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# Detection of dermatophytes through multiplex PCR and loop-mediated isothermal amplification (LAMP)

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## Abstract
Dermatophytes are zoonotic fungal agents that are infectious and widespread among animals globally. The new loop-mediated isothermal amplification (LAMP) method and Multiplex PCR are tools for identifying these agents. Both offer high specificity and sensitivity, and are simple and quick to execute. The aim is to develop a LAMP and a rapid multiplex PCR method to detect *Microsporum* spp and *Trichophyton* spp, the most commonly isolated fungal genera from cats and dogs. Both methods focus on the CHS-1 gene. They were evaluated for specificity and sensitivity using 34 and 43 cultures obtained from dogs and cats, in that 21 *Microsporum* and 8 *Trichophyton* strains from dogs, and 29 *Microsporum* and 12 *Trichophyton* strains from cats. The specificity was 85.29% and 95.34% for dogs and cats, the sensitivity for 89% *M. canis* and 85% *T. mentagrophytes* respectively. The LAMP and multiplex PCR required 60 and 140 minutes, respectively, for both targets. The limit of detection (LOD) for both was 10 spores/mL for LAMP and 1 spore/mL for multiplex PCR.

**Keywords:** *Microsporum* spp., *Trichophyton* spp., LAMP assay, multiplex PCR

## Introduction
Dermatophytes, belonging to the phylum Ascomycota, class Euascomycetes, order Onygenales, and family Arthrodermataceae, are categorized into the genera *Microsporum, Trichophyton,* and *Epidermophyton* based on their asexual reproduction traits. These fungi target body parts like skin, hair, feathers, horns, hooves, nails, and claws. The most commonly isolated dermatophyte species from animals include *M. canis* (typically from cats and dogs), *T. verrucosum* (from cattle and small ruminants), *M. equinum* (from equidae), *T. mentagrophytes* (from cats and dogs), and *T. mentagrophytes* var. *erinacei* (from hunting dogs). Notably, *Microsporum canis* and *Trichophyton mentagrophytes* are the dermatophytes most frequently found in cats and dogs, presenting zoonotic significance (Garg *et al.,* 2009; Cabanes *et al.,* 1997) [2, 1]. In this work, we devised two techniques for the early and quick identification of *T. mentagrophytes* and *M. canis*: a multiplex qPCR method and a LAMP method (Mustak, 2022) [5].

## Materials and Methods
### Standard strains isolates
Reference strains of *M. canis* (3270) and *T. mentagrophytes* (8476) were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC) to serve as positive controls in the optimization and validation of the developed methods. For validation, 21 *M. canis* and 8 *T. mentagrophytes* strains were isolated from dogs, and 29 *M. canis* and 12 *T. mentagrophytes* strains were isolated from cats. All strains were confirmed by conventional and one-step PCR methods.

### DNA Extraction
40 µl of SDS, 10 µl of proteinase K, and 200µl of the sample were incubated at 56 °C for 30 minutes in a water bath. Subsequently, 200µl of a mixture of saturated phenol, chloroform, and isoamyl alcohol (25:24:1) was added, followed by vortexing for 10 seconds. The solution was then centrifuged at 10,000 RPM for 5 minutes at 4 °C. The supernatant was carefully

transferred to a new tube. To this, 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. Afterward, it was centrifuged at 12,000 RPM for 15 minutes at 4 °C, and the supernatant was removed. The remaining pellet was washed with 500µl of 70% ethanol and centrifuged at 12,000 RPM for 2 minutes. The ethanol was discarded, and the pellet was dried at 37 °C, then reconstituted in 50µl of nuclease-free water. This method is referenced from Garg *et al*., 2009 [2], and Koneman and Roberts, 1985 [4].

**Primers for Multiplex**

Taq DNA polymerase targets the Der ITS1-2(Internal transcribed spacer 1 & 2) gene with the primers Der ITS1-2 Fw "5'-ATCATTAACGCGCAGGC-3'" and ITS1-2 Rv "5'-TGGCCACTGCTTTTCGG-3'". The alignment of sequences has facilitated the design of a common set of positive and negative sense primers capable of amplifying genomic products ranging from 400-600 base pairs for dermatophytes (Kim *et al.,* 2011) [3].

**Primers for LAMP assay**

Suitable primers for LAMP were designed for the chitin synthase-1 (CHS-1) gene, a conserved 3592 bp region encoding a cellulose-like polysaccharide in the cell wall. This gene was targeted to detect *M. canis* and *T. mentagrophytes*. The sequences obtained were aligned using Clustal-Omega, and conserved regions specific to each species were identified within these alignments (Mustak, 2022) [5].

| Organisms | | Sequences | Size (bp) |
|---|---|---|---|
| *M.canis* | F3 | GCATTGCCAAACAGCAGGT | 619 |
| | B3 | AGGATGCGGCCGAAGG | |
| | FIP | GCCCTTGACCTCCATGCCGATTTTAAGGACGTCACCGCCCA | |
| | BIP | GCCCGTCCAGCTCCTCTTCTTTTACCGGTGCGAGTTGATCT | |
| | L-F | CTGGGTGGTGTACTCGTAGAT | |
| | L-B | TGCCTCAAGGAGAAGAACCAG | |
| *T.mentagrophytes* | F3 | AGCAGCAAGACATGGGGTA | 504 |
| | B3 | TAACCTGGGTGCCCTTGA | |
| | FIP | GAACAGCTCTCGTACGCGGATTTTTTAGCCTGGAAGAAGATTGTCG | |
| | BIP | GATGGCATTGCCAAACAGCAGGTTTTCCATGCCTATCTGGGTGGTA | |
| | L-F | CGACCGTCTGAGACGATACAAA | |
| | L-B | TCAACGGTAAAGACGTCACTGC | |

**Optimization of Multiplex and LAMP**

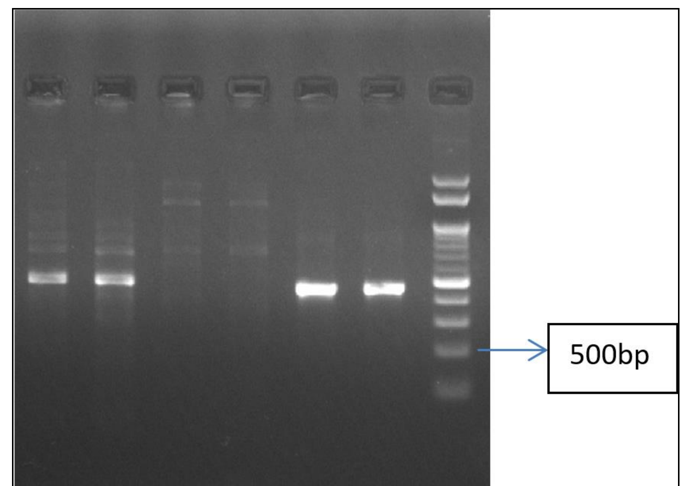
The PCR amplification reaction mixture was set to 50µl, comprising 0.1 mM dNTPs, 10X PCR buffer, 0.5 mM primer, 0.6 U Taq polymerase (Enzynomics, Daejeon, Korea), and 50 ng of genomic DNA solution. For ITS 1 and 2, the reaction conditions were as follows: an initial hot step at 94 °C for 7 minutes, denaturation at 95 °C for 1 minute, annealing at 60 °C for 1 minute, and extension at 72 °C for 1 minute. This cycle was repeated 35 times, ending with a final extension at 72 °C for 7 minutes. For the 28S ribosomal RNA, the conditions were: an initial hot step at 94 °C for 7 minutes, denaturation at 94 °C for 1 minute, annealing at 50 °C for 30 seconds, and extension at 72 °C for 1 minute. This process was also repeated for 35 cycles, concluding with a final extension at 72 °C for 7 minutes.

The concentrations of LAMP primers and the analysis duration were determined based on the Bst polymerase enzyme, as indicated by New England BioLabs. The LAMP reaction mixture, with a total volume of 25 µL, included 6 mg/mL BSA, 20 mg/mL PEG 400, 0.25% Tween 20, 15 mM peptide complex, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTP mix, 0.1 U Bst DNA polymerase, 120 µM HNB, and primers at various concentrations (40 µM FIP, 40 µM BIP, 5 µM F3, 5 µM B3, 10 µM Loop-F, 10 µM Loop-B1), along with 10 µl (50 ng/µL) of template nucleic acid. The primer sets amplification conditions were optimized to 65 °C for 60 minutes, following the enzyme manufacturer guidelines.

**Results**

The PCR analysis results using dermatophyte-specific primers (ITS1 & ITS2) showed bands ranging between 400-600 bp (Fig. 1). Field isolates numbered 34 and 43, derived from dogs and cats, were utilized for validation. Of these, 21 *Microsporum* and 8 *Trichophyton* strains were from dogs, while 29 *Microsporum* and 12 *Trichophyton* strains were from cats.

The LAMP assay identifies color changes within 60 minutes at 60 °C, observable with the naked eye (Fig. 2). It detected 31 *M. canis* isolates and 11 *T. mentagrophytes* isolates.




Ladder 100 pb; N- Negative control;
Sample code :- 1 - +ve *M. canis*, 2 – field isolate
Sample code :- 5 - +ve *T. mentagrophytes*, 6 – field isolate
Positive bands will visualized between 400-600bp

**Fig 1:** Multiplex PCR

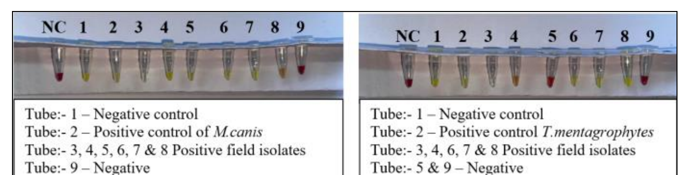



**Fig 2:** LAMP assay of Dermatophytes

## Discussion

Compared to the gold standard method, the new methods demonstrated 85.29% and 95.34% specificity for the Multiplex and LAMP assays, respectively; the sensitivity was 89% for *M. canis* and 85% for *T. mentagrophytes*. Ohst *et al*. revealed that a modular singleplex qRT-PCR assay for detecting the most prevalent dermatophytes had higher specificity (88.2%) and sensitivity (60.7%) than both microscopy and culture methods.

Garg *et al.,* (2009) [2] suggested that nested PCR targeting the CHS1 gene could be the gold standard for detecting dermatophytes in patients. They also noted that the nested-PCR technique is not only quick but also straightforward and cost-effective compared to other molecular methods for detecting dermatophytes. Nevertheless, the methods developed in our study are less expensive and faster than other PCR-based techniques.

## Conclusion

*M. canis* and T. *mentagrophytes* are the most commonly isolated fungal dermatophytes from cats and dogs. They pose a serious public health concern due to their potential to cause zoonotic infections in humans. In this study, we introduced the LAMP method in India for the rapid identification of *M. canis* and *T. mentagrophytes*, along with a multiplex PCR method for the simultaneous detection of both pathogens at genus level. While mycological culturing remains the gold standard for identifying these fungal agents, it requires approximately seven to 21 days to yield results. The newly developed methods significantly reduce this time, providing objective and accurate results in 60 minutes with LAMP and 140 minutes with multiplex PCR.

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