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In-vitro Antifungal Susceptibility of *Malassezia* Dermatitis in Dogs

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Authors' contributions

This work was carried out in collaboration among all authors. All the authors have played role in relation to conceptualization, its methodology, original draft preparation, investigation, data curation, supervision, editing, validation and resources. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Malassezia dermatitis is a highly pruritic type of dermatitis which mainly produces itching, erythema as its primary skin lesion and secondary skin lesions includes scaling, seborrhoea, maldour, lichenification and hyperpigmentation. The present study was conducted to compare the antifungal efficacy of Ketoconazole, Itraconazole, and Terbinafine against *Malassezia* spp. and to evaluate the antifungal sensitivity of *Malassezia* species isolated from dermatitis cases in dogs in and

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around Jabalpur, Madhya Pradesh. A total of 100 skin swabs were collected from dogs with dermatological symptoms suggestive of *Malassezia* spp., out of which 87 swabs were successfully isolated and showed good growth on Sabouraud's Dextrose Agar (SDA) during primary isolation from the skin swabs. For this, commercially available antifungal discs of Ketoconazole and Itraconazole were used whereas for Terbinafine antifungal disc was made due to lack of its availability. Based on *in-vitro* antifungal susceptibility studies, it can be concluded that Ketoconazole, Itraconazole, Fluconazole and Terbinafine were found to be effective against *Malassezia* dermatitis and can be advocated as the drug of choice for the treatment of *Malassezia* dermatitis in dogs.

Keywords: Dermatitis; Malassezia spp. Ketoconazole; itraconazole; terbinafine; fluconazole; dogs.

1. INTRODUCTION

Skin problems in dogs are one of the most common and difficult problems encountered by veterinarians in small-animal practice [1]. Various skin problems such as parasitic, fungal, bacterial, viral skin diseases and allergies occur in dogs. Since allergic dermatitis is one of the most common underlying causes, diagnostic investigation for allergy is often indicated [2]. Factors related to climate change like cold, heat, light, sunshine, and humidity determine the incidence of skin diseases [3,4]. Several studies from India and abroad have indicated that skin affections make up to 12-75% of the small animal population [5].

Malassezia spp. is one of the infectious agents of concern that might harm pets and pet owners. *Malassezia* dermatitis in dogs typically develops as a secondary issue as a result of underlying skin conditions including canine atopic dermatitis and flea allergy dermatitis, recurring bacterial pyoderma, and endocrine problems (particularly hypothyroidism) [6]. *Malassezia* dermatitis is an inflammatory skin condition that affects dogs and is characterized by an overgrowth of the fungus *M. pachydermatis* on the skin. This opportunistic pathogen infects the commensal skin in humans and the saprophytic yeast in animals and birds. These fungi are present in the stratum corneum layer of skin which is rich in lipid and may vary in size between 1 to 8 micrometers in diameter. *Malassezia* dermatitis is also known as elephant skin disease and it occurs usually when the protective barrier of the skin is either disrupted or when the immune system is suppressed. Breeds with skin folds are predisposed to *Malassezia* overgrowth. *Malassezia* dermatitis is the most common cutaneous mycotic infection in dogs. This fungus exists in both yeast and mycelial forms, with the mycelial form known as *Malassezia* and the yeast form known as *Pityrosporum*. The genus *Malassezia* belongs to the Phylum *Basidiomycota* and comprises 14 species based on their morphology, biochemical features and molecular analysis [7-10].

Malassezia yeast is known to produce proteolytic enzymes, which can cause damage to the epithelium, leading to the enlargement of ceruminous glands and hyperplasia. The disease often starts in the summer with an increase in humidity, and persists throughout the winter. Excessive sebum production, epidermal barrier disruption, moisture accumulation, concurrent and atopic dermatitis, bacterial skin infections in domestic animals etc. can all favour yeast proliferation. It can affect dogs of any breed, age or sex [11]. *Malassezia* dermatitis has zoonotic potential as it is a commensal infection in human being which is transmitted by human health-care workers from their pet dog to neonatal patients [12].

Antifungal treatments can cause systemic disturbances, when used for a long time. Resistance developed by yeast to conventional drugs, reoccurrence of the condition, long duration of treatment, and high treatment costs pose significant challenges for both pet owners and veterinarians. Therefore, it is necessary to develop more economical antifungal agents with minimal side effects to address these challenges. Therefore, the present study aimed to compare the antifungal efficacy of itraconazole and terbinafine against *Malassezia* isolates from dogs with dermatitis.

2. MATERIALS AND METHODS

2.1 Sample Collection

Hundred skin swabs from various lesions were collected from the dogs suspected for malasseziosis based on the clinical signs of pruritus, macule, papule, pustule, alopecia, erythema, rancid odour, scaling, hyperpigmentation and lichenification from the

dogs which were brought to the Veterinary Clinical Complex (V.C.C.), Department of Veterinary Medicine, College of Veterinary Science and A.H., N.D.V.S.U., Jabalpur (M.P.).

Sterile cotton swabs moistened with sterile distilled water were used to collect the samples from the suspected dogs. The cotton swab was rolled and rubbed firmly against the entire skin area for 10 seconds from the dogs showing dermatological problems suggestive of *Malassezia* dermatitis [13]. The swab was then kept in a sterile test tube and stored at -20ºC until further laboratory procedure.

2.2 Antifungal Agents

Ketoconazole, Itraconazole, Fluconazole and Terbinafine were selected for the present study due to their widespread use in veterinary medicine. Commercial discs are available. Typical concentrations of 10 µg/disc, 25 µg/disc and 50 µg/disc might be used depending on the testing requirements. Commercially available discs of Ketoconazole (10 µg), Itraconazole (10 µg) and Fluconazole (25 µg) were used in the present study whereas for Terbinafine antifungal discs were prepared by the following procedure.

2.2.1. Preparation of antifungal discs

A. Materials Required: For the preparation of antifungal discs, antifungal agents such as Itraconazole, Terbinafine, Ketoconazole, or Fluconazole, either in tablet or powder form were used. Solvents like sterile water, ethanol, or saline were required to dissolve the antifungal agents. Whatman filter paper discs (sterile) of 6 mm were used for the discs with sterile forceps needed to handle them. Sterile petri-plates were used to place the discs and a micropipette with sterile tips for accurate measurement and dispensing of solution. A laminar flow cabinet is also required to maintain a sterile environment during the preparation process.

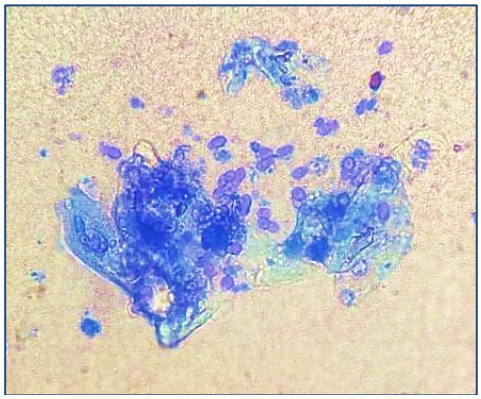

Fig. 1. Microscopic examination of acetate tape impression positive for *Malassezia* organism (Methylene blue staining x1000)

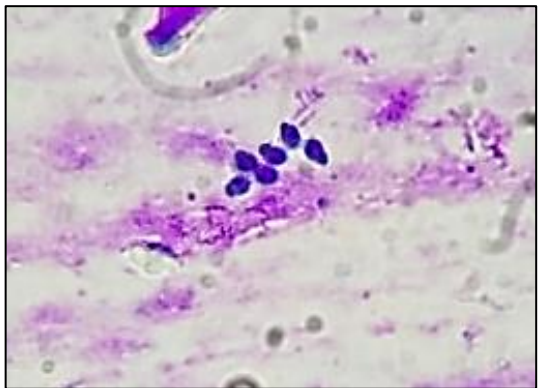

Fig. 2. Microscopic examination of sterile cotton swab smear positive for *Malassezia* organism (Diff-Qik Stain x1000)

B. Procedure: A suspension of the test organism was prepared by the direct colony suspension method. Four distinct and morphologically similar colonies were taken with a sterile cotton swab and transferred into test tubes containing sterile deionized water. After homogenization of the yeast suspension, comparisons were made with the 0.5 McFarland turbidity standards, which is equal to 1-5 x 10^6 CFU/ml [14]. A sterile cotton swab was dipped into the suspension and rotated several times. Excess fluid was removed from the swab by pressing firmly against the inside wall of the tube. The lawn culture was stripped on the SDA plates and the plates were placed in the refrigerator for 2 hours for better absorption of the culture.

1. Preparing Stock Solutions

- Dissolve the antifungal powder in an appropriate solvent to make a stock solution. For this, weigh an accurate amount of Terbinafine (powder/tablet) – 250 mg in 25 ml of saline/distilled water to prepare a 10 mg/ml stock solution.

2. Soaking Antifungal Discs

- Place sterile Whatman filter paper discs in a sterile petri-plate. Using a micropipette, carefully apply a specific volume of the stock solution onto each filter paper disc. For example, to prepare a 10 µg/disc of Terbinafine concentration, pipette 1 µl of a 10 mg/ml stock solution onto the disc ensuring the solution to be evenly distributed across the disc.

3. Drying the Discs

- Allow the impregnated discs of Terbinafine and incubated at 37ºC overnight and later dried under the laminar airflow. The produced discs have the ability to absorb about 0.01 ml.

4. Storing the Discs

- Once dry, store the antifungal discs of Terbinafine in sterile petri dishes or vials at an appropriate temperature (typically 4°C) until use. Ensure that the discs are protected from light and moisture.

5. Labelling the Discs

- Clearly label each petri-dish or vial with the type of antifungal drug, its concentration and date of preparation.

2.3 Isolation and Identification of *Malassezia* Yeast

Sterile cotton swabs were used for the collection of samples from dogs which were found positive for *Malassezia* organism. The swabs were inoculated in Sabouraud's Dextrose Agar (SDA). The plates were then incubated at 37ºC for 24-72 hours. Colony characters were studied by observing shape, size, colour and consistency of colonies. Identification of species *Malassezia pachydermatis* was based on the macroscopic and microscopic appearance of colonies and its ability to grow on the medium with no lipid supplementation. Pure cultures were preserved at 4ºC and the organisms were sub-cultured at two weeks interval [15]. Out of 100 clinical swabs, 87 swabs showed good growth of *Malassezia* organism on SDA.

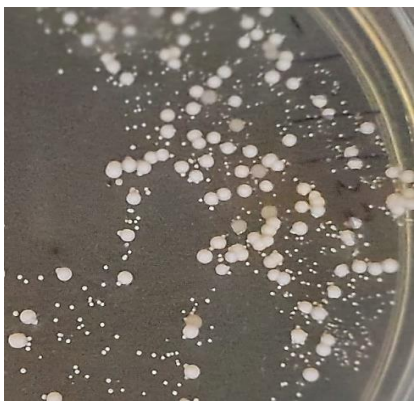

Fig. 3. Colony morphology of *Malassezia* on Sabouraud's Dextrose Agar (SDA) showing smooth, convex, white or creamy coloured colonies

3. RESULTS AND DISCUSSION

"Samples collected from dogs with symptoms suggestive of *Malassezia* dermatitis were incubated at 37ºC for 7 days on SDA. Growth of *Malassezia* was observed from 4-7 days on SDA. Based on the above results, it can be concluded that SDA is a preferable medium for the isolation of *Malassezia* organism" [16]. "The colonies of *Malassezia* spp. were macroscopically visible over 3-5 days when incubated at a temperature of 37ºC whereas, the growth was weak when incubated at room temperature (25ºC). The colonies were raised or high convex and smooth with cream colour initially and later became dry, wrinkled and orange to brown in colour" [16].

For microscopic examination, individual colonies were picked up, smeared over clean glass slides stained with Methylene blue solution, air dried and examined under an oil immersion objective (x1000) for the presence of characteristic footprint or peanut shaped organisms (Fig. 4).

3.1 Antibiogram for *Malassezia spp.* Isolates

"Antifungal susceptibility test was carried out as per the standard disc diffusion method" as described by Bauer et al. [17]. Sabouraud's Dextrose Agar (pH 5.6 ± 0.2) was employed for antifungal susceptibility tests. Following antifungal discs with the mentioned concentrations were applied over the plated SDA. Itraconazole (It) 10 µg, Ketoconazole (Kt) 10 µg and Fluconazole (Flu) 25 µg discs were commercially available whereas Terbinafine disc (T) 10 µg was prepared by the procedure of preparation of antifungal discs.

The discs produced were placed at an equal distance from each other over the agar surface and pressed gently to ensure full contact. The antifungal impregnated plates were incubated in the inverted position at 37ºC for 48 hrs. The diameter of the bacterial inhibition zone around each applied disc was measured. The interpretation of zone of diameter was carried out according to the standards laid down by Clinical Laboratory Standards Institute (CLSI), formerly known as National Committee for Clinical Laboratory Standards (NCCLS). The diameter of zone of inhibition was translated into sensitive or resistance.

Details of the antifungal susceptibility test of *M. pachydermatis* to antifungal drugs are presented in Table 1.

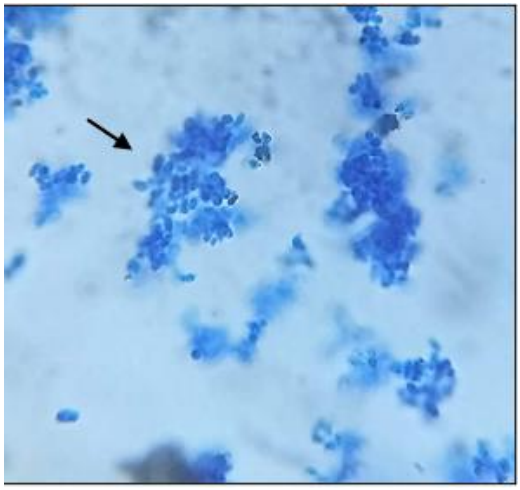

Fig. 4. Microscopic examination of smear from pure colony showing peanut or footprint shaped blue coloured budding yeasts (Methylene Blue Staining (1000X)

Table 1. *In-vitro* antifungal sensitivity pattern of *Malassezia pachydermatis*

S.no.	Name of the antifungal disc	Zone of inhibition
1.	Ketoconazole	33 mm, 37 mm
2.	Itraconazole	26 mm, 28 mm
3.	Terbinafine	36 mm, 40 mm
1.	Fluconazole	23 mm, 28 mm

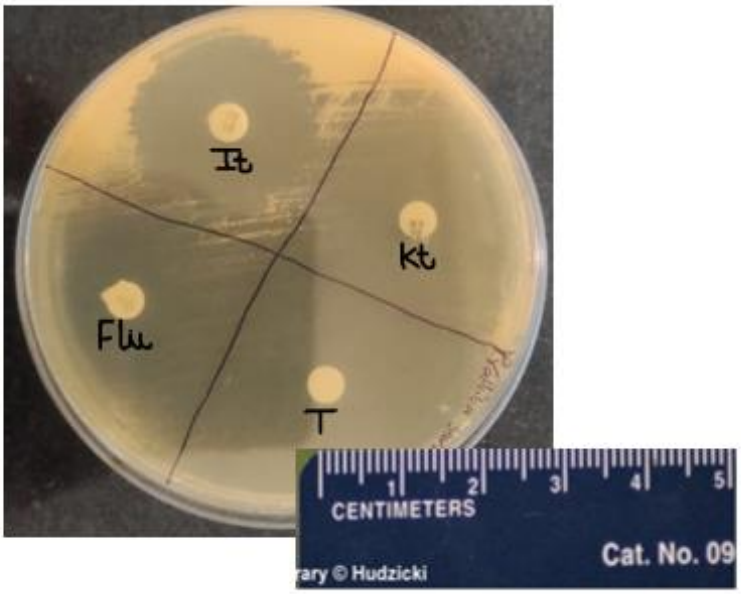

Plate 1. Antifungal sensitivity pattern against *Malassezia* spp. using standard antifungal agents i.e. Terbinafine (T), Ketoconazole (Kt), Itraconazole (It), and Fluconazole (Flu), measure the zone of inhibition around each disc. If the zones of inhibition from adjacent discs overlap, measure the radius from the center of the disc to a point on the circumference where a distinct edge is present. Multiply this radius by 2 to obtain the diameter of the zone of inhibition. In this, the radius of the zone of Terbinafine is 18 mm, therefore, the diameter of the zone of inhibition would be 36 mm [17]

Among the different antifungal agents, the zone of inhibition was highest in Terbinafine (T) followed by Ketoconazole (Kt), Itraconazole (It) and Fluconazole (Flu) respectively as shown in Table 1 and Plate 1. These findings are in agreement with [18,13,19] who reported that the *Malassezia pachydermatis* isolates showed sensitivity to Ketoconazole, Fluconazole and Itraconazole.

"Ketoconazole, Fluconazole and Itraconazole (Imidazoles and triazoles) inhibit the cytochrome P-450 enzyme lanosterol 14 alpha demethylase, preventing the demethylation of lanosterol to ergosterol and thus inhibiting the synthesis of ergosterol, the main sterol in fungal cell membranes. It also leads to complete disappearance of oxidative enzymes in the fungal cell membranes. It also leads to complete disappearance of oxidative enzymes in the fungal cells resulting in intracellular accumulation of toxic levels of hydrogen peroxide. By preventing the synthesis of ergosterol, the agents alter the cell's permeability, resulting in its death" [13]. As Ketoconazole can be effective in treating fungal infections, its potential for serious side effects necessitates careful consideration and monitoring. Alternative antifungal agents with better safety profiles are often preferred, especially for long-term use.

Terbinafine is an allylamine antifungal drug. It acts as an inhibitor of enzyme squalene epoxidase. It inhibits the conversion of squalene-to-squalene epoxide and decrease the synthesis of ergosterol. Due to excessive intracellular accumulation of squalene, fungal cell death occurs due to disruption of cell membrane [20]. Terbinafine has a high safety margin in humans [21]. It has no inhibitory effect on cytochrome P450 systems therefore, it is considered to be more selective than azole derivatives such as Ketoconazole.

In the present study, none of the isolates were found resistant to antifungal drugs. All the isolates were susceptible to Ketoconazole, Itraconazole, Fluconazole and Terbinafine. A cent percent sensitivity recorded in this study indicated that any of the above drugs can be suggested for the

treatment of *Malassezia pachydermatis* dermatitis in dogs.

Nowadays resistant strains of *Malassezia* yeasts are increasingly detected. Resistance to antifungal drugs in clinical cases for *Malassezia* species is sometimes reported in both human and veterinary medicine. “Most studies have shown little evidence for *in-vitro* antifungal resistance, multiple reports have demonstrated occasional very high anti-fungal MICs in individual *Malassezia* species and strains” [22]. The results obtained with respect to the susceptibility of *M. pachydermatis* field isolates indicate that the resistance of the yeasts to the commonly used antifungals is not alarming. Nevertheless, we recommend starting the therapy only after testing the isolates to sensitivity to specific antimycotics. The correct choice of antifungal medication after previous testing can accelerate the treatment of yeast infection and reduce the risk of resistance.

3.2 Clinical Implications

The results of the present study suggest that Terbinafine was found to be most effective than as compared to Ketoconazole, Itraconazole and Fluconazole against *Malassezia* dermatitis in dogs, based on *in vitro* susceptibility tests. This finding is significant for veterinary practitioners seeking the most efficacious treatment for canines. Thus, they may consider Terbinafine as a first-line treatment option, especially in cases resistant to Ketoconazole and Itraconazole. However, individual variability in response to Terbinafine and Ketoconazole necessitates careful selection and monitoring. Routine antifungal susceptibility testing can guide veterinarians in choosing the most appropriate antifungal therapy, thereby improving clinical outcomes and minimizing the risk of treatment failure.

Due to the increasing resistance to antifungal medications, the therapy of yeast infection is an increasing problem. Therefore, in the actual treatment of yeast infections, it is important to choose the most effective antifungal drug. The correct treatment can only be initiated after testing the sensitivity of isolates to selected antimycotics. Several methods are available to test the sensitivity of isolates. The most commonly used is disc diffusion method as it is fast and commonly used in laboratories.

3.3 Future Research

Further clinical trials are required to validate these findings *in vivo.* Investigations into the pharmacokinetics, safety profiles, and long-term efficacy of Itraconazole and Terbinafine in dogs with *Malassezia* dermatitis are essential for developing comprehensive treatment guidelines.

4. CONCLUSION

Terbinafine exhibits superior antifungal activity against *Malassezia pachydermatis* as compared to Itraconazole, Fluconazole and Ketoconazole suggesting that it may be a more effective treatment option for *Malassezia* dermatitis in dogs. These findings support the need for further clinical research to optimize the treatment protocols in veterinary dermatology.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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