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ARTICLE

Rapid PCR based identification of two medically important dermatophyte fungi, *Microsporum canis* and *Trichophyton tonsurans*

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ABSTRACT PCR-based species specific protocols have been worked out for two important pathogens *M. canis* and *T. tonsurans* responsible for dermatophytosis with various clinical appearances. Reactions were designed to use a common reverse primer and 2 specific forward primers and were optimised to be efficient under the same PCR conditions allowing the detection of these two fungi from one reaction volume. The specific PCR reactions were evaluated both on collection strains of other dermatophytes and on clinical samples. In connection with these methods, different DNA extraction techniques were also tested to assist for an efficient PCR based detection.

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KEY WORDS

dermatophytes
DNA extraction
ITS
polymerase chain reaction

The dermatophytes are morphologically and physiologically related moulds widely distributed throughout the world. The most frequently encountered species belong to the genera *Epidermophyton*, *Microsporum*, and *Trichophyton*: as keratin utilizing microorganisms they frequently infect the hair, skin and nails. An atypical manifestation with more severe and more extensive lesions can evolve in immunocompromised patients. Direct contact or exposure to infected desquamated cells can promote transmission (Santos et al. 2006). A number of exoenzymes, such as elastase, keratinases and proteinases, which can facilitate their invasion into keratinized tissues, are the important virulence factors of these fungi (Weitzman and Summerbell 1995). Though the daily routine in the drug selection against these fungi rarely relies on the exact recognition of the microroganism, however, future trends for more efficient treatments and the need to know more about their epidemiology underline the importance of the molecular species identification. Nowadays, this is a relatively neglected field for dermatophytes. While there is a plethora of various molecular methods for most of the groups of human pathogenic fungi, in case of dermatophytes, only sporadic attempts appear in the literature (e.g.: Jackson et al. 1999, 2000; Liu et al. 2000; Arabatzis et al. 2007).

In the absence of a functional dermatophyte-specific polymerase chain reaction (PCR), current diagnosis of dermatophytoses relies on microscopy and culture. However, the combination of these techniques is particularly time-consuming and low in sensitivity.

The aim of the present study was to develop a molecular method allowing the species specific identification of *Microsporum canis* and *Trichophyton tonsurans*. In connection with this, different DNA extraction methods were tested as well as specific polymerase chain reaction (PCR) based methods were worked out and evaluated for these species.

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Materials and Methods

Strains

The following culture collection strains were used in this study: *Microsporum canis* (American Type Culture Collection, USA; ATCC 36299), *Microsporum gypseum* (ATCC 24102), *Trichophyton mentagrophytes* (ATCC 9533), *Trichophyton rubrum* (ATCC 28188) and *Trichophyton tonsurans* (ATCC 28942). Seventeen dermatophytes isolated from randomly selected clinical samples (nail and skin) were also used in the evaluation of the method. Clinical samples were collected at the Department of Dermatology, University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj Napoca (Kolozsvár), Romania. All these isolates were maintained on potato dextrose agar (PDA, Sigma, 0.4% potato starch, 2% glucose, 1.5% agar) slants at 4°C.

DNA extraction

For DNA extraction, dermatophytes were grown in potato dextrose liquid medium (PDB) under continuous shaking

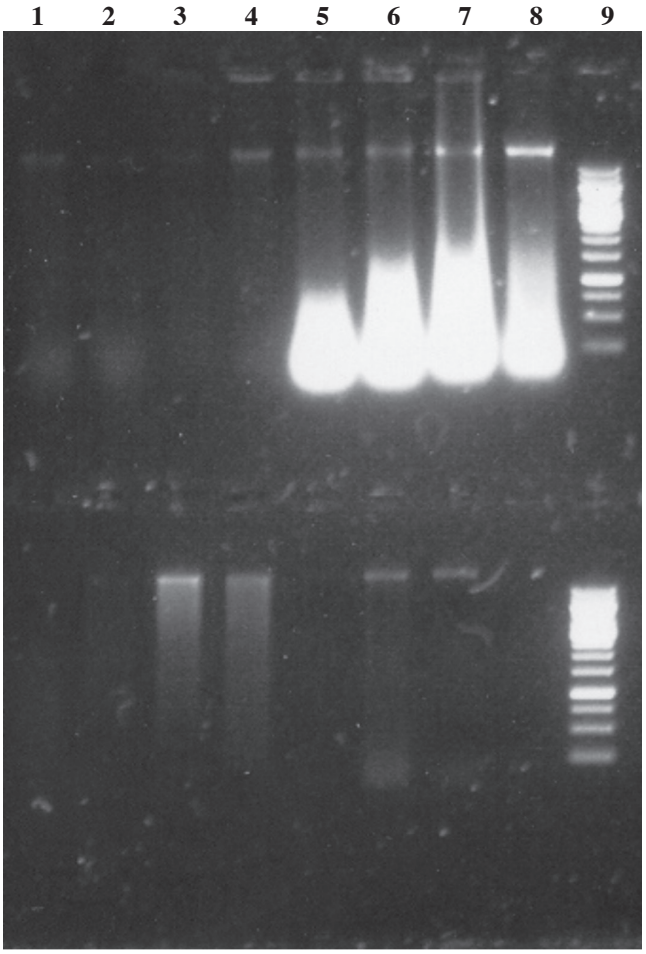

Figure 1. Comparison of DNA fragment size distributions and relative DNA yields obtained with the four DNA extraction procedures described in the Materials and Methods section. Five-microliter portions from these extractions were electrophoresed on a 0.8% (wt/vol) agarose/TAE gel. Upper and lower row, lane 9: GeneRuler 1 kb DNA Ladders (Fermentas). Lanes 1-4 and 5-8 (in both rows) contains DNA from *T. mentagrophytes*, *T. tonsurans*, *M. canis* and *M. gypseum*, respectively. Upper row, lanes 1-4 and 5-8: E.Z.N.A.® Yeast DNA Kit and MasterPure Yeast DNA Purification Kit, respectively. Lower row, lanes 1-4 and 5-8: ZR Fungal/Bacterial DNA Kit and Qiagen DNeasy Plant Mini Kit, respectively.

(200 rpm) at 30°C for 7 days. Four commercially available DNA extraction kits were tested and used in the total DNA extraction experiments: ZR Fungal/Bacterial DNA Kit (Zymo Research, Orange, CA, USA), MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), Qiagen DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands) and E.Z.N.A.® Yeast DNA Kit (Omega Biotek, Norcross, GA, USA). All these kits were used according to the instructions of the manufacturers. DNA samples were stored at -20°C.

PCR reactions and electrophoresis

Species specific PCR reactions were performed as follows: each 20 µl of reaction mixture contained 2 µl of 10 x PCR buffer (Double-Taq, ZenonBio), 4 µl of dNTP solution (200 µM each of dATP, dCTP, dGTP and dTTP), 3 µl of 25 mM $MgCl_2$ (ZenonBio), 4 - 4 µl (0.2 - 0.2 µM) of primers, 0,2 µl (0.5 U) of Double-Taq DNA polymerase (ZenonBio), 1 µl of genomic DNA extract (50 ng/µl) and 1.8 µl distilled water. Control reactions, without genomic DNA extract, were also run.

Amplifications were performed with a PTC-0148 Mini48 DNA thermocycler (BioRad, USA). The first cycle involved a denaturation step at 94°C for 2 min. This was followed by 35 amplification cycles, involving a denaturation step at 94°C for 10 sec, an annealing step at 67°C for 20 sec and a chain extension step at 72°C for 1 min.

Identification of clinical samples has been performed by the amplification of the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) followed by the sequencing of the amplification products (Luo and Mitchell 2002). The PCR primers used were ITS1 and ITS4 which amplify the variable ITS1 and ITS2 sequences surrounding the 5.8S-coding sequence and situated between the Small SubUnit-coding sequence and the Large SubUnit-coding sequence of the ribosomal operon (White et al. 1990). The conditions of the PCR were as described by Saiki et al. (1988). The reaction mixture (20 µl) contained 2 µl of 10 x PCR buffer (Double-Taq, ZenonBio), 4 µl of dNTP solution (200 µM each of dATP, dCTP, dGTP and dTTP), 3 µl of 25 mM $MgCl_2$ (ZenonBio), 4 - 4 µl (0.2 - 0.2 µM) of ITS1 and ITS4 primers, 0.2 µl (0.5 U) of Double-Taq DNA polymerase (ZenonBio), 1 µl of template DNA (50 ng/µl), 1 µl BSA (Fermentas) and 0.8 µl sterile distilled water.

Ten µl of each amplification product was separated by electrophoresis on 1% agarose/TAE (4.84 g Tris base, 1.14 glacial acetic acid, 2 ml 0.5 Na EDTA pH=8) gels and visualized by UV fluorescence (UVP, BioDoc-It™) after ethidium bromide (0.5 mg/ml) staining, using GeneRuler 100 bp Plus DNS marker (Fermentas) as size standard.

Results and Discussion

Nucleic acid extraction from dermatophytes

Protocols for extraction of DNA of fungal cells either are very time-consuming or show poor yield of DNA compared to methods of extraction of DNA, e.g. of human cells. Other protocols require additional lysis steps like sonification or mechanical disruption or harmful chemicals such as phenol-chloroform or guanidine thiocyanate. From these reasons, commercial nucleic acid extraction and purification kits are more and more popular. Nucleic acid samples with proper quality are important prerequisite for any further DNA associated identification method. This especially important for the dermatophytes providing minuscule samples from clinical sources and growing very slowly under laboratory conditions.

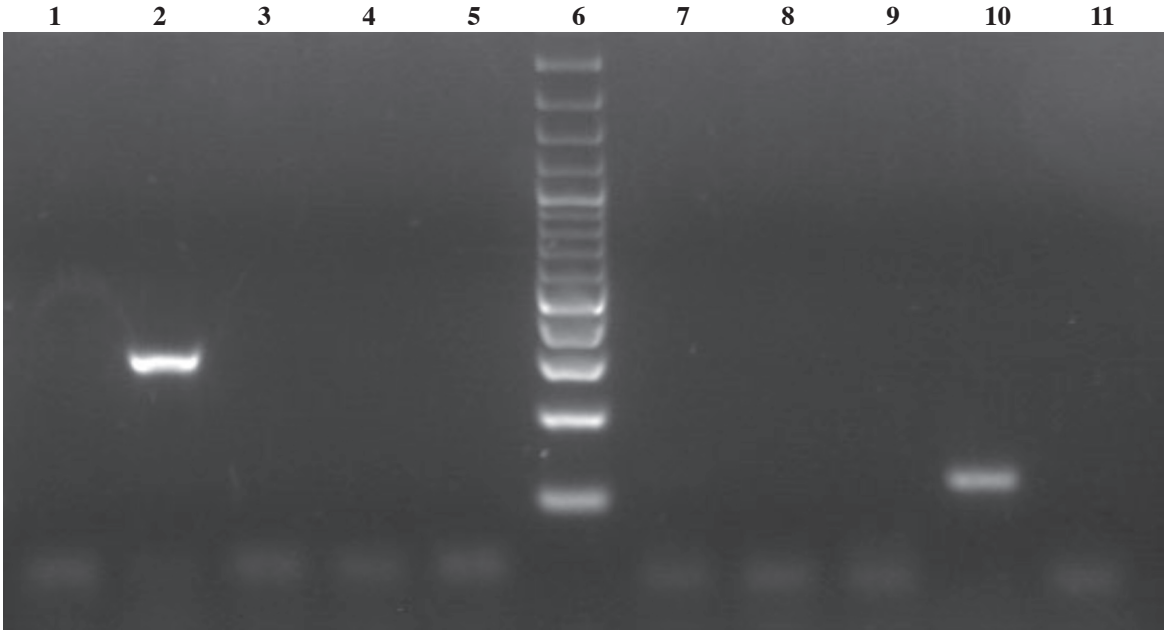

Figure 2. Amplification products of the PCR reactions with species specific primers for *T. tonsurans* (TTF/CR; lanes 1-5) and *M. canis* (MCF/CR; lanes 7-11), electrophoresed on 3% agarose gel. Lanes 1-5 and lanes 7-11: *T. mentagrophytes*, *T. tonsurans*, *T. rubrum*, *M. canis* and *M. gypseum*, respectively. Lane 6: GeneRuler 100 bp Plus DNA marker (Fermentas).

Commercial kits tested in this study utilise little bit different approaches for nucleic acid isolation. The E.Z.N.A.® Yeast DNA Kit combines the reversible nucleic acid-binding properties of HiBind® matrix with spin column technology. The MasterPure™ Yeast DNA Purification Kit's protocol involves nonenzymatic cell lysis at 65°C, followed by removal of protein by precipitation, and nucleic acid precipitation and resuspension. No lyticase, proteolytic enzymes, or bead-beating are used in the procedure. The ZR Fungal/Bacterial DNA Kit™ disrupts cells by bead beating; nucleic acids are further purified by spin column technology (ZR BashingBead™ Lysis/Filtration Tube) without using organic denaturants or proteinases. The DNeasy Plant Kits (Qiagen) provides silica-based DNA purification in spin columns: no organic extraction or ethanol precipitation is necessary for the procedure.

Among the four kits tested, MasterPure Yeast DNA Purification Kit (Epicentre) proved to be a very efficient tool for isolation DNA for dermatophytes. When tested on 4 different species (*T. mentagrophytes*, *T. tonsurans*, *M. canis* and *M. gypseum*) this protocol provided the highest yield and reproducibility (Fig. 1).

Table 1. Primers used in PCR reactions.

Primer	Sequence (5′-3′)
CR (*common reverse*)	5′ – TCGCTGCGTTCTTCATCGATG – 3′
MCF	5′ – CGCTCGCCGGAGGATTACTC – 3′
TTF	5′ – AGGATAGGGCCAAACGTCCGT – 3′

Species specific identification of *M. canis* and *T. tonsurans*

Traditionally, identifications of dermatophytes were performed using conventional methods of mycological identification which are based on the analysis of characteristic macroscopic and microscopic features of these fungi. However, such identification is difficult to perform due to the polymorphic character of these traits, additionally increased by variations in temperature, media composition, and other parameters of cultivation. Likewise, in some instances, the dermatophytes fail to produce any obvious reproductive structure in culture (sterile mycelia) which makes it impossible for ultimate diagnosis.

Molecular methodologies provide powerful new tools for the identification of various pathogenic fungi (White et al.1990; Luo and Mitchell 2002; Borman et al. 2008; Nyilasi et al. 2008). Among these, PCR-based methods are particularly promising in connection with the identification of dermatophytes because of their simplicity, specificity, and sensitivity (Jackson et al. 1999; Mochizuki et al. 2003; Ohst et al. 2004; Arabatzis et al. 2007).

Whereas in the genus *Epidermophyton*, *E. floccosum* is the only species which is pathogenic, the genera *Microsporum* and *Trichophyton* are made up of multiple anthropophilic and zoophilic species. Furthermore, besides the several species

of *Trichophyton*, several different variants are also identified within the species *T. mentagrophytes* (Gräser et al. 1999; Liu et al. 2000).

In the present study, PCR-based species specific protocols have been worked out for two important pathogens, *M. canis* and *T. tonsurans*. The sizes of amplicons from these 2 specific reactions are substantially different (128 bp, 285 bp; Fig. 2). Reactions were designed to use a common reverse primer (CR) and 2 specific forward primers (MCF, TTF; Table 1) and were optimised to be efficient under the same PCR conditions allowing the detection of these two fungi in one reaction.

When this set of primers was tested for other frequently encountered dermatophytes no false amplifications were detected (Fig. 2). They were also evaluated on clinical samples where the identity of the pathogens was later proved by sequencing the ITS gene regions. These 17 samples contained the following fungi: *T. tonsurans*, *T. rubrum* (9), *T. mentagrophytes* (3), *M. canis* (2), *M. gypseum*, and one unidentified fungus. Both *T. tonsurans* and *M. canis* were reproducible detected from these samples without the cross-reaction with other fungi (results not shown).

In our study specific PCR reactions were developed for two dermatophytes, *M. canis* and *T. tonsurans*. Further investigations with the aim to establish similar identification reactions for other frequently encountered dermatophytes are in progress now.

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