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Molecular diagnosis of dermatophyte isolates from canine and feline dermatophytosis in Northeast Iran

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Abstract

Background: Dermatophytes are the most common causes of cutaneous fungal diseases. Dermatophytosis is a common skin disorder in dogs and cats. Species identification of these fungi is important from a therapeutic and epidemiological aspect. Conventional methods used to identify dermatophyte species are often lengthy and may be inefficient in many circumstances. Recently broad varieties of several molecular DNA-based techniques were successfully utilised for species detection of dermatophytes.

Objectives: The aim of this study was to determine the molecular detection of dermatophyte isolates from canine and feline dermatophytosis in Mashhad, Iran.

Methods: Thirty dermatophytes isolated from dogs and cats with skin lesions and one standard strain of *Microsporum canis* were cultured onto Mycosel agar, and then internal transcribed spacer (ITS) region of the ribosomal DNA was amplified using the universal fungal primers ITS1 and ITS4. PCR products were subjected to sequencing and sequence analysis.

Results: Based on the sequencing of the ITS1-5.8S-ITS2 region on all samples, all the studied strains were *M. canis* and their sexual stage (teleomorph) was *Arthroderma otae*.

Conclusions: *Microsporum canis* was the only species found among dogs and cats, and its high prevalence can increase the rate of transmission to humans. In practice, ITS-PCR, with sequence analysis, is a useful and reliable method to identify and differentiate various pathogenic species, and it can be used in clinical and epidemiological fields, even for the rapid diagnosis of dermatophyte species that are closely interrelated.

KEYWORDS
cat, dermatophytosis, dog, *Microsporum canis*, PCR, ribosomal DNA

1 | INTRODUCTION

Dermatophytosis in companion animals is an infection of keratinised tissues (i.e., skin, hair and/or nails) caused by dermatophytes and are classified in three anamorphic (asexual or imperfect) genera, *Epidermophyton*, *Microsporum* and *Trichophyton*, of anamorphic class Hyphomycetes of the Deuteromycota (fungi imperfecti; Moriello et al., 2017; Seker & Dogan, 2011; Weitzman & Summerbell, 1995). Some dermatophytes are also capable of reproducing sexually and producing ascomata with asci and ascospores. These species are classified in the teleomorphic genus *Arthroderma*, family Arthrodermataceae of the Onygenales, phylum Ascomycota (Kac, 2000; Weitzman & Summerbell, 1995).

Microsporum canis, *M. ferrugineum* and *M. audouinii* are three phylogenetically closely related dermatophytes in the *Arthroderma otae* complex (Gräser et al., 2008).

Dermatophytes are usually categorised into anthropophilic, zoophilic and geophilic species, according to their habitat (Cafarchia et al., 2012). The studies on the isolation of dermatophytes from dogs and cats have shown that the prevalence of infections is approximately 4%–20% in dogs and higher than 20% in cats (Brilhante et al., 2003; Cabanes et al., 1997; Cafarchia et al., 2004; Lewis et al., 1991; Seker & Dogan, 2011). Although a wide variety of dermatophytes have been isolated from animals, a few zoophilic species including *M. canis*, *Trichophyton mentagrophytes*, *T. quinum* and *T. verrucosum* and also geophilic species such as *M. gypseum* are responsible for the majority of serious infections (Seker & Dogan, 2011).

Conventional approaches for laboratory diagnosis of dermatophytes are based on demonstration of hyphae/arthroconidia by direct microscopic examination of clinical samples, followed by an examination of gross morphological characters of their colonies (e.g., rate of growth, colony topography and pigmentation of the surface and reverse sides) as well as microscopic morphology (e.g., shape and size of macroconidia, microconidia and hyphae) and physiological criteria (Mohammadi et al., 2015). However, identification of dermatophytes sometimes remains difficult or uncertain due to their overlapping phenotypic characteristics, variability and pleomorphism (Li et al., 2008).

Recent advances in molecular biology and progress in technology have allowed the development of new techniques for species determination and strain typing in microbiology.

The routine approaches used for the laboratory diagnosis of dermatophytosis are direct microscopic examination and culture. Nonetheless, the identification of dermatophytes is usually based on cultural examinations, which are time-consuming (taking up to 14 days), and its application is often complicated by the growth of other fungi, which are normally present in skin or hair samples from animals (Cafarchia et al., 2012). From a clinical point of view, in order to define species or to perform an epidemiological study, it is important to have a reliable method for the identification of dermatophyte species.polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and/or direct sequencing techniques have been used to identify and differentiate species or genotypes of dermatophytes recovered in culture. However, only a few studies have attempted to specifically characterise dermatophytes from hair-coat samples of dogs or cats, and all of the techniques have relied on sequence-based analysis of the selected genetic markers. The phylogeny of dermatophytes, however, remains unclear because their members are phylogenetically and taxonomically related; however, their phenotypic features are sometimes poor, and many isolates from medical and veterinary samples have lost their sexual activity (Makimura et al., 1999). ITS-1 and ITS-2 of rDNA and a part of chs-1 have shown particular promise as markers for the specific identification of dermatophytes (Cafarchia et al., 2009; Gräser et al., 2008; Kano et al., 1999). The epidemiological features of dermatophytosis have not been described in many parts of the country. In this survey, we aimed to delineate the distribution profile of canine and feline dermatophytosis in Mashhad, a city located in the northeast of Iran, using rDNA internal transcribed spacer (ITS) regions.

2 | MATERIAL AND METHODS

2.1 | Clinical isolates

Isolates were obtained from the mycology laboratory at the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. A total of 30 dermatophytes isolated from dogs and cats with skin lesions and one standard strain of *M. canis* (PTCC 5069) were inoculated onto Mycosel agar (Merck Co.). Plates were then incubated aerobically at 25°C and examined daily for 14 days.

2.2 | DNA preparation

DNA was extracted from each isolate according to a previously described method by Liu et al. (2000a). In brief, a small piece of each fresh colony (~50 mg wet weight) was harvested through filtration and washed three times with sterile saline. The sample was transferred to 1.5-ml microcentrifuge tubes and subjected to centrifugation at 5000 × g at room temperature for 1 min. Then, it was ground by glass beads and 500 μl of lysis buffer (400 mM (hydroxymethyl)aminomethane hydrochloride (Tris/HCl) (pH 7.5), 60 mM ethylene-diamine-tetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS) and 150 mM sodium chloride (NaCl)) was added, and the mixture was left at room temperature for 10 min, then it was vortexed twice and after each step in order to break the cell wall, rapid freezing was performed at −70°C. After that, 10 μl of proteinase *K* was added and incubated at 60°C for 1 h. The solution was then incubated for 10 min to inactivate the enzyme at 95°C. Subsequently, 150 μl of sodium acetate buffer (60 cc sodium acetate 5 M, 11.5 cc acetic acid glacial, 28.5 cc distilled water. pH 4.8) was added to the tubes and shaken gently and then centrifuged at 10,000 × g for 2 min. An equal volume of cold isopropyl alcohol was added to the supernatant and mixed by inversion spin at 12,000 g for 2 min and the supernatant was discarded.

The DNA pellet was washed three times with 500 μl of 70% (v/v) ethanol, air-dried and dissolved in 50 μl of distilled water. In order to measure the concentration and purity of the DNA, the optical density was read in 1.5% agarose gel.

2.3 | ITS PCR sequencing

The ITS region of ribosomal DNA was amplified using universal fungal primers. The following primer sets were used: ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC; White et al., 1990). PCR mixture contained 25 μl of PCR Master Mix (2X; Taq DNA polymerase 0.08 U/μl, MgCl2 3 mM, 0.4mMof each dNTPs), 1.5 μl of each primer (ITS1 and ITS4) and 5 μl (40 ng) of DNA template solution in a total volume of 50 μl.

The PCR cycling conditions included 33 cycles of 94°C for 5 min, 94°C for 40 s, 58°C for 45 s and 72°C for 60 s followed by an extension step of 72°C for 10 min. PCR was carried out using a thermal cycler.

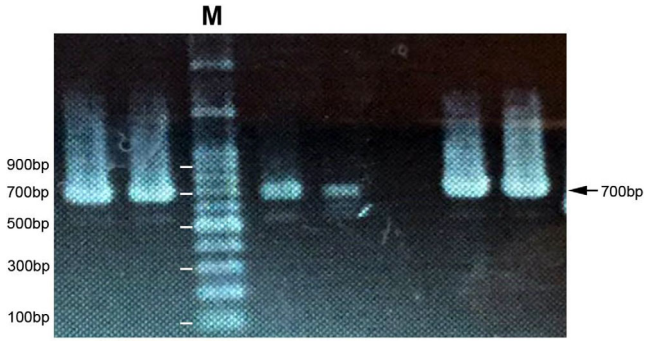

FIGURE 1 Agarose gel electrophoresis of internal transcribed spacer-PCR products of *Microsporum canis* isolates. M: 100 bp molecular size marker

The resulting PCR products were separated in 1.5% agarose gels in 0.5x Tris-borate-EDTA buffer and stained with ethidium bromide. The products were detected as a single band of approximately 700 bp. The PCR products were sent to Takapouzist Co. (Bioneer) for one direct sequencing with ITS1 primer.

2.4 | Phylogenetic analysis

Chromatograms were analysed by Chromas software (Version 3.1). Forward sequences were edited using MEGA6 (Tamura et al., 2007) software. Sequence analysis was performed by comparison of the test nucleotide sequences with reference dermatophyte nucleotide sequences obtained from the Central Bureau of Fungal Cultures database at the Westerdijk Fungal Biodiversity Institute (http://www.cbs.knaw.nl/dermatophytes/BioloMICSID.aspx). Accession numbers were also obtained from GenBank for the dermatophyte sequences (https://www.ncbi.nlm.nih.gov/genbank/). MEGA6 software was also used for further investigations on intra-species differences and genetic association between the isolates.

3 | RESULTS

Most of the grown colonies corresponded to the typical *M. canis* colonies (colony with smooth, cotton or granular surface, yellow and white colony surface and golden yellow colony border). However, some colonies also had atypical appearance, they also lacked pigmentation, and in some cases, they lacked macroconidia; in these cases, the diagnosis was difficult on the basis of phenotypic characteristics, and the exact identification of the species was dependent on molecular detection. Therefore, the molecular method was used to accurately detect the samples. PCR and sequencing assay have confirmed the identification of strains as *M. canis*. A specific product was visible only at the annealing temperature of 58°C. PCR amplification of the target region yielded single bands of approximately 700 bp in all clinical and standard strains (Figure 1). In addition to ITS region, primers also amplify portions of the small and large subunits on both sides of ITS region, in order to improve detection accuracy. Sequence analysis indicated that DNA products amplified by primers ITS1 were 739 and 720 bp for *M. canis*. All typical and atypical isolates with similarity of > 99% to the reference ITS sequences belonged to *M. canis* and *A. otae* (teleomorph of *M. canis;* Figure 2).

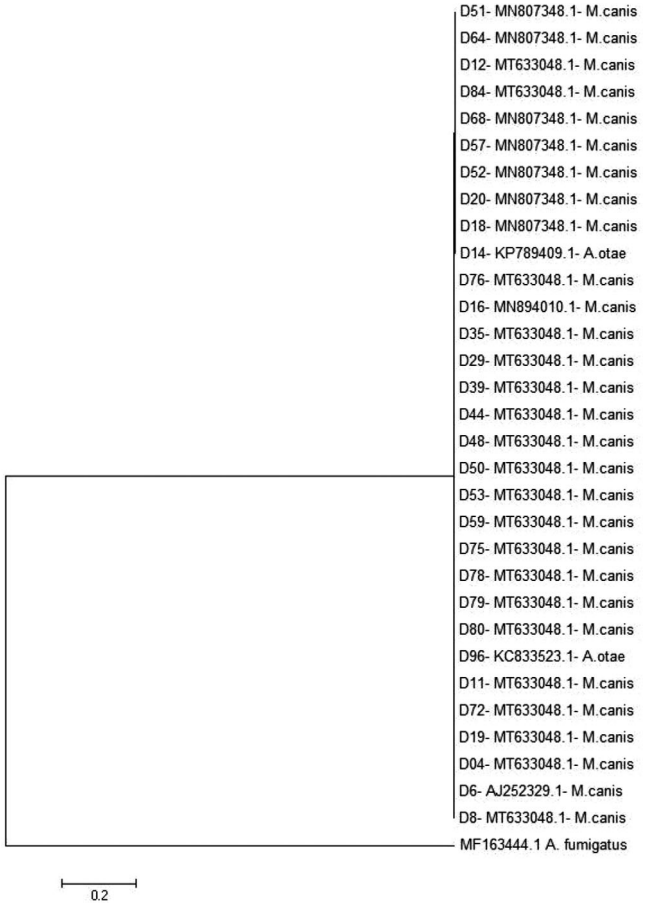

FIGURE 2 Phylogenetic tree of 31 dermatophyte isolates with accession numbers (D50 is the standard strain of *M. canis* (PTCC 5069) and the others are clinical isolates) using sequence of the rDNA ITS regions; *Aspergillus fumigatus* is shown as a negative control

4 | DISCUSSION

Precise species-level identification of dermatophytes is mandatory to know the source of infection, whether zoophilic, anthropophilic or geophilic, and to apply proper treatment and control measures (Gohar et al., 2019). Conventionally, the identification of dermatophytes including *Microsporum* spp., especially *M. canis*, usually depends on phenotypical features, such as direct microscopy and in-vitro culture. Morphological and physiological characteristics can frequently vary; in fact, the phenotypic features can be easily influenced by external factors such as temperature and the medium used (Taha et al., 2017).

However, phenotypic methods are time-consuming, requires experienced personnel due to identification overlap between species

(Arabatzis et al., 2007; De Baere et al., 2010; Gohar et al., 2019), particularly in species with a degenerate appearance such as *M. ferrugineum*, because colonies may be similar to atypical (dysgonic) variants of *M. canis* (Gräser et al., 2008). In addition, the phenotypical features of dermatophytes may predominantly show variations, which often complicate the diagnosis; these alternations may be affected by numerous factors such as temperature, media, host and user therapy. In order to select the best therapeutic procedure, it is important to identify dermatophytes at the genus or species level. Since conventional laboratory procedures for the identification of dermatophytes are either slow or lack enough specificity, molecular technologies, such as DNA sequencing techniques, which can rapidly and precisely identify dermatophytes, are required (Liu et al., 2000b). Sequencing of the hypervariable ITS region, including ITS1, ITS2 and the intermediary 5.8S rDNA, allow the elucidation of the phylogeny of closely interrelated fungi (Kac, 2000). Currently, the sequence database of rDNA ITS region is considered the gold standard for dermatophytes (Makimura et al., 1999; Gräser et al., 2008). The present study aimed to use molecular methods to detect dermatophytes from canine and feline dermatophytosis.

In different parts of the world, zoophilic dermatophytes are among the major causes of dermatophyte infections in humans. Similar to other studies, the current study indicates that *M. canis* is the most common dermatophyte isolate of cats and dogs suffering from dermatophytosis; therefore, further scrutiny and monitoring is required. In this regard, Katiraee et al. (2021) in Tabriz, Iran, Cafarchia et al. (2012) in Italy, Nweze (2011) in Nigeria and Viani et al. (2007) in Brazil showed that *M. canis* was the most dominant aetiologic agent of dermatophytosis in dogs and cats (Cafarchia et al., 2012; Katiraee et al., 2021; Nweze, 2011; Viani et al., 2007).

Molecular studies of dermatophytosis in human specimens have also shown that *M. canis* has been isolated from clinical specimens as a zoophilic dermatophyte. Earlier studies conducted in Iran by Fallahi et al. (2017) in Gilan, Ansari et al. (2016) in southern Iran, Falahati et al. (2016) in Tehran, Mohammadi et al. (2015) in Isfahan, Diba et al. (2014) in Urmia, Rezaei-Matehkolaei et al. (2013) in Tehran reported *M. canis* with rates of 4.9%, 10%, 9%, 2.2%, 32.1% and 4%, respectively (Ansari et al., 2016; Diba et al., 2014; Falahati et al., 2016, 2017; Mohammadi et al., 2015; Rezaei-Matehkolaei et al., 2013).

As noted, some zoophilic dermatophytes, such as *M. canis*, have close phylogenetic relationships with anthropophilic species such as *M. ferrugineum* and *M. audouinii* (Moriello et al., 2017). These species are difficult to be identified based on their phenotypic characteristics because macroscopic and microscopic characteristics of the colonies may be similar to the atypical strains of *M. canis*. The appearance of these features have long incubation periods and might also be unstable. Therefore, for rapid and accurate identification of these species, genotypic and molecular methods are required (Rezaei-MatehKolaei et al., 2012). Various studies have used molecular methods to identify and discriminate these close species (Hassan, 2018; Kobylak et al., 2016; Rezaei-Matehkolaei et al., 2012).

In our study, PCR and ITS sequencing were used for the specific detection of dermatophyte isolates from dogs and cats with skin lesions. The present method can be completed within approximately 24 h from isolated colonies. Based on the results obtained here, it was found that *M. canis* was the only dermatophyte isolate identified using the sequence of the rDNA ITS regions.

As mentioned earlier, few molecular studies have been performed in order to identify aetiologic agents of dermatophytosis in animals (Cafarchia et al., 2012; Katiraee et al., 2021; Nweze, 2011; Tartor, Damaty, &Mahmmod, 2016; Viani et al., 2007; Ziolkowska et al., 2015). DNA-based diagnostic techniques, such as sequence analysis of specific gene regions, are more reliable than classical identification methods of dermatophytes.

5 | CONCLUSION

Dermatophytosis is not a fatal disease; however, it is important to consider the fact that it is transmitted between animals and humans and therefore rapid and precise identification of dermatophytes is essential in order to apply appropriate preventive measures and to direct empirical antifungal therapy. Our study provided useful insights into the reliability of the ITS-PCR method for the identification of dermatophytes. This is the first molecular study of feline and canine dermatophytosis in the northeast of Iran and affords knowledge of the epidemiology of the disease in companion animals. The reported method is simple and convenient, but the main issue that would reduce the application of this approach in routine clinical laboratories is the added cost. However, it is likely that many laboratories would find a use for this approach to identify a minority of atypical isolates or those leading to ambiguous results with conventional identification.

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AUTHOR CONTRIBUTION

Performing experiments, *formal analysis*: Atena Lavari. *Conceptualisation*, *funding acquisition*, *investigation*, *methodology*, *project administration*, *supervision*, *validation*, *visualisation*, *writing-original draft*, *writing-review and editing*: Samaneh Eidi. *Supervision*, *data curation*, *investigation*, *methodology*, *formal analysis*: Minoo Soltani.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Please contact the corresponding author for all reasonable data requests.

PEER REVIEW

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