungal agents. In vitro, antifungal susceptibility testing could therefore, prove helpful in the better management of dermatophytosis because effective antifungal agents for the optimization of antifungal therapy can be selected by this method by determining minimum inhibitory concentrations (MICs) of these agents. Broth macro- and micro-dilution methods, agar dilution and disc diffusion methods are routinely used for this purpose (Bueno et al, 2010) (Nwezeet al, 2007). For determining MICs, Clinical and Laboratory Standards Institute (CLSI) approved protocol M38-3rd edition for filamentous fungi including dermatophytes has been recommended in its guidelines of (CLSI, 2017).The present study is the antifungal resistance pattern of an isolate from the skin, hair and nails of human patients to itraconazole, ketoconazole, terbinafine, and fluconazole.

2. Materials and Methods

Study Area

It is a prospective study of clinically suspected cases of superficial fungal infection from the Rama hospital which is located in Mandhana, Kanpur Nagar (U.P.) from February 2017 to May 2018. The study was also approved by the ethical committee of Rama Medical College. The hospital provides medical services to the town population and the surrounding rural and peri-urban areas. A total of 118 isolates (T. mentagrophyte-80, T. rubrum-31, T. interdigitale-05 and M. gypseum-02) were tested for MIC against 4 antifungal drugs in this study. Patients already under antifungal treatment were excluded from the study groups. After the detailed history, a clinical examination of the patient was made in good light which will include the site of the lesion, number of lesions, types, presence of inflammatory margin, etc. The infected skin, hair and nail sample were collected, KOH mount of the samples was prepared and cultured on Sabouraud's dextrose agar with 0.05% chloramphenicol and 0.5% cycloheximide (Hi-Media) incubate at 25°C and 37°C up 2-4week. Positive cultures were slide culture, urease test, hair perforation teats, gram stain and L.P.C.B. stains were done for the identification of the dermatophytes.

Antifungal agents

The antifungal agents used in this study were itraconazole, fluconazole, (Metro Chem API Pvt. CTD Erragadda, Hyderabad, India), ketoconazole (Arti drugs Ltd., Thane, Maharashtra, India), and terbinafine (Shreeji Pharma International, Sarabhi, Vadodara, Gujarat, India) in powdered form were used in the study.

Determination of antifungal susceptibility testing

Broth micro dilution method M38-3rd edition approved protocol of (CLSI, 2017) for filamentous fungi will be followed for determining the susceptibility of dermatophyte species. Drug dilutions: Stock dilutions of itraconazole, ketoconazole and terbinafine were prepared in dimethyl sulfoxide (HiMedia) and fluconazole in sterile distilled water according to the standard protocol. The two-fold dilutions of the stock solution were further prepared in RPMI 1640 medium with L-glutamine and without sodium bicarbonate (HiMedia). These dilutions were used in the test at a pH of 7.0 ± 0.1 with 3- (N-morpholino) propane sulfonic buffer (HiMedia) along with 1N NaOH. The antifungal final concentrations will be 0.5 µg/ml to 64 µg/ml for fluconazole and 0.0078 µg/ml to 128 µg/ml for itraconazole, ketoconazole and terbinafine.

Preparation of inoculums of dermatophyte species

Cultures of dermatophyte species (7–8 days old) grown on SDA slants at 25°C were used to prepare inoculums. The fungal growth was covered with 5

ml of sterile normal saline (0.9%) and suspensions prepared by scraping the growth from the surface of the slants with a sterile swab that contain conidia and hyphal fragments. The heavy particles were allowed to settle down for 10–15 min. The upper clear suspension was transferred to a fresh tube, and its optical density was set equal to 0.5 McFarland standards. The final cell density was set between 2 × 103 and 6 × 103 colony-forming units per ml. which was used in the assay.

Testing procedure

Flat-bottomed, 96 well microtitre plates having 8 rows and 12 columns were used to perform the susceptibility tests. Eight test organisms in a volume of 100 µl each were placed in the wells of 8 rows of the plates (one test organism in each row). The dilutions (100 µl) of the drugs were added in each well of ten columns of the plate from left to right. The concentration of the drug was highest in the first column and decreases from left to right. The contents were incubated at 35°C for 4–5 days. The 11th and 12th columns will contain inoculated positive controls and un-inoculated negative control respectively.

Quality control reference strains

Candida parapsilosis strain ATCC-22019, C. krusei strain ATCC-6258, T. rubrum ATCC-28188,T. mentagrophytes ATCC-9533 and M. canis ATCC-36299 were used as quality control reference strains as approved by the CLSI and their susceptibilities to itraconazole, ketoconzole terbinafine and fluconazole were also tested. The plate containing these strains was incubated at 25°C for 48 h, as recommended by CLSI.

Determination of minimum inhibitory concentration values

The MIC values of a drug were defined as the lowest antifungal concentration at which no growth is visible in the wells when detected visually (80–100% inhibition). These values for each drug were read and recorded.

Figure 1: Determination of minimum inhibitory concentrations (MICs) of itraconazole by micro-broth dilution method against different dermatophyte isolates.

Statistical analysis

The mean values, MIC range, MIC50 and MIC90 values were determined for all antifungal agents, used in the assay, as per the standard protocol. The statistical analysis was done by t-test using SPSS 20 software to find the independence of the variables or whether they had similarities in their MIC values with $P < 0.005$.

3. Results

A total of 118 isolates (T. mentagrophyte-80, T. rubrum-31, T. interdigitale-05 and M. gypseum-02) were entered in this study after clinical identification with conventional diagnostic approaches such as KOH microscopy and culture method.

Table 1: Antifungal susceptibility test result of Itraconazole and Ketoconazole by MIC method

Dermatophytes Species	MIC (µg/mL)												MIC 50	MIC 90
	0.008	0.0156	0.0312	0.0625	0.125	0.25	0.5	1	2	4	8	16		
Itraconazole														
T. mentagrophyte	**10**	**12**	**15**	**33**	**9**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0.0625**	**0.125**
T. rubrum	**0**	**3**	**9**	**14**	**2**	**2**	**1**	**0**	**0**	**0**	**0**	**0**	**0.312**	**0.25**
T. interdigitale	**2**	**2**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.008**	**0.312**

M. gypseum	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.0156**	**0.0625**
Ketoconazole														
T. mentagrophyte	**5**	**9**	**16**	**42**	**4**	**4**	**0**	**0**	**0**	**0**	**0**	**0**	**0.0312**	**0.125**
T. rubrum	**0**	**2**	**6**	**13**	**5**	**2**	**3**	**0**	**0**	**0**	**0**	**0**	**0.0625**	**0.25**
T. interdigitale	**0**	**0**	**1**	**3**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.0625**	**0.125**
M. gypseum	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.0312**	**0.125**

Table 2: Antifungal susceptibility test result of Terbinafine and Fluconazole by MIC method

Dermatophytes Species	**MIC (µg/mL)**												**MIC 50**	**MIC 90**
	0.0312	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**		
Terbinafine														
T. mentagrophyte	**3**	**2**	**10**	**18**	**12**	**10**	**8**	**5**	**3**	**4**	**2**	**3**	**0.25**	**2**
T. rubrum	**1**	**3**	**4**	**3**	**9**	**3**	**4**	**0**	**1**	**1**	**2**	**0**	**0.5**	**2**
T. interdigitale	**0**	**0**	**1**	**0**	**1**	**0**	**2**	**1**	**0**	**0**	**0**	**0**	**0.125**	**2**
M. gypseum	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0.0312**	**2**
Fluconazole														
T. mentagrophyte	**0**	**0**	**0**	**2**	**7**	**8**	**15**	**30**	**8**	**5**	**2**	**3**	**0.5**	**16**
T. rubrum	**0**	**0**	**0**	**0**	**0**	**0**	**5**	**18**	**3**	**2**	**2**	**1**	**2**	**32**
T. interdigitale	**0**	**0**	**0**	**0**	**2**	**0**	**2**	**1**	**0**	**0**	**0**	**0**	**0.5**	**4**
M. gypseum	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0.5**	**4**

The majority of the itraconazole was observed to be inhibiting T. mentagrophytes effectively and also observed lower MIC which ranged from 0.008 µg/ml to 0.25 µg/ml. Ketoconazole also observed similar MIC range from 0.008 µg/ml to 0.25 µg/ml as that of itraconazole. Terbinafine was also observed with higher MIC ranging from 0.0312 µg/ml to 64 µg/ml. T. mentagrophytes were also observed with higher MIC values for fluconazole which ranged from 0.25 µg/ml to 64 µg/ml. Itraconazole showed lower MIC values for T. rubrum which was 0.015 µg/ml to 0.5 µg/ml. Ketoconazole also observed a similar MIC range between 0.015 µg/ml to 0.5 µg/ml as that of itraconazole. MIC range of terbinafine was 0.0312 µg/ml to 32 µg/ml which was also observed higher than that of itraconazole and ketoconazole. MIC range of T. rubrum for fluconazole was 2 µg/ml to 64 µg/ml.Isolates of T. interdigitale, were also observed and subjected to antifungal MIC testing. Itraconazole and ketoconazole were also observed in MIC towards the lower side. MIC range for itraconazole and ketoconazole were 0.008 µg/ml to 0.0312 µg/ml and 0.0312 µg/ml to 0.125 µg/ml, respectively. Similar to other isolates, fluconazole and terbinafine exhibited higher MIC ranging from 0.125 µg/ml to 4 µg/ml and 0.5 µg/ml to 4 µg/ml respectively.Isolates of M. gypseum MIC ranges for Itraconazole and ketoconazole again showed MIC towards the lower side. MIC range for itraconazole and ketoconazole were 0.0156 µg/ml to 0.0625 µg/ml and 0.0312 µg/ml to 0.125 µg/ml, respectively. Similarly, to other isolates, fluconazole and terbinafine exhibited higher MIC values from 0.0312 µg/ml to 2 µg/ml and 0.5 µg/ml to 4 µg/ml, respectively.Dermatophyte isolates is presented MIC values of >1 µg/ml for itraconazole, ketoconazole, terbinafine and >2 µg/ml for fluconazole and were classified as resistant (Ghannoum MA et al, 2004) (Sardana Ket al, 2018) and (European Committee on Antimicrobial Susceptibility Testing Antifungal Agents Breakpoint, 2015) Isolates resistant to terbinafine and fluconazole were 31.36% and 64.41%, respectively. While isolates that were sensitive to terbinafine and fluconazole were 68. 64% and 35.59%, respectively. While isolates of MIC values for itraconazole and ketoconazole were 100%, respectively. [Table 3]

Table 3: Table showing number of isolates as per cut-off value

Antifungals	No. of isolates below cut-off value	No. of isolates above cut-off value
Itraconazole	118 (100%)	0 (0%)
Ketoconazole	118 (100%)	0 (0%)
Terbinafine	81 (68. 64%)	37 (31.36%)
Fluconazole	42(35.59%)	76 (64.41%)

4. Discussion

Dermatophytosis form over 50% to 75% of all the mycological infections. The diagnosis of a dermatophytic infection is mostly done clinically, but often confused with other skin infections due to the topical application of steroid ointments and creams, leading to further misdiagnosis and mismanagement (Panda S and Verma S, 2017). Hence, there arises the need for the correct, efficient, and rapid laboratory diagnosis of dermatophytes(Mercy et al, 2014) (Bhatia and Sharma , 2014)The most common dermatophyte species which were isolated in our study were T. mentagrophytes and T. rubrum which are predominant worldwide, but less frequently reported in Africa (Havlickovaet al, 2008). M. gypseum was less frequently isolated in the present study.

Some researchers followed the protocol M38-A2 by CLSI in 2008 for determining the susceptibility of dermatophytes that was intended for filamentous fungi (Motaet al, 2009). Later, the document was modified to the M38-3rd edition of (CLSI, 2017). This document also includes the protocolfor dermatophyte which has been followed by us for determining the MIC values of itraconazole, ketoconazole, terbinafine and fluconazole against different dermatophyte species. The unavailability of such reference method previously was due to the difficulty in the standardization of some parameters such as temperature, incubation time, selection of growthmedium etc., for different species of dermatophytes (Jessup et al, 2000). In the present study, we incubated T. rubrum, T. mentagrophyte, T. interdigitale and M. gypseum at 25°C and 35°C as mentioned in the M38-3rd edition of CLSI, 2017 protocol. Some researchers have obtained better growth of dermatophyte species at 28°C (Pujol et al, 2002) (Da Silva et al, 2007) and (Araújo et al, 2009). For determining the MICs of itraconazole, ketoconazole, terbinafine and fluconazole the cultures of T. mentagrophyte, T. interdigitale and M. gypseum were incubated for 3-4 days and T. rubrum for 5-7 days as good growth was observed after incubation of specific period. Good inhibitory activity of all the four antifungal agents against T. mentagrophyte, T. rubrum, T. interdigitale and M. gypseum was demonstrated in the present study [Table 1, 2]

In this study all dermatophytic isolates T. mentagrophytes, T. rubrum, T. interdigitale, and M. gypseum recorded higher MIC 90 values against fluconazole and terbinafine indicating higher chances of treatment failure when treated with these drugs. These higher MIC values for fluconazole have also been reported by other authors previously (Pathaniaet al, 2018) (Sardana et al, 2018) , (Sabtharishi et al, 2017) and(Aktaset al, 2014)Clinical inefficacy with the treatment of terbinafine has been reported by many authors (Marcoux et al, 2018) ,(Singh and Shukla , 2018) and (Majid , 2016) . Similar to our study, higher MIC values against terbinafine have as well been reported from India (Pathaniaet al, 2018)and (Dabas et al, 2017) . Itraconazole and ketoconazole had lower MIC values for all species of dermatophytes, which indicates that these drugs could be the better choice for the successful treatment of dermatophytic infections. Many authors from India and abroad have reported similar findings with itraconazole and ketoconazole (Pathaniaet al, 2018) (Sardana et al, 2018), (Sabtharishi et al, 2017) and (Aktaset al, 2014).37 isolates (31.36%) showed higher MIC against terbinafine (i.e. cut-off MIC > 1 μg/ml) and 76 isolates (64.41%) against fluconazole (i.e. cut-off MIC >2 μg/ml). Patients with these isolates were switched over to itraconazole, as it carried fewer adverse effects compared to others. No patient was switched over to ketoconazole. Patients with isolates having lower MIC values for fluconazole or terbinafine were advised to continue the same treatment and were advised to keep personnel hygiene and affected area dry. With the implementation of the above strategies all treatment failure cases of dermatophytosis were treated successfully.

5. Conclusion

This study highlights the prevalent dermatophytes in Kanpur, India and their antifungal susceptibility. Antifungal susceptibility testing also helps to realize the epidemiological pattern of drug resistance in some given regions, and thus may help to prefer more effective antifungal agents for standard treatment. Only the broth micro dilution method is currently accepted to determine the in-vitro susceptibility of dermatophytes. When this method is laborious and requires expertise, only few mycology laboratories can perform this test. In the present scenario ofincreasing resistance to dermatophytes, these studies will go a long way tohelp clinicians choose the most suitable therapy.

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Conflicts of interest

The authors declare that there is no potential conflict of interest associated with this study.

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