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Antifungal Susceptibility Testing of Dermatophytes by Agar Based Disk Diffusion Assay in Tertiary Care Hospital, Nepal

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SK, JBS, BMP and BR were responsible for study design and supervision of work. Authors SK and SD were responsible for laboratory work. Authors SK, SD and RM were responsible for data analysis and manuscript preparation. All authors read and approved the final manuscript.

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ABSTRACT

Background: Dermatophytes are fungi, which cause most of superficial fungal infections. Several antifungal agents are commercially available to treat these dermatophytes.
Methods: We adopted a newly developed agar based disk diffusion assay to test susceptibility of 72 clinically isolated dermatophytes belonging to 5 species *Trichophyton interdigitale* **(**44), *Trichophtyon rubrum* (13), *Trichophyton tonsurans* (6), *Microsporum canis* (6), and *Epidermatophyton floccosum* (3). The process involved the application of four commercially available anti-fungal disks namely Ketoconazole (15 µg), Miconazole (10 µg), Fluconazole (25 µg) and Clotrimazole (10 µg) for antifungal susceptibility testing.
Results: The study shows miconazole is the most effective antifungal drugs against dermatophytes followed by ketoconazole and clotrimozazole.

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Conclusions: The disk diffusion assay method for antifungal susceptibility testing of dermatophytes species in this *in vitro* study may give insights into the application in therapeutic strategy against dermatophytic infections. Disk diffusion method is a simple and cost-effective for susceptibility testing of dermatophytes.

Keywords: Dermatophytes; antifungal test; disk diffusion method.

ABBREVIATIONS

KOH mount : Potassium Hydroxide Mount
SDA : Sabourad's Dextrose Agar
MHA : Mueller Hinton Agar
DTM : Dermatophyte Test Medium
PDA : Potato Dextrose Agar
LPCB : Lacto Phenol Cotton Blue

1. BACKGROUND

Dermatophytes are a homogenous group of fungi that live on the keratin of the stratum corneum, nails, and hair, which have the ability to utilize keratin as a nutrient source in living animals including man. They are important cause of superficial infections (dermatophytosis) affecting several millions of people worldwide and the risk of acquiring a dermatophyte infection in lifetime is estimated between 10–20% [1]. The type and severity of the host response is often related to the species and strain of dermatophytes causing the infection. Patients who have compromised epidermis, poor hygiene, live in crowded conditions, have co-morbidities, and have close contact with people having skin and soft tissue infections are at high risk of acquiring a skin and soft tissue infection themselves.

There are several antifungal agents to treat these dermatophytes. Azole based antimycotics agents block the conversion of lanosterol to ergosterol by inhibiting ezymelanosterol 14 α-demethylase. Finally, it disrupts structure and function of fungal membrane leading to inhibition of fungal growth. In general selection of antifungal agents will be dependent on the probable microorganisms causing infections. Some of infections respond well to topical anti- fungal agents but more extensive or severe diseases require a systemic therapy while in some cases, antifungal therapy is a failure because of resistance to the antifungal drugs by the fungi. As the availability of various antifungal drugs to treat dermatophytosisis increasing, it is important to evaluate the resistant dermatophytes using standardized, simple and reproducible *in vitro* assay to determine the antifungal activity of drugs against isolates. There are several methods for antifungal susceptibility of dermatophytes such as micro and macro dilution, agar dilution, E test, sensititre, colorimetric dilution and disc diffusion are available globally among which dilution tests are widely used in micro and macro assays but these methods are difficult to use in most laboratories [2].

The disk diffusion *in vitro* assay is a simple, easy to perform and economical method that can be used to evaluate antifungal susceptibility testing of dermatophytes in developing countries which in general shows a good correlation with the reference method for micro dilution antifungal susceptibility testing [3]. Advantages of a standardized disk diffusion-based assay for evaluating the antifungal susceptibility of dermatophytes include the ease of use, reproducibility, accuracy, and low cost [3,4,5].

This study was carried out to determine the antifungal susceptibilities pattern of dermatophytes from clinical specimens by using simple, inexpensive, accurate method of agar based disk diffusion assay.

2. METHODS

The present study was conducted on 72 clinically diagnosed patients with dermatophytes strains belonging to 5 species *T. interdigitale* **(**44), *T. rubrum* (13), *T. tonsurans* (6), *M. canis* (6), and *E .floccosum* (3) who visited department of Dermatology, Tribhuvan University Teaching Hospital, Kathmandu over a period of six months from January to June 2014. The samples from patients were collected in aseptic conditions from infected areas such as skin, nail and hair [6,7]. Specimens were processed at department of clinical Microbiology for direct microscopic examination (KOH mount) and fungal culture as per standard protocol [8]. Culturing of organisms from skin, nail and hair was done on selective medium as Sabouraud Dextrose Agar with or without cycloheximide for identification of dermatophytes species. Isolation and identification of dermatophytes was done based on macroscopic observation of fungal colonies as

well as lactophenol cotton blue (LPCB) mount microscopic examination as shown in Figs. 1-4. Antifungal susceptibility testing was performed after identifying them on cultural, morphological and biochemical characteristics [9,10]. A total of 4 commercially available drugs namely Ketoconazole (15 µg), Miconazole (10 µg), Fluconazole (25 µg) and Clotrimazole (10 µg) were used for antifungal susceptibility testing.

2.1 Inoculum Preparation

Dermatophytes were sub cultured on potato dextrose agar & incubated at 30℃ for 7 days to enhance sporulation. Following the fungal growth, Culture was harvested in 1ml distilled water and colonies were probed with the help of pipette to obtain mixture of mycelium and conidia. Dense inoculum suspension of conidia and hyphal elements was transferred to sterile test tube and allowed to sediment for 30 minutes. After the settlement of heavy particles, the upper homogeneous suspensions were transferred to another sterile tube and were adjusted with a spectrophotometer set at 65% transmittance and 530 nm [4].

Fig. 1. Macroscopic view of *M. canis* (Forward view)

2.2 Disk Diffusion Assay

Plates of Muller Hinton Agar (MHA), with 2% glucose were inoculated using a cotton swab dipped in the standardized conidial and hyphal suspension and are exposed to air dry. The four antifungal drugs were then applied to MHA plates and after which were incubated at 28℃ for 5-10 days. After the growth of colonies on plates, the sizes of zone of inhibition around the antifungal disks were measured. Antifungal criteria of sensitive, intermediate and resistance pattern of antifungal disks were reported by measuring zone of diameter in mm according to Pakshir et al. [11]. The data were analyzed using Microsoft SPSS.

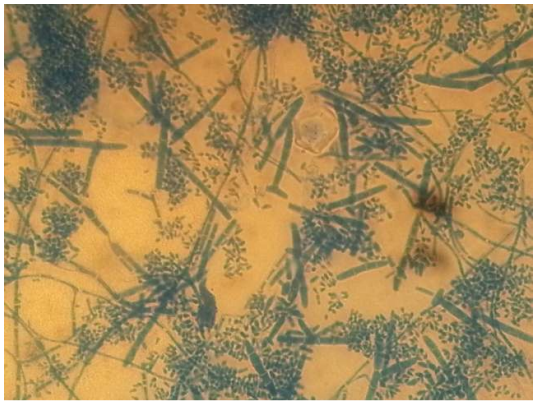

Fig. 2. Microscopic view of *T. mentagrophytes* (LPCB mount, 400X)

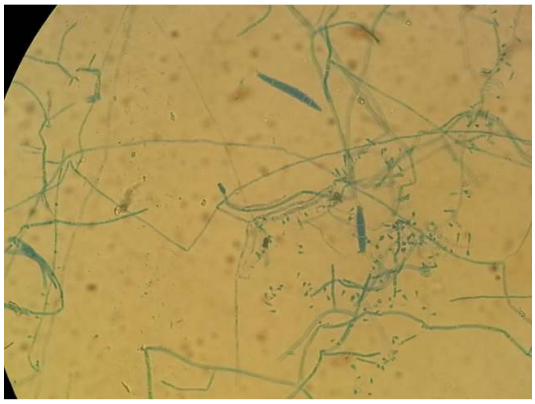

Fig. 3. Microscopic view of *M. canis* (LPCB mount, 400X)

Fig. 4. Macroscopic view of *T. mentagrophytes* (Forward view)

Table 1. Antifungal susceptibility test of dermatophytes

Results	Antifungal drugs			
	Ketoconazole (15µg)	Miconazole (10 µg)	Fluconazole (25 µg)	Clotrimazole (10 µg)
Sensitive	60(83.4%)	68(94.5%)	-	63(87.5%)
Intermediate	8(11.1%)	1(1.3%)	72(100%)	-
Resistance	4(5.5%)	3(4.2%)	-	9(12.5%)

3. RESULTS

A total of 72 clinically diagnosed dermatophytes were isolated and identified. The isolates belong to three genera and five species *T. interdigitale* **(**44), *T. rubrum* (13), *T. tonsurans* (6), *M. canis* (6), and *E. floccosum* (3). These isolates subjected to susceptibility testing by agar based disk diffusion method. The test results of the susceptibility to antifungal drugs were as follows: Ketoconazole (15 µg): 60(83.4%) sensitive, 8(11.1%) intermediate, 4(5.5%) resistance. Miconazole (10 µg): 68(94.5%) sensitive, 1(1.3%)intermediate, 3(4.2%) resistance. Fluconazole (25 µg): 72(100%) intermediate. Clotrimazole (10 µg): 63(87.5%) sensitive, 9(12.5%) resistance. It was showed that miconazole was the most sensitive drugs followed by ketoconazole and clotrimazole. The results of the susceptibility to antifungal drugs with disk diffusion method are summarized in Table 1.

4. DISCUSSION

A standardized disk diffusion based assay for determining the antifungal susceptibility testing of dermatophytes is desirable and has number of advantages. In developing countries disk diffusion assay will provide an aid to antifungal susceptibility test as several studies shows that this assay is not only reproducible and accurate but also economical and very easy to perform [5,12,13].

Antifungal susceptibility testing by disk diffusion has become an important means in treatment of patients with fungal infections in developing countries. In line with the availability of an increasing array of antifungal agents, both intrinsic and emergent antifungal drug resistance are encountered [14]; There is a need for accurate, reproducible and predictive susceptibility testing of fungal isolates in order to help inform clinical choice.

The standard disk diffusion assay can be adapted for assessment of dermatophyte resistance against antifungal drugs. Some studies suggest that disk diffusion method is reproducible and reliable method that has good correlation with reference method for micro-dilution antifungal susceptibility test [15,16,17]. However, study done by Singh et al. could not find a significant correlation between micro-dilution and disk diffusion methods, probably due to their use of Dermasel agar medium [18]. This medium is unacceptable for antifungal susceptibility testing. In our study we found that ketoconazole and miconazole had larger inhibition zones around the disks whereas fluconazole had poor activity against isolates, almost no zone of inhibition around the disks. There are some other studies, which indicate that fluconazole had less activity against dematophytes [14,16]. *In vitro* determination of antifungal activity of fluconazole against dermatophytes has variable results because of the use of different methods and media [4,18].

5. CONCLUSIONS

We have concluded that miconazole is most effective antifungal drugs against dermatophytes followed by ketoconazole and clotrimozazole. Disk diffusion testing provides a simple and cost-effective way for laboratories to accomplish *in vitro* susceptibility testing for dermatophytes in comparison with micro dilution methods, which plays an increasingly important role in decision making for choice of drugs.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The ethical approval for study was taken from Institutional Review board, Institute of Medicine (IOM), Tribhuvan University Teaching Hospital, Nepal.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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