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Antifungal susceptibility testing of dermatophytes isolated from animals by disc diffusion assay

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Abstract
Dermatophytosis is a common cause of cutaneous mycoses in animals and humans. The most commonly encountered dermatophytes are *Microsporum*, *Trichophyton* and *Nannizia* species. In the study, we evaluated the antifungal susceptibility of 10 *T. mentagrophytes* complex isolates, 8 *Nannizzia* isolates, 4 *Arthroderma* isolates, and 30 *M. canis* isolates against seven commercially available antifungal discs (HiMedia) including miconazole (30 µg) fluconazole (10 µg), nystatin (50 µg), ketoconazole (30 µg), itraconazole (30 µg), clotrimazole (10 µg) and amphotericin B (20 µg). Clotrimazole was found to have the highest mean diameter of the zone of inhibition in *Microsporum*, *Trichophyton* and *Nannizzia* isolates. The lowest zone of inhibition was exhibited by fluconazole in all the tested isolates. Even though disc diffusion assays are not commonly performed, they will provide preliminary information about the most useful antifungal agent before initiating the therapeutics.

Keywords: *Trichophyton*, *Microsporum*, *Nannizzia*, *Arthroderma*, disc diffusion, antifungal agents

1. Introduction
Dermatophytosis is a superficial skin disease commonly affecting the animals and humans. The dermatophytes comprises of the genera *Microsporum*, *Trichophyton*, *Epidermophyton*, *Nannizzia*, *Arthroderma*, *Praphyton* and *Lophohyton* (De Hoog *et al*., 2017) [1]. It is a zoonotic disease and can be transmitted to humans from pet and livestock animals. A very limited spectrum of antifungal agents is commercially available for the treatment of dermatophytosis. The most commonly used drugs are azoles such as ketoconazole and itraconazole and allyamines such as terbinnafine. However, like antibiotic resistance, antifungal resistance is also an emerging concern among dermatophytes (Yamada *et al*., 2017; Salehi *et al*., 2018; Hsiao *et al*., 2018) [2, 3, 4]. Antifungal drug resistance is a main obstacle in the treatment of dermatophytosis among humans and animals as it may lead to refractory and recurrent infections. (Yamada *et al*., 2017; Salehi *et al*., 2018) [2, 3].
For the antifungal susceptibility testing of dermatophytes, broth microdilution is commonly performed to assess the minimum inhibitory concentration values (MIC 50 and MIC 90). But the assay is time consuming and the observations are prone to variations with the individuals. The disc diffusion assay is not generally performed for dermatophytes and guidelines are not provided by Clinical Laboratory and Standards Institute (CLSI). However, certain researchers have attempted disc diffusion assay for dermatophytes (Esteban *et al*., 2005) [5]. In the present study, we evaluated the antifungal susceptibility of dermatophytes isolated from animals using disc diffusion assay.

2. Materials and Methods
2.2 Preparation of inoculum
The dermatophyte isolates were recovered from samples collected from the farms and teaching veterinary clinical complex of Indian Veterinary Research Institute; Bihar Veterinary College, BASU; College of Veterinary Science, GADVASU and other private enterprises from different states of India. A suspension containing conidia and hyphae was used as the test inoculum in the disc diffusion assay for dermatophytes. The isolates were sub-cultured on potato dextrose agar and incubated at 28 ºC for 7-14 days or till sufficient growth was visible. The culture plates were flooded with 0.85% sterile normal saline solution and with the help of a sterile micro tip the surface was gently agitated.

The dense suspension containing hyphal fragments and conidia was collected in a 15 ml Eppendorf tube. The tubes were allowed to stand for 15-20 minutes to settle down the heavy particles. The upper homogenous suspension was collected in a fresh 15 ml Eppendorf tube and adjusted to a transmittance of 65% with the help of a spectrophotometer at 530 nm.

2.3 Inoculation of the test medium

Antifungal assay agar (HiMedia) was used to perform the disc diffusion assay. A sterile cotton swab was dipped in the tubes containing the transmittance-adjusted suspension. The swabs were used to streak the surface of the agar plates along with rotating the plates. The procedure was repeated three times and special care was taken to streak the edges of the agar plates.

2.4 Appliation of antifungal discs

Seven commercially available antifungal discs (HiMedia) including miconazole (30 µg) fluconazole (10 µg), nystatin (50 µg), ketoconazole (30 µg), itraconazole (30 µg), clotrimazole (10 µg) and amphotericin B (20 µg) were used in the study. The discs were applied on the agar surface using sterile forceps. The discs were distributed as a set of 4 discs on one plate and 3 discs on another plate. The discs were gently pressed into the agar using forceps. The plates were then incubated at 28 ºC for 4-7 days. The zone of inhibition was measured and expressed in millimetres.

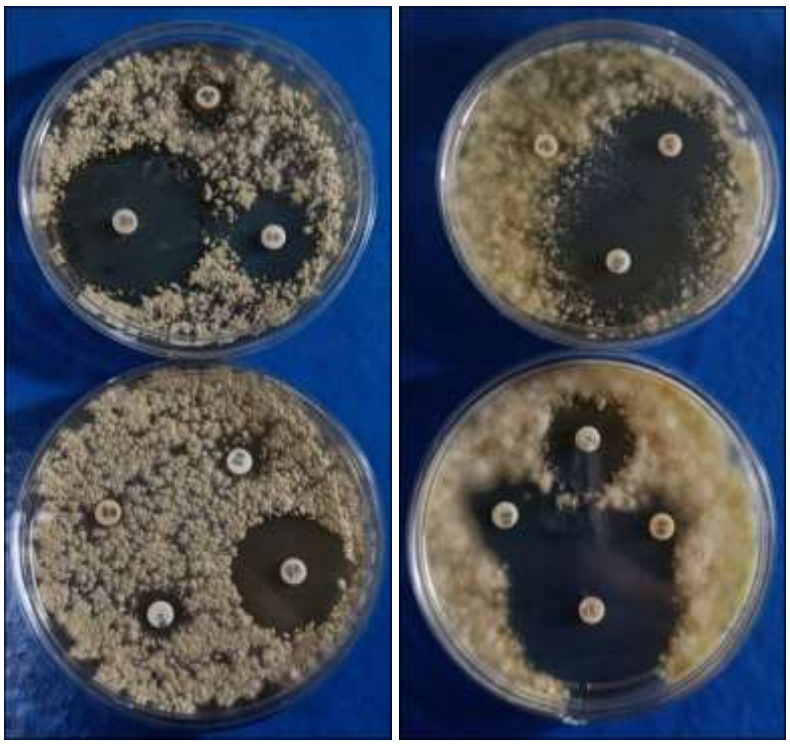
3. Results and Discussion

Disc diffusion assay was performed for 10 *T. mentagrophytes* complex isolates (Isolate TM1 to TM10), 8 *Nannizzia* isolates (Isolates N1 to N8), 4 *Arthroderma* isolates (Isolates A1 to A4) and 30 *M. canis* isolates (Isolates MC1 to MC30) (Figure 1 and 2). The zone of inhibition of different antifungals against the dermatophyte isolates are mentioned in Table No. 1. Fluconazole (FLC 10) was found to have the least zone of inhibition with all the tested isolates. In the case of *T. mentagrophytes* complex isolates, clotrimazole (CC 10; 20.7mm) and miconazole (mic 30 17.5 mm) were having the largest mean zone of inhibition. Similarly, clotrimazole (18.625 mm) and miconazole (16.5 mm) were also having the highest mean zone of inhibition against *Nannizzia* isolates. In the case of *M. canis* isolates clotrimazole (22.46 mm) and miconazole (21.43 mm) were having an almost similar mean zone of inhibition. A study conducted by Begum and Kumar, 2021also observed similar findings in the antifungal susceptibility of dermatophytes using the disc diffusion method. The highest zone of inhibition was exhibited by clotrimazole and the lowest by fluconazole. In our study also the mean zone of inhibition in *T. mentagrophytes* complex, *Nannizzia* spp. And *M. canis* was highest for clotrimazole. In the study conducted by Esteban *et al*., 2005 [5] clotrimazole was found to have a high zone of inhibition after terbinafine.

Table 1: Zone of inhibition of antifungal discs against *Trichophyton*, *Microsporum*, *Nnanizzia* and *Arthroderma* species isolates

Sl. No.	Isolate	CC 10 (mm)	FLC 10 (mm)	KT 30 (mm)	AP 20 (mm)	NS 50 (mm)	IT 30 (mm)	MIC 30 (mm)
	***Trichophyton mentagrophytes* complex**							
1	Isolate TM1	35	0	15	6	8	14	14
2	Isolate TM2	30	0	8	0	6	17	13
3	Isolate TM3	15	0	8	5	8	6	18
4	Isolate TM4	9	6	14	5	10	15	21
5	Isolate TM5	25	0	15	0	5	5	17
6	Isolate TM6	9	0	14	0	9	6	20
7	Isolate TM7	30	0	20	4	9	25	19
8	Isolate TM8	25	0	30	0	10	16	25
9	Isolate TM9	13	0	21	5	5	9	20
10	Isolate TM10	16	0	11	4	5	7	8
	Mean	20.7	0.6	15.6	2.9	7.5	12	17.5
	Standard Deviation	9.44	1.89	6.65	2.55	2.06	6.48	4.79
	Standard Error	2.98	0.6	2.10	0.80	0.65	2.04	1.51
	***Nannizzia* spp.**							
1	Isolate N1	20	0	17	4	8	8	18
2	Isolate N2	21	0	16	5	10	10	17
3	Isolate N3	19	0	9	5	9	7	15
4	Isolate N4	18	0	7	0	7	7	16
5	Isolate N5	15	0	10	4	8	8	17
6	Isolate N6	20	5	11	0	0	6	14
7	Isolate N7	16	0	10	5	6	8	16
8	Isolate N8	20	0	10	5	7	8	19
	Mean	18.625	0.625	11.25	3.5	6.875	7.75	16.5
	Standard Deviation	2.13	1.76	3.45	2.20	3.04	1.16	1.60
	Standard Error	0.75	0.625	1.22	0.77	1.07	0.41	0.56
	***Arthroderma* spp.**							
1	Isolate A1	30	0	20	5	10	10	25
2	Isolate A2	19	0	25	8	13	10	25
3	Isolate A3	20	0	21	14	17	10	24
4	Isolate A4	21	0	18	19	15	7	22
	Mean	22.5	0	21	11.5	13.75	9.25	24
	Standard Deviation	5.06	0	2.94	6.24	2.98	1.5	1.41
	Standard Error	1.96	0	1.14	2.41	1.15	0.58	0.54

	Microsporum canis							
1	Isolate MC 1	29	0	27	6	0	0	20
2	Isolate MC 2	21	0	22	5	0	12	22
3	Isolate MC 3	25	0	23	13	6	13	22
4	Isolate MC 4	16	0	18	5	0	11	22
5	Isolate MC 5	27	0	21	5	0	6	20
6	Isolate MC 6	18	0	16	8	0	10	21
7	Isolate MC 7	28	0	15	7	0	0	21
8	Isolate MC 8	19	0	16	7	0	11	22
9	Isolate MC 9	20	0	21	11	8	14	26
10	Isolate MC 10	24	0	19	6	0	7	21
11	Isolate MC 11	24	0	20	6	0	6	22
12	Isolate MC 12	26	0	18	10	0	0	22
13	Isolate MC 13	28	0	24	7	0	10	21
14	Isolate MC 14	22	0	7	6	9	5	15
15	Isolate MC 15	21	0	10	6	6	6	24
16	Isolate MC 16	25	0	12	7	10	6	22
17	Isolate MC 17	21	0	15	5	7	5	21
18	Isolate MC 18	23	0	14	5	6	4	20
19	Isolate MC 19	20	0	14	5	8	0	25
20	Isolate MC 20	26	0	20	6	7	21	20
21	Isolate MC 21	18	0	15	0	5	10	21
22	Isolate MC 22	25	0	10	0	7	0	21
23	Isolate MC 23	25	0	11	0	0	5	20
24	Isolate MC 24	25	0	14	0	0	0	22
25	Isolate MC 25	16	0	12	5	8	12	23
26	Isolate MC 26	25	0	11	6	0	0	25
27	Isolate MC 27	25	0	10	0	7	10	20
28	Isolate MC 28	17	0	13	0	9	10	20
29	Isolate MC 29	15	0	11	4	7	10	20
30	Isolate MC 30	20	0	10	0	6	10	22
	Mean	22.46	0	15.63	5.03	3.86	7.13	21.43
	Standard Deviation	3.91	0	4.98	3.38	3.80	5.23	2.01
	Standard Error	0.71	0	0.91	0.61	0.69	0.95	0.36
	Control strains							
1	*T. mentagrophytes* ATCC	21	0	13	7	11	12	20
2	*M. canis* ATCC	32	0	25	12	14	10	30



(A) (B)

Fig 1: Disc diffusion assay of *Nannizzia* spp. isolate (A) and *M. canis* isolate (B)

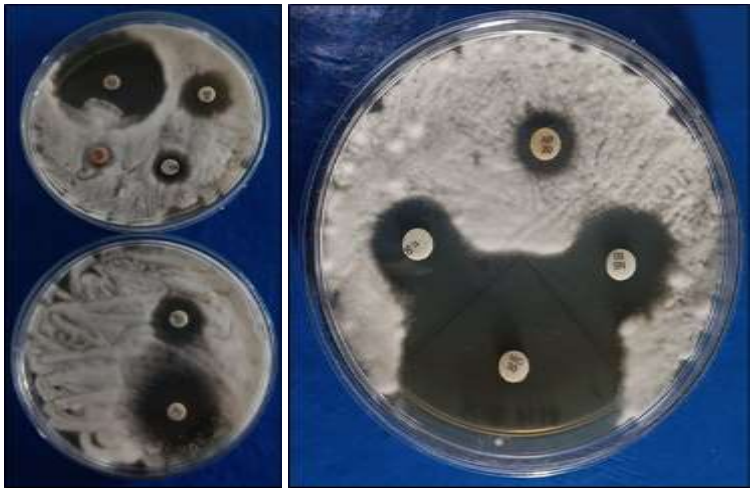


Fig 2: Disc diffusion assay of *T. mentagrophytes complex* isolate (A) and *Arthroderma* spp. isolate (B)

4. Conclusion

In this study, clotrimazole was identified as the most effective drug against commonly encountered dermatophytes. The disc diffusion assay can be used to test dermatophytes but the variation in the conidia production and the growth rate among the dermatophyte species necessitates further streamlining of the protocols. Since there are no established guidelines for interpreting the results of the disc diffusion assay for dermatophytes, the isolates cannot be classified as resistant, intermediate sensitive or sensitive. Nevertheless, the disc diffusion assay can be performed in laboratories lacking facilities for the broth microdilution assay, providing initial information on the most sensitive antifungal agent that can be used for treating tinea or ringworm.

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