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Isolation and identification of dermatophytes from dogs and cats in Chennai by conventional and UniPlex PCR assay

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Abstract
The present study was aimed to isolate and identify dermatophytes of dogs and cats in Chennai by conventional and molecular assay. 100 samples were collected from each dogs and cats brought to madras veterinary college teaching hospital with the symptoms of dandruff, alopecia. Initially screened by wood lamp technique, phenotypic identification was done by morphological character in Sarbourad dextrose agar and Dermatophyte test medium. Molecular analysis by Uniplex PCR was performed by targeting 18s-RNA gene of fungi belonging to genera *Microsporum* and *Trihophyton*. As a result, 34 pure cultures of dermatophytes from dogs and 43 pure cultures of dermatophyte from cats respectively. Three different species were identified from culture as *M. canis, M. gypseum* and *T. mentagrophytes* by morphological characters. A total of *Microsporum* (21%), *Trichophyton* (8%) genus in dogs, a total of *Microsporum* (29%), *Trichophyton* (12%) genus in cats were confirmed by Uniplex PCR.

Keywords: Isolation, identification, sarbourad dextrose agar, dermatophyte test medium, uniplex-PCR

Introduction
A fungal infection of the skin called dermatomycosis is brought on by dermatophytes, specifically *Microsporum, Trichophyton*, and *Epidermophyton* spp. Spores of dermatophyte can spread widely and remain in the environment for a long time, contaminating surfaces, bedding, tools, dust, and air (Devi and Vijayakumar, 2013) [5]. *Candida* can be isolated from the skin and mucous membranes of healthy dogs and cats, infection must be confirmed with cytology or histopathology when *Candida* is isolated from these sites.

Illness is more likely in young people or under stress, as those who live in densely populated areas. Any combination of hair loss, scaling, crusting, erythema, papules, hyperpigmentation, and fluctuating pruritus can be a clinical indication (Cafarchia *et al*., 2006; Copetti *et al*., 2006) [2, 3]. Skin biopsies or direct inspection of hairs or scales from lesions can be used to confirm the diagnosis. A Wood's light or dermoscopy can be used to identify hairs for direct examination or culture. Fungal culture must be utilized in conjunction with the results of the clinical examination in order to ascertain whether spores are present on the hair coat. The presence or lack of fungal DNA on the hair coat is verified by PCR testing. The ability to discern between viable and nonviable spores is lacking. Topical antifungal therapy disinfects the hair coat and gets rid of infection in animals who require treatment.

Materials and Methods
Collection of samples
The present study was conducted at Madras Veterinary College teaching hospital during January 2023 – May 2024. Dogs and cats showing skin infection and dandruff were screened under wood lamp technique. Skin samples were obtained by scraping from the rim to the center of the suspected lesions with a sterile scalpel blade, following the cleansing of the affected areas with 70% isopropyl alcohol. The collected samples were then placed in sterile container. Accompanying epidemiological data related to the animal were recorded. The samples were transported to the laboratory without refrigeration, duly labelled, ensuring no transport medium was used and all moisture was excluded.

Conventional method of Identification

The samples were inoculated into Dermatophyte Test Medium (DTM) and Sabouraud's Dextrose Agar (SDA) medium, which includes 0.05%. For around four weeks, the SDA plates were incubated at 28°C inn BOD incubator, and the growth of fungi was periodically monitored. After four weeks, if there was no growth, the outcome was deemed to be fungal-free. In a similar manner, DTM plates were incubated for up to three weeks at 28°C, and any color changes were observed (Weitzman and Summerbel, 1995; Robert and Pihet, 2008) [18, 13]. Microscopic examination of the cultures involved removing a fragment of aerial mycelium using an inoculation loop and staining it with a drop of lacto-phenol cotton blue (Lacaz *et al*., 2002; Kawasaki, 2011) [14, 12].

Molecular method of identification

40µl of SDS, 10µl of proteinase K, and 200µl of the sample were incubated at 56 °C for 30 minutes in a water bath. Then, 200µl of saturated phenol, chloroform, and isoamyl alcohol (25:24:1) were added, and the mixture was vortexed for 10 seconds. It was centrifuged at 10,000 RPM for 5 minutes at 4 °C. The supernatant was transferred to a new tube. One-tenth volume of 3M sodium acetate (pH 5.5) and 1ml of chilled 100% ethanol were added. The mixture was incubated at -20 °C overnight. It was then centrifuged at 12,000 RPM for 15 minutes at 4 °C, and the supernatant was discarded. The pellet was washed with 500µl of 70% ethanol and centrifuged again at 12,000 RPM for 2 minutes. The ethanol was discarded, the pellet was dried at 37 °C, and reconstituted in 50µl of nuclease-free water (Garg *et al*., 2009; KONEMAN and ROBERTS, 1985) [7, 13] PCR was performed with 25µL reaction buffer, which consisted of DNA template 5µl, master mix 10µl, forward primer 1µl, reverse primer 1µl an Nuclease Free Water 8µl. Taq DNA polymerase targeting 18s-RNA gene and 50 picomole (pmol) each of VDLDr-F "CAACAACGGATCTCTTGGTTCC" and VDLDr-R "TACCTGATCCGAGGTCAACC". The alignment of sequences facilitated the design of a common set of positive and negative sense primers, which are capable of amplifying genomic products of 351 bp for *Trichophyton* and 366 bp for *Microsporum*, respectively. Conditions for thermal cycling, the reaction mixture was first denatured for 4 minutes at 95 °C. It then underwent 35 cycles of denaturation for 20 seconds at 95 °C, annealing for 25 seconds at 56 °C, and extension for thirty seconds at 72 °C. A final extension step was then performed in a thermal cycler for 10 minutes at 72 °C. To genotype the PCR products on 1.5% Agarose Gel, the specific PCR products were separated depending on size using electrophoresis at 100 volts for 45 minutes.

Results

In this study of 100 each dogs and cats cases, three different species of dermatophytes were isolated, resulting in an overall dermatophytosis prevalence of 36.5%. Out of the 77 isolates, *M. canis* was identified in 11% cases, *T. mentagrophytes* in 7% cases, *and M. gypseum* in 9% cases in dogs and *M. canis* was identified in 19% cases, *T. mentagrophytes* in 9% cases, and *M. gypseum* in 7% cases in cats respectively, using a range of conventional methods. All isolates exhibited a pink color change in the DTM medium within the expected timeframe of seven to ten days post-inoculation. The *M. canis* isolates also produced the characteristic cottony to wooly aerial mycelium that became powdery and light brown in the center on the obverse side (Fig. 1a), while the reverse side showed brilliant brown surrounded by the yellow pigment(Fig. 1b), *M. gypseum* also cottony or powdery, they grow rapidly with a colour range of white to buff on obverse side (Fig. 2a), with a reverse that can range from pink, to red, to yellow (Fig. 2b), *T. mentagrophytes* showed Characterized by a flat appearance, white to cream in color, with a surface ranging from powdery to granular on obverse side (Fig 3a), The reverse pigmentation typically presents as yellow-brown to reddish-brown in reverse (Fig 3b). In uniplex PCR assays, all positive clinical samples and controls yielded 351bp bands for *Trichophyton* and 366bp for *Microsporum*. While the majority of samples showed infection by a single dermatophyte genus, some revealed mixed infections, as evidenced by bands for both *Microsporum* and *Trichophyton* (see Figure 4). Of the seventy-seven culture-positive samples tested for dermatophytosis via uniplex PCR, seventy (90.90%) returned positive results. Of these, 21 samples (30%) were infected with *Microsporum,* and 8 samples (11.42%) with *Trichophyton* in dogs. Additionally, 29 samples (41.42%) tested positive for *Microsporum,* and 12 samples (17.14%) for *Trichophyton* in dogs. In cats, two samples (2.85%) showed mixed infections with both *Microsporum* and *Trichophyton.* The assay achieved a sensitivity rate of 93.50%.

Fig 1a: Growth of *M. canis* on Dermatophyte Test Medium (obverse side)

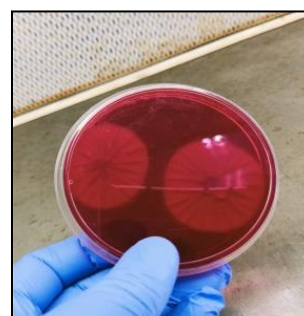

Fig 1b: Growth of *M. canis* on Dermatophyte Test Medium (obverse side)

Fig 2a: Growth of *M. gypseum* on Dermatophyte Test Medium (obverse side)

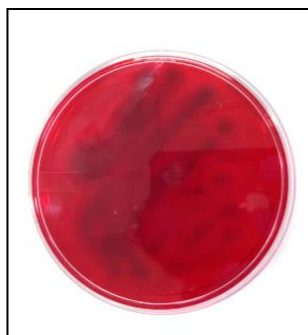

Fig 2b: Growth of *M. gypseum* on Dermatophyte Test Medium (obverse side)

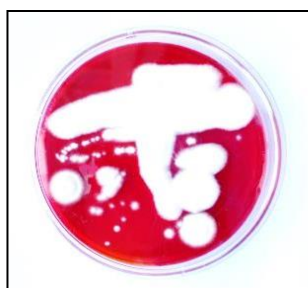

Fig 3a: Growth of *T. mentaggrophytes* on Dermatophyte Test Medium (obverse side)

Fig 3b: Growth of *T. mentaggrophytes* on Dermatophyte Test Medium (obverse side)

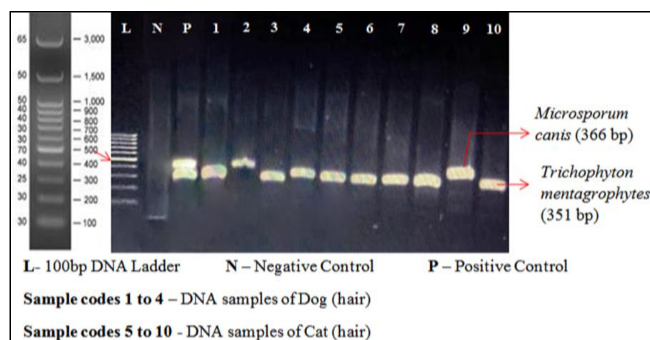

Fig 4: Agarose gel based genotyping of PCR products. Ladder (L), Negative Control (N), Positive Control (P), *Microsporum* positive samples 366 bp (L2, L9), *Trichophyton* positive sample 351 bp (L1, L3, L4, L5, L6, L7, L8, L10)

Discussion

As global demographic patterns shift, with rising numbers of individuals undergoing chemotherapy, those who are immunocompromised, and organ transplant recipients, the prevalence of zoonotic fungal infections is also on the rise (Hayette and Sacheli, 2015; Verma and Madhu, 2017) [9, 17]. This trend is exacerbated by the growing issue of antifungal resistance (Alcazar-Fuoli and Mellado, 2014; Singh *et al*., 2018) [1, 16]. Given that traditional diagnostic methods are time-intensive, require specialized expertise, and lack sensitivity and specificity, molecular tools have become valuable for the identification of dermatophytes.

In this study, *M. canis* was identified in 11% cases, *T. mentagrophytes* in 7% cases, and *M. gypseum* in 9% cases in dogs and *M. canis* was identified in 19% cases, *T. mentagrophytes* in 9% cases, and *M. gypseum* in 7% cases in cats respectively, displayed phenotypic characteristics. The identification of dermatophytes based solely on phenotypic traits is often complicated by their pleomorphism, as highlighted (Deng *et al*., 2008; Gnat *et al*., 2019) [4, 8]. Additionally, the commonality of overlapping features and variability among dermatophytes was reported (Diongue *et al*., 2019) [6]. Consequently, for accurate identification of dermatophytes, employing species-specific primers and/or sequencing the internal transcribed spacer (ITS) region of ribosomal RNA is advised. The uniplex PCR results showed 90.90% specificity to cultures, the estimates indicated that the uniplex PCR is efficient in diagnosing disease (Saeed *et al.,* 2018) [15].

Conclusion

Dermatophytes have become a significant zoonotic pathogen, posing a risk to human health, particularly for pet owners, due to their easy transmission from dogs and cats. The study resulted in the development of a rapid, efficient, reliable, and cost-effective molecular-based assay to diagnose and distinguish between two genera of dermatophytes. Validating the differential diagnosis of dermatophytes can substantially assist clinicians in treating animal cases with targeted drugs, thereby minimizing the risk of zoonotic transmission.

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