

The use of a one-step PCR method for the identification of *Microsporum canis* and *Trichophyton mentagrophytes* infection of pets

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Introduction: Dermatophytes are a closely related group of keratinophilic fungi. They encompass important etiological agents of superficial fungal infections. These fungi are able to invade keratinized tissues of humans and animals, causing dermatophytosis (ringworm) of hair, nails or skin. **The aim:** Traditional diagnostics of ringworm is based on morphological identification of cultured fungi and is time-consuming. **Materials and methods:** In this study, we applied a method patented by Brillowska-Dąbrowska and coworkers (Brillowska-Dąbrowska A, Saunte DM, Arenderup MC, 2007, Five-hour diagnosis of dermatophyte nail infections with specific detection of *Trichophyton rubrum*. *J Clin Microbiol* 45: 1200–1204) which involves extraction of fungal DNA and PCR amplification with pan-dermatophyte primers to confirm the presence of dermatophytes. **Results:** The method used here is able to confirm the presence of dermatophyte DNA in pure cultures in less than 5 hours.

Key words: dermatophytosis, animals, pan-dermatophytes, PCR,

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INTRODUCTION

Dermatophytes and yeasts are the major etiological factors of superficial fungal infections. They are common worldwide and their incidence continues to increase (Ameen, 2010). Superficial infections or colonization of keratinized tissue caused by dermatophytes — dermatophytoses, (ringworm or tinea) occur in both healthy and immunocompromised patients (Jaya *et al.*, 2009). These cosmopolitan mycoses afflict an extensive range of mammals, including humans, but rarely concern birds. Pets are often infected as well as livestock. Because of the contagiousness, difficulties in identification and costly treatment there is a high occurrence of dermatophytoses, especially in animal herds. Animal ringworm can be easily transmitted to humans (Chermette *et al.*, 2008). The main lesions of dermatophytoses are alopecia, erythema, papules, scaling or crusting. The classic clinical changes are well separated, with active inflammation at the periphery — hence ringworm.

Dermatophytes are keratinophilic pathogenic fungi that utilize keratin as a nutrient source. These fungi invade the stratum corneum of the epidermis and keratinized tissues derived from it (e.g., skin, hair, nails, fur)

(Chermette *et al.*, 2008; Putignani *et al.*, 2010). Taxonomically, these filamentous fungal pathogens belong to three related anamorphic (imperfect or asexual) genera: *Microsporum*, *Trichophyton*, and *Epidermophyton* (pathogenic for humans only). Individual species capable of reproducing sexually are classified in the teleomorphic genus *Arthroderma* of the *Ascomycota*. (Weitzman & Summerbell, 1995).

Animals can be infected by a variety of dermatophytes. These include zoophilic, geophilic (soil-adapted) as well as anthropophilic (adapted to human hosts) species. The most frequently isolated dermatophyte species from animals are: *Microsporum canis*, *Microsporum gypsum*, *Microsporum nanum*, *Trichophyton mentagrophytes*, *Trichophyton verrucosum* and *Trichophyton equinum* (Weitzman & Summerbell, 1995; Chermette *et al.*, 2008).

Routine, conventional laboratory diagnostics of dermatophytoses (“the gold standard”) involve direct microscopic examination of clinical specimens followed by *in vitro* culture techniques (Liu *et al.*, 2000; Mahoney *et al.*, 2003). Microscopic identification of fungal elements directly in clinical samples using potassium hydroxide 10% (KOH) is a quick method, but its specificity and sensitivity is low and proper execution depends on the researchers’ experience. Moreover, false negative results are possible. According to Levitt and coworkers (2010) the sensitivities for KOH smear and culture were 73.3% (CI: 66.3 to 79.5%) and 41.7% (34.6 to 49.1%) respectively. Although somewhat specific, *in vitro* culture as a diagnostic procedure is time-consuming. (Jaya *et al.*, 2009). It might take up to 4 weeks or longer to give the final results or are not successful. Furthermore, morphological identification may be confusing due to polymorphism of dermatophytes (Putignani *et al.*, 2010).

Polymerase chain reaction (PCR)-based techniques shorten the diagnostic procedure and generally have high sensitivity and specificity compared to conventional methods (Jaya *et al.*, 2009, Dworecka-Kaszak, 2011). Many PCR-based techniques such as PCR fingerprinting (Graser *et al.*, 2000; Faggi *et al.*, 2002), Random Amplification of Polymorphic DNA (RAPD) (Hrynciewicz *et al.*, 2011), Restriction Fragment Length Polymorphism (RFLP) (Arabatzis *et al.* 2006) and real-time PCR (Wiselink *et al.*, 2011) have been used recently to identify dermatophytes from *in vitro* cultures.

Further, TRFLP (PCR-terminal restriction fragment length polymorphism) (Verrier *et al.*, 2012), nested PCR (Verrier *et al.*, 2013) or PCR-ELISA (Tchernev *et al.*,

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Abbreviations: PCR, polymerase chain reaction

2013) have also found their use in identification of previously cultured dermatophytes. However, there is still need to design a rapid, reliable and reproducible molecular-based protocol that can be easily implemented in diagnostic laboratories to handle large volumes of clinical specimens in a reasonable time. The main problem, however, is the lack of a reliable procedure to extract intact fungal DNA directly from clinical specimens.

The aim of this work was to apply PCR to detect veterinary ringworm-causing species based on a technique described earlier by Brillowska-Dąbrowska and coworkers (2007) for identification of human onychomycosis. It involves a fungal DNA extraction step followed by pan-dermatophyte PCR and electrophoresis. Our results confirm the presence of dermatophyte-specific DNA in specimens derived from cultured clinical samples. Adaptation of this procedure indicates a possibility of designing a reliable protocol for quick detection of dermatophytes-specific DNA directly in veterinary clinical samples.

MATERIALS AND METHODS

Clinical isolates. Clinical specimens were obtained from the microbiology diagnostic laboratory at the Faculty of Veterinary Medicine, Warsaw University of Life Sciences-SGGW (Warsaw, Poland). Fifteen hair samples from 8 cats and 7 dogs (Fig. 1) suspected of ringworm were initially examined by direct microscopy and cultured on Sabouraud dextrose agar (SDA). Ringworm was identified in all specimens. Culture conditions were as follows: Sabouraud dextrose agar supplemented with chloramphenicol (0.05 g/l) and cycloheximide (0.4 g/l), at 30°C for up to 14 days. Pure cultures were derived basing on macro- and micromorphology (Fig. 2A and B).

DNA preparation from dermatophyte cultures. The method proposed by Brillowska-Dąbrowska and coworkers (2007) was used to isolate DNA from dermatophytes: Briefly, fungal colonies were picked and incubated for 10-min in 100 µl of extraction buffer A (60 mM sodium bicarbonate (NaHCO₃), 250 mM potassium chloride (KCl)] and 50 mM Tris, pH 9.5) at 95°C. Subsequently, a 100 µl of 2% bovine serum albumin, (BSA) was added and mixed thoroughly. The DNA-containing solution was used for PCR assay.

Pan-dermatophyte PCR. A set of primers detecting a DNA fragment encoding chitin synthase1 of dermatophytes, panDerm_for (5'GAAGAAGATTGTCGTTT-

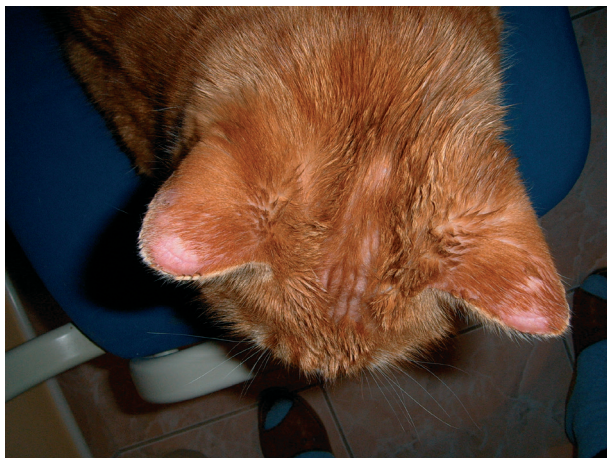


Figure 1. Typical ringworm lesions in a cat's ears due to *Microsporum canis* (Photo: W. Dardzińska)

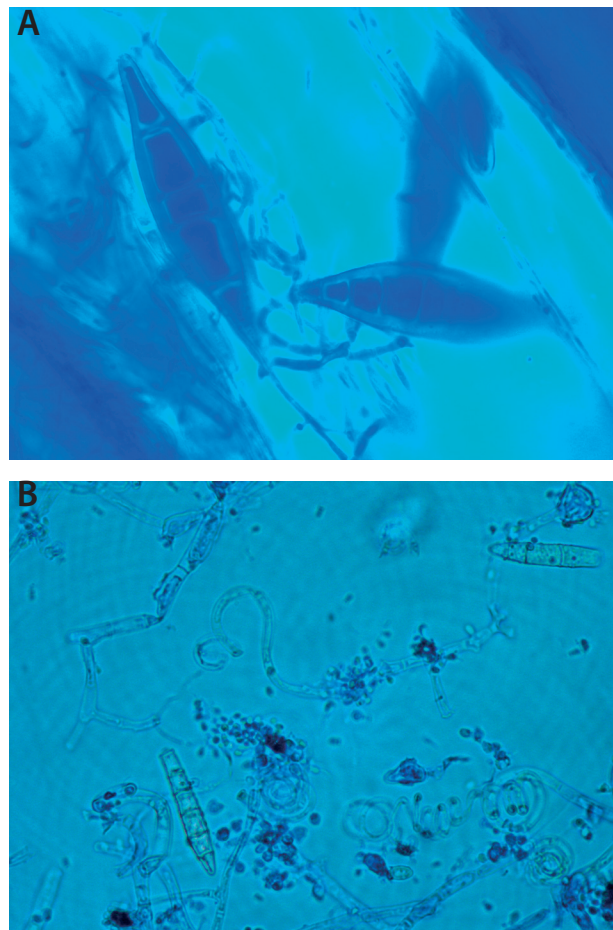


Figure 2. Photomicrograph of:

(A) macroconidia of *Microsporum canis*. Microconidia typically are absent. Macroconidia are fusoid, verrucose, and thick walled. They have a recurved apex and contain 5–15 cells.

(B) spiral hyphae of *Trichophyton mentagrophytes*. Microconidia are numerous, unicellular, round to pyriform and found in grape like clusters. Spiral hyphae are often present. Macroconidia are multiseptate, club-shaped (methylene blue, x1000) (Photo: I. Dąbrowska)

GCATCGTCTC3') and panDerm_rev (5'CTCGAGGT-CAAAAGCACGCCAGAG3'), was used for PCR assay. Two different PCR mixtures were compared. The first was a commercial preparation (2xPCR Master Mix Plus High GC [A&A Biotechnology]: Taq DNA polymerase 0.1 U/µl, MgCl₂ 4 mM, dNTPs (dATP, dCTP, dGTP, dTTP) 0.5 mM of each dNTP) and the second was a self-composed. Both contained 10 µl of PCR Mix, 0.1 µl of each primer (panDerm_for and panDerm_rev) and 2 µl of DNA in a total volume of 20 µl. PCR was performed in an Eppendorf Mastercycler thermocycler.

The time-temperature profile for PCR was; initial denaturation for 3 min at 95°C followed by 45 s at 94°C, 45 s at 54°C or 56°C or 58°C and finally 45 s at 72°C for a total of 35 cycles. The presence of a specific PCR product of approximately 366 bp was determined by electrophoresis on a 2% agarose gel containing ethidium bromide.

RESULTS

All of clinical specimens analysed by conventional 'gold standard' diagnostics (direct microscopy and culture) yielded positive results. The isolates obtained in culture, identified on the basis of their morphology in-

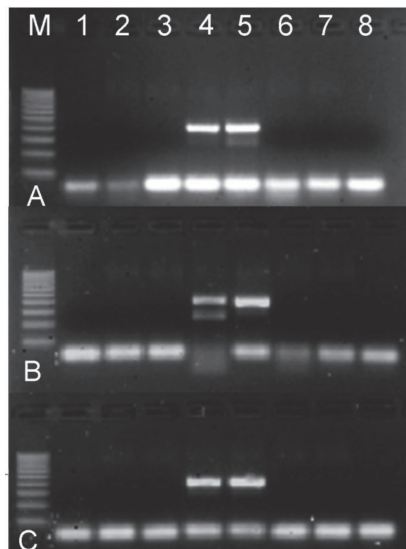


Figure 3. Electrophoretic patterns of PCR products with pan-dermatophyte primers and 2xPCR Master Mix Plus High GC (A&A Biotechnology) at:

(A) 54°C, (B) 56°C and (C) 58°C annealing temperature. Lanes 1 and 8: negative control with water, lines 2 and 3: *Microsporium canis*, lines 4–7: *Trichophyton mentagrophytes*, line M: 100–1000 bp Ladder (A&A Biotechnology).

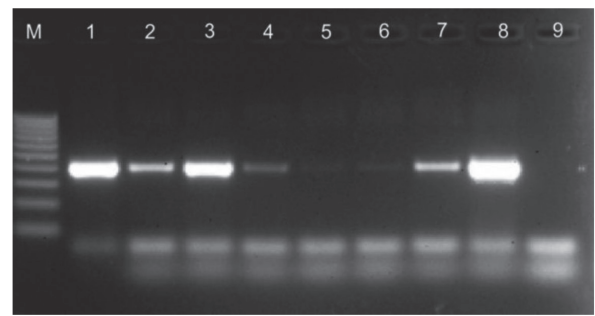


Figure 4. Electrophoretic patterns of PCR with pan-dermatophyte primers and at 58°C annealing temperature and homemade PCR mix.

Lanes 1 and 8: *Trichophyton mentagrophytes*, lines 2–7: *Microsporium canis*, line 9 negative control with water, line M: 100–1000 bp Ladder (A&A Biotechnology).

cluded seven representatives of the *Trichophyton mentagrophytes* complex strains and eight *Microsporium canis*. PCR assay confirmed correct identification of strains as dermatophytes (Table 1).

Using the pan-dermatophyte PCR primers and cycling conditions described by Bsrillowska-Dabrowska and coworkers (2007), both specific and unspecific products were obtained at annealing temperature under 58°C in the presence of commercial PCR mix (Fig. 3A, B). A

Table 1. PCR results for the fifteen clinical specimens with growth

Number of sample	Species of infected animal	Clinical diagnosis	Traditional method identification	PCR result (commercial mix; annealing temperature 58°C)	PCR result (homemade mix; annealing temperature 58°C)
1.	dog	skin lesions, alopecia	<i>Trichophyton mentagrophytes</i>	lack of specific product	specific product, 366 bp
2.	cat	skin lesions, alopecia	<i>Microsporium canis</i>	lack of specific product	specific product, 366 bp
3.	dog	skin lesions, alopecia	<i>Trichophyton mentagrophytes</i>	specific product, 366 bp	specific product, 366 bp
4.	cat	skin lesions, alopecia	<i>Microsporium canis</i>	lack of specific product	specific product, 366 bp
5.	dog	skin lesions, alopecia	<i>Trichophyton mentagrophytes</i>	lack of specific product	specific product, 366 bp
6.	cat	skin lesions, alopecia	<i>Microsporium canis</i>	lack of specific product	specific product, 366 bp
7.	dog	skin lesions, alopecia	<i>Trichophyton mentagrophytes</i>	lack of specific product	specific product, 366 bp
8.	cat	skin lesions, alopecia	<i>Microsporium canis</i>	lack of specific product	specific product, 366 bp
9.	dog	skin lesions, alopecia	<i>Trichophyton mentagrophytes</i>	specific product, 366 bp	specific product, 366 bp
10.	dog	skin lesions, alopecia	<i>Trichophyton mentagrophytes</i>	lack of specific product	specific product, 366 bp
11.	cat	skin lesions, alopecia	<i>Microsporium canis</i>	lack of specific product	specific product, 366 bp
12.	cat	skin lesions, alopecia	<i>Microsporium canis</i>	lack of specific product	specific product, 366 bp
13.	dog	skin lesions, alopecia	<i>Trichophyton mentagrophytes</i>	lack of specific product	specific product, 366 bp
14.	cat	skin lesions, alopecia	<i>Microsporium canis</i>	lack of specific product	specific product, 366 bp
15.	cat	skin lesions, alopecia	<i>Microsporium canis</i>	lack of specific product	specific product, 366 bp

specific product (approx. 366 bp) was visible only at the annealing temperature of 58°C (Fig. 3C). Commercial PCR mix yielded a specific product only when *T. mentagrophytes* DNA was used in the reaction, while the self-composed PCR mix (with lower concentration of KCl) produced specific products from both *T. mentagrophytes* and *M. canis* (Fig. 4). No contamination of negative control samples (water) was observed under the experimental conditions described here.

Although the one-step procedure described here cannot distinguish individual fungal species, the results indicate that the PCR approach may allow detection of dermatophyte specific DNA from *M. canis* and *T. mentagrophytes*.

DISCUSSION

By far the most frequently diagnostic techniques used for confirmation of dermatophyte infection are direct microscopy and cultivation on Sabouraud's dextrose agar (Tchernev *et al.*, 2013). So far this procedure has permitted identification of the etiological agent thus facilitating the selection of appropriate treatment. However, traditional methods have a diagnostic sensitivity of 50–70%, while 30–50% of fungal agents cannot be identified by conventional means (Weinberg *et al.*, 2003). In laboratory practice there are cases when precise identification of dermatophyte species is impossible due to lack of sporulation. A solution to this problem could be isolation of DNA with subsequent sequence determination followed by alignment with fungal databases. Unfortunately, such an approach is time consuming and rather too expensive for routine work. The method proposed here shows good detection and agreement with the traditional method.

Although the use of self-composed PCR mix allowed a 100% detection of dermatophyte DNA, nonetheless, there is a need to optimize the protocol. The necessity to optimize arises from using different reagents than those used in the Brillowska-Dąbrowska approach (2007). Moreover, it should be emphasized that the gold standard methods permit correct identification only if performed by a qualified mycologist with a good knowledge of dermatophyte morphological features such as micro- and macroconidia, the presence of chlamydospores etc. In this study, detection was possible in less than five hours (not including the time of culture) and results were more objective and independent of the investigator. Mycological knowledge is not required for implementation and interpretation of the results. In addition, the analysis requires a small amount of sample as would be in clinical specimens.

Only a limited number of diagnostic laboratories use modern molecular techniques such as PCR in routine diagnostics due to lack of a universally accepted diagnostic algorithm (Tchernev *et al.*, 2013). However, the method of fungal DNA extraction proposed by Brillowska-Dąbrowska and coworkers (2007) brings hope that simple, fast, and low-cost diagnoses of dermatophytosis of animals may become available as a standard in routine veterinary laboratory diagnosis.

This is the first step of research with the object of designing an optimal, simple and fast method of detection and identification of dermatophytes on the level of species directly in clinical veterinary samples.

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