Comparative study of Microsporum canis isolates by DNA fingerprinting

Shabnam Shafiee,1 Ali Reza Khosravi1 and Iradj Ashrafi Tamai2

1Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran and 2Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Summary

Microsporum canis is a zoophilic fungus and it is an important agent of dermatophytosis. Cats act as important reservoirs. Clinically, it is too difficult to differentiate dermatophytosis caused by various species, also this fungus loses its morphological characteristics easily because of subculture; so using of rapid and accurate laboratory techniques for identifying the dermatophytes is important, therefore, RAPD-PCR was applied for the differentiation of the isolates. In this study, 10 M. canis isolates were detected in cats, dog, human, fox and rabbit at the Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran. For running the RAPD-PCR, PCR set system and three random primers OPU 15, OPU 13 and OPA 04 were used. Then phylogenetic tree and similarity coefficient table were drawn. The results showed that there were some common bands between M. canis isolates. There were some specific bands for each isolates, as well. Our study showed, despite the typical morphology of the whole isolates, they were placed in different branches in molecular typing.

Key words: Dermatophytosis, DNA fingerprinting, isolates differentiation, Microsporum canis.

Introduction

Dermatophytosis is the most general fungal disease in different animals.1 Microsporum canis is a zoophilic fungus and it is widely isolated from the hair coat of cats with dermatophytosis, sometimes cats infected with M. canis without clinical signs.2 Human only is infected after direct or indirect contact with canine. Furthermore, M. canis in human, tend to be mild to severe inflammatory, whereas other agents make severe kerion like tinea capitis or violently inflammatory reaction.3

Specification of the dermatophytes is their potency to grow patently in the hair, nail or stratum corneum and they seldom infect the dermis and subcutaneous tissues in exact conditions. In addition, they can digest components of the cornified cell cover.4 As fungal morphology frequently change, so identification of the agent through phenotypic characteristics is not enough and accurate and it is time consuming.5,6

It was shown that the genotypic characteristics are beneficial for dermatophytes.7 There are some molecular typing for the identification of these fungi, like: restriction fragment length polymorphism analysis of mitochondrial DNA,8 random amplification of polymorphic DNA (RAPD),9 polymerase chain reaction (PCR) fingerprint,5 gene-specific PCR10 and RFLP analysis.11

The aim of this study was to evaluate the genetic diversity of M. canis isolates obtained from human and animals, at the Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Iran.
Material and methods

Isolates

Isolates of *M. canis* were obtained from the Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Iran, from 10 subjects with dermatophyte infections (5 isolates from cats, 1 from fox, 1 from dog, 1 from rabbit and 2 from humans).

Mycological laboratory tests were done consist of KOH preparations and fungal cultures on sabouraud’s dextrose agar with chloramphenicol (SDA). Cultures were incubated at 30 °C for 3 weeks. After that, the macromorphology of *M. canis* was analysed in SDA. Surface and texture of the colony and presence or absence of pigmentation were analysed. Then the grown mycelium was harvested from the SDA surface and transferred to Erlenmeyer flasks containing 250 ml of sabouraud’s dextrose broth and rotated every day, also the isolates were incubated at 30 °C for 1 month (rotation was done to encourage formation of a mycelial mat rather than a hyphal ball). After 20 days, the biomass of cultures was washed with deionised water and ground to powder in liquid nitrogen, then transferred to −20 °C until the DNA extraction.

Extraction of fungal DNA

Fungus ground biomass of 100 mg was added to 500 μl lysis buffer (STES: 0.2 mol l⁻¹ Tris-Hcl, 0.5 mol l⁻¹ Nacl, 0.1% SDS and 0.01 mol l⁻¹ EDTA) that was preheated at 60 °C, then the samples were shaken vigorously, after that 20 μl proteinase K was added and incubated at 65 °C for 20 min.

The samples were submitted to a chloroform isoomyl alcohol (24 : 1, V/v) and mixed well, then they were left on ice for 30 min and separated by centrifugal force (800 g) for 30 min, the supernant was removed and 200 μl deionised water was added and the samples were left at room temperature until nucleic acid was mixed in water, then 100 μl NH₄ AC (7.5 mol l⁻¹) was added and they were left on ice for 1 hour until proteins were precipitated, after that separated by centrifugal force (800 g) for 30 min. The supernatant was transferred to a new tube and the equal volume of isopropanol was added, after 10 min at room temperature the nucleic acid was seen like white filaments. After being separated by centrifugal force (800 g) for 15 min, the supernatant was removed and pellet was washed in 200 μl ice cold 70% ethanol; then, separated by centrifugal force (800 g) for 5 min, the supernatant was removed and pellet was left at room temperature for few minutes. After that pellet was resuspended in 50 μl deionised water, then extracted DNA was kept at −20 °C.

Random primer amplification polymorphic DNA method

DNA typing of the strains by RAPD method was done with three random primers: OPU 15: 5’-ACGGGC-CAGT-3’, OPU 13: 5’-GGCTGGTTCC-3’, OPA 04: 5’-ACCGACCTG-3’.

Random amplification of polymorphic DNA reactions were performed with 2 μl of sample DNA, 10 mmol l⁻¹ of each (dATP, dCTP, dTTP, dGTP), 30 μmol l⁻¹ of primers, 3 μl of 10× PCR buffer (100 mmol l⁻¹ Tris-HCL, pH: 9), 50 mmol l⁻¹ of Mgcl₂, 2.5 U of Taq DNA polymerase and sterile water was added to a final volume of 30 μl.

The amplification programme included of one cycle of 4 min of denaturation at 94 °C, 35 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min and extension at 72 °C for 1 min, and one final extension step at 72 °C for 10 min. PCR products were separated by 1.5% (w/v) agarose gel electrophoresis in 0.5× TBE buffer (Tris-borate, 0.045 mol l⁻¹ and EDTA, 0.001 mol l⁻¹) at 85 V for 90 min, and stained with ethidium bromide (0.5 μg ml⁻¹), finally visualised under UV light.

Statistical analysis

The pattern of RAPD products was separately gotten for every primer, amplified DNA fragments were marked as 0 (fragment absent) and 1 (fragment present) in an information matrix. A phenogram was made by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) after assessing the association coefficients by the simple matching method.

Antifungal drugs

Standard powders of antifungal drugs, griseofulvin (Sigma Chemical Co., St Louis, MO, USA), itraconazole (Janssen Pharmaceutica, Beerse, Belgium) and terbinafine (Novartis, E. Hanover, NJ, USA), were used to indispose stock solutions. Itraconazole and griseofulvin
were solvated in 100% dimethyl sulfoxide (Curtin Matheson Scientific Inc., Houston, TX, USA) and terbinafine was solvated in dimethyl sulfoxide with 5% Tween 80 (Curtin Matheson Scientific Inc.).

**Inoculums preparation**

Each isolate was subcultured onto a potato dextrose agar (PDA, Merck Co., Darmstadt, Germany) slant and incubated at 30°C for 7 days. Sterile normal saline (85%) was added to the slant and the culture was slightly swabbed with a cotton tip applicator to drive the conidia from the hyphal mat. The suspension was transferred to a sterile centrifuge tube and the volume was formulated to 5 ml with sterile normal saline. The suspension was formulated to make a conidial concentration of $1 \times 10^3$ cell ml$^{-1}$ by counting with a haemocytometer.

**In vitro susceptibility testing**

Micro dilution plates were set-up in accordance with the NCCLS M38-A$^{12}$ reference method for dermatophytes with modifications. Column 1 was filled with 200 $\mu$l of RPMI 1640 (American Biorganics Inc., Niagara Falls, NY, USA) with L-glutamine and 3-(N-morpholino) propanesulfonic acid (MOPS) to serve as a sterility control. Columns 2 through 11 were filled with 100 $\mu$l of the inoculum and 100 $\mu$l of the serially diluted antifungal drugs. Column 12 was filled with 200 $\mu$l of the inoculum and served as a growth control. Serial twofold dilutions, ranging from 0.13 to 64 $\mu$g ml$^{-1}$ for griseofulvin and 0.06 to 32.0 $\mu$g ml$^{-1}$ for itraconazole and terbinafine were tested. The microdilution plates were incubated at 30°C for 4 days then read them visually. The minimum inhibitory concentration (MIC) was defined as the point at which the organism was inhibited 80% compared with the growth in the control well. The minimum fungicidal concentration (MFC) was prescribed by subculturing a 10 $\mu$l aliquot from all MIC wells indicating no visible growth onto a PDA plate.

**Results**

Genotyping diversity of *M. canis* isolates amplified by primers of OPU 15, OPU 13 and OPA 04 is presented in Figs. 1, 2 and 3 respectively. Approximately DNA bands were observed in the electrophoresis gel of PCR products with all primers. There were some common bands between understudy *M. canis* isolates, and each isolates had some specific bands.

Similarity coefficients among 10 genotypes of *M. canis* isolates are summarised in Table 1. Maximum and minimum similarity coefficients were observed between isolates 1 and 2, and isolates 4 and 8 respectively. The yield phenogram caught by UPGMA is shown in Fig. 4.

In this study, the similarities and differences among the isolates were characterised by drawing phenogram for PCR results obtained by using each primer and all primers. The isolates were divided into seven groups by OPU 15, six groups by OPU 13 and six groups by OPA 04. According to this breakdown, maximum similarity was observed between primers OPU 15 and OPA 04. There was genetic similarity relevance between isolates 3 and 5.

Determination of the MICs and MFCs of griseofulvin, itraconazole and terbinafine against understudy *M. canis* isolates is presented in Table 2. The highest MIC against griseofulvin was observed in *M. canis* isolates 7, 8 and 9 which were 0.9 ± 0.1, 1.2 ± 0.4 and 0.95 ± 0.4 $\mu$g ml$^{-1}$ respectively.
Discussion

*Microsporum canis* is a zoophilic fungus with worldwide distribution. It survives on hair and skin of cats without any clinical symptoms, as a result, infected cats can transfer the infection directly or indirectly to human and other animal species. *M. canis* is known as an important causative agent of tinea capitis in children in urban areas of Iran and other countries. Actually, the infected cats act as an important reservoir. According to Khosravi [15] this dermatophyte did not create any clear signs in the adult cats and they are able to act as normal carriers.

Morphology and physiology characteristics of dermatophytes, especially *M. canis*, may frequently illustrate variations that entangle the diagnosis. According to the results of this study, in spite of the typical similarity in the morphology of the whole isolates, they were placed in different branches in molecular typing. It seems that some factors could influence genetic patterns, like: environmental condition, laboratory and culture techniques and conditions, the type of host (human or animal), media components, incubation temperature, using medicine and the maintenance techniques of fungal colonies.

The methods for identification of *M. canis* based on morphological analysis are not always sufficient. According to the results obtained by Brilhante et al. [7], the macromorphological analysis of *M. canis* in SDA and PA revealed four types of colonies. This variety of morphologies may be due to effort in adapting the strains to different environmental terms.

Baeza et al. [16] examined ten isolates of *Trichophyton rubrum* by RAPD and claimed that RAPD analysis can be used in epidemiological studies.

In the present study, 10 isolates of *M. canis* which obtained from human and animals were compared based on genetic diversity. The results demonstrated that there were some common bands among the isolates by using three primers. Also they showed genetic

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Figure 2 Amplification profiles of PCR products from isolates of *Microsporum canis* with primer OPU13. Molecular size marker (100 bp) is in the line marked M and corresponding sizes as base pairs are given on the right. Lanes 1, 2, 5, 6 and 10: *M. canis* isolates obtained from cats. Lane 3: *M. canis* isolate obtained from fox. Lanes 4 and 7: *M. canis* isolates obtained from human. Lane 8: *M. canis* isolate obtained from rabbit. Lane 9: *M. canis* isolate obtained from dog.

Figure 3 Amplification profiles of PCR products from isolates of *Microsporum canis* with primer OPA04. Molecular size marker (100 bp) is in the line marked M and corresponding sizes as base pairs are given on the right. Lanes 1, 2, 5, 6 and 10: *M. canis* isolates obtained from cats. Lane 3: *M. canis* isolate obtained from fox. Lanes 4 and 7: *M. canis* isolates obtained from human. Lane 8: *M. canis* isolate obtained from rabbit. Lane 9: *M. canis* isolate obtained from dog.
Some investigators studied the molecular typing of *M. canis* strains which isolated from an outbreak of tinea capitis in schools, after RAPD-PCR technique, the patterns of all the strains of *M. canis* amplified by OPI 07 and OPK 20 primers were similar and each strain had six bands, genotyping map of all strains were identical, as well. Their results are different from the results of our study; this difference can be due to genetic characteristic differences among isolates, geographical and regional differences, trial conditions and the number of studied samples.17

Dobrowolska et al. [14] evaluated the differentiation of *M. canis* isolates from patients and animals in Central Poland, the results showed that there were not any differences in the analysed *M. canis* strains by using (GACA)₄ and (ACA)₅ typing.

Liu et al. [6] used the arbitrary primed PCR (AP-PCR) technique and demonstrated that the most dermatophytes generated different DNA band patterns on gel electrophoresis; the constitution of specific DNA bands in the AP-PCR was independent of morphological variation. Based on their obtained results, the

Table 1 Similarity coefficients among ten genotypes of *Microsporum canis* isolates; ('1', '2', '3', '4', '5', '6', '7', '8', '9', '10').

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Figure 4 The yield phenogram caught by UPGM depicting relationships among 10 operational taxonomic units of *Microsporum canis* isolates using 34 characters.

Table 2 Determination of the MICs and MFCs of antifungal agents (μg ml⁻¹) against understudy *Microsporum canis* isolates; ('1', '2', '3', '4', '5', '6', '7', '8', '9', '10').

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<th>Griseofulvin</th>
<th>Itraconazole</th>
<th>Terbinafine</th>
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above-mentioned technique can differentiate dermatophytes at the genetic level.

Khosravi et al. [18] evaluated molecular typing of *Epidermophyton floccosum* isolated from patients with dermatophytosis. They analysed 13 clinical isolates of *Epidermophyton floccosum* by RAPD with seven arbitrary primers. Among these primers, OPN 16 created banding patterns from all isolates. Some of them indicated very close relation. They showed that dermatophytes lightly lose their normal phenotypical characteristics in culture and sometimes morphological characteristics can be differed in aspects of growth, colour and consistency.

Faggi et al. [5] applied RAPD-PCR for identifying different dermatophyte species, but they could not observe any interspecies differences among dermatophytes, except *Trichophyton mentagrophytes*.

Our study showed that isolates can be placed in different branches by molecular fingerprinting methods, although they were approximately identically and typical based on morphological characteristics, thus some factors can impress genetic patterns that our study was seconder.

To show the possible correlation between the antifungal susceptibility and genotypical pattern of *M. canis* isolates, microdilution test by using griseofulvin, itraconazole and terbinafine was done. In general, itraconazole and terbinafine showed good activities against understudy *M. canis* isolates (itraconazole: ranging from 0.25 ± 0.1 to 0.6 ± 0.4 μg ml⁻¹, terbinafine: 0.5 ± 0.1 to 0.6 ± 0.7 μg ml⁻¹). However, griseofulvin had more MIC levels against some isolates (7, 8 and 9) than the others (0.9 ± 0.1, 1.2 ± 0.4 and 0.95 ± 0.4 μg ml⁻¹ respectively). So this study showed that itraconazole and terbinafine were effective against the *M. canis* isolates. It is agreed with Brillhant et al. [19] results although in their study the all *M. canis* strains.

In conclusion, based on RAPD data analysis, we obtained different fingerprinting patterns of understudy *M. canis*. Interestingly, the three isolates (7, 8 and 9) with high levels of MIC to griseofulvin revealed various genetic patterns in comparison to the other ones, but further studies with a large number of *M. canis* isolates with different susceptibilities to griseofulvin should be used to approve our findings.

These three isolates have been obtained from animals and human in which they had been repeatedly treated by griseofulvin. Consequently, they showed resistance against griseofulvin.

Regarding the results, it is necessary to continue this study, with more isolates by RAPD analysis and various primers, into the future.

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