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Regular paper

Real-time PCR approach in dermatophyte detection and *Trichophyton rubrum* identification

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Dermatophytes are keratinophilic molds that infect human hair, nails and skin. Diagnosis of dermatophytosis is based on morphological, serological and biochemical features. However, identification is difficult and laborious due to similarities between microorganisms. Thus, there is considerable interest to develop mycological diagnostic procedures based on molecular biology methods. In this study, fast, two-step DNA extraction method and real-time PCR was used for detection of dermatophytes DNA using pan-dermatophyte primers and identification of *Trichophyton rubrum* from pure cultures. The applied method allowed correct detection of all dermatophytes and correct identification of *Trichophyton rubrum* in less than 2 hours.

Key words: fungal infections, dermatophytosis, DNA extraction, real-time PCR

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INTRODUCTION

Dermatophytes are infectious agents causing superficial fungal infections of the skin, nails and hair of humans and animals. They are comprised of three genera: *Epidermophyton*, *Microsporum* and *Trichophyton*. The genus *Epidermophyton* is represented by one pathogenic species, *E. floccosum*, whereas *Microsporum* and *Trichophyton* are comprised of many pathogenic species (Weitzman & Summerbell, 1995). Dermatophytosis requires long-term therapy with antifungal drugs that have potential side effects, hence, the correct diagnosis is very important (Hainer, 2003; Weinberg *et al*., 2003). Typically diagnosis is based on microscopy of the clinical specimens followed by *in vitro* culture and morphological identification of fungus. Therefore, making the proper diagnosis requires specialized skills. Microscopy directly on clinical specimens is a fast method allowing the diagnosis of fungal infection, however, do not provide differentiation between dermatophytes and other molds and also gives false-negative results in 5 to 15% of the cases (Gentles, 1971; Petrini & von Rosen, 2002; Singh *et al*., 2003). *In vitro* culture is capable to provide species-specific identification based on morphological and biochemical criteria. However, it is not only costly as it requires a range of culture media, but also time-consuming because of the slow growth and the need for additional physiological tests. Additionally, the culture is negative in up to 40% of microscopy-positive cases (Petrini & von Rosen, 2002; Weinberg *et al*., 2003).

The perfect diagnostic procedure for dermatophytes infections should be simple, specific, rapid and associated with low cost. It should provide identification – to the genus as well as to the species level in order to guide appropriate treatment, provide knowledge of likely source of infection and risk of transmission and finally, report data for epidemiological studies. Several PCR tests for dermatophytes infections are described in the literature (Ebihara *et al*., 2009; Bontems *et al*., 2009; Bergmans *et al*., 2010; Wisselink *et al.,* 2011; Bergman *et al*., 2013; Miyajima *et al*., 2013; Verrier *et al*., 2013; Abastabar *et al*., 2014), of which, however, only few have been reported implemented in routine testing (Brillowska-Dąbrowska *et al*., 2007; Alexander *et al.,* 2011; Kondori *et al*., 2013; Mehlig *et al.,* 2014). This may be because of the limitations associated with the available diagnostic tests, including limited number of species identified to the species level (Berk *et al*., 2011; Brillowska-Dąbrowska *et al.,* 2013), complicated or multi-step DNA extraction procedures from patient specimen (Garg *et al*., 2009; Litz & Cavagnolo, 2010; Berk *et al*., 2011; Bergman *et al*., 2013; Miyajima *et al.,* 2013), the need of automated systems that are not available in small laboratories (Wisselink *et al*., 2011) or necessity of a pre-PCR culture step.

Here, we present a real-time PCR test for detection of any of the dermatophytes (pan-dermatophyte) without their differentiation and identification of fungal DNA from *T. rubrum* isolates. This involves two-step DNA extraction procedure followed by real-time PCR. This method may be applied in routine diagnostic laboratories due to 100% specificity and selectivity as well as significant reduction of analysis time, as the whole procedure — DNA extraction and real-time PCR, for 30 analysed samples, is 1.5 h method.

MATERIALS AND METHODS

Microorganisms and controls. A total number of 55 isolates form collection of Department of Microbiology, Gdańsk University of Technology (Poland) and 9 from Department of Dermatology, Venereology and Allergology, Medical University of Gdańsk (Poland) were included in the study: (9) *Epidermophyton floccosum,* (2) *Microsporum audouinii,* (11) *Microsporum canis,* (1) *Microsporum gypseum*, (13) *Trichophyton mentagrophytes*, (14) *Trichophyton rubrum*, (1) *Trichophyton soudanense*, (7) *Trichophyton terrestre*,

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Abbreviations: PCR, polymerase chain reaction

Table 1. Real–time PCR results for dermatophyte and non–dermatophyte isolates.

Isolates	Results			
	panDermatophyte		*Trichophyton rubrum*	
Dermatophytes	positive results/specimens number	T_m (°C)	positive results/specimens number	T_m (°C)
Epidermophyton floccosum	9/9	88.5–90	0/2	–
Microsporum audouinii	2/2	92	0/2	–
Microsporum canis	11/11	87.5–92	0/2	–
Microsporum gypseum	1/1	89–92	0/1	–
Trichophyton interdigitale	1/1	89	0/1	–
Trichophyton mentagrophytes	13/13	88–89	0/13	–
Trichophyton rubrum	14/14	87.5–88.5	14/14	92.5
Trichophyton soudanense	1/1	88	0/1	–
Trichophyton terrestre	7/7	88.5–89	0/7	–
Trichophyton tonsurans	3/3	88.5–89	0/3	–
Trichophyton verrucosum	2/2	87.5–88	0/2	–
Trichophyton violaceum	1/1	87	–	–
Non–dermatophytes				
Acremonium charticola	0/1	–	0/1	–
Acremonium kiliense	0/1	–	0/1	–
Alternaria alternata	0/1	–	0/1	–
Alternaria species	0/1	–	0/1	–
Candida albicans	0/1	–	0/1	–
Candida glabrata	0/3	–	0/1	–
Candida krusei	0/1	–	0/1	–
Fusarium culmorum	0/1	–	0/1	–
Mucor circinelloides	0/1	–	0/1	–
Mucor racemosus	0/1	–	0/1	–
Penicillium digitatum	0/1	–	0/1	–
Penicillium verrucosum	0/1	–	0/1	–
Penicillium paneum	0/1	–	0/1	–
Rhizosporus oligosporus	0/1	–	0/1	–
Rhizosporus oryzae	0/1	–	0/1	–
Scopulariopsis brevicaulis	0/1	–	0/1	–

(3) *Trichophyton tonsurans*, (2) *Trichophyton verrucosum,* (1) *Trichophyton violaceum*. Identification was done by observation of macro- and micromorphology.

DNA extraction. DNA was extracted as described before (Brillowska-Dąbrowska, 2007). Shortly, small fragment of mycelium was resuspended in 100 µl of extraction buffer (60 mM $NaHCO_3$, 250 mM KCl and 50 mM Tris, pH 9.5), followed by 10 min incubation at 95°C. Next, 100 µl of neutralization buffer was added (2% bovine serum albumin). After vortex mixing DNA-containing solution was stored at +4°C for subsequent analysis.

Pan-dermatophyte real-time PCR. The specific primers detecting a DNA fragment encoding chitin synthase 1, panDerm1 (5' GAA GAA GAT TGT CGT TTG CAT CGT CTC 3') and panDerm2 (5' CTC GAG GTC AAA AGC ACG CCA GAG 3') (Brillowska-Dąbrowska *et al.,* 2007) were used. PCR mixture consisted of 10 µl SybrA (A&A Biotechnology, Poland), 0.2 µl of each primer (panDerm1 and panDerm2) at 100 µM and 2 µl of DNA in a volume of 20 µl. The real-time PCR was performed in LightCycler® Nano (Roche). The time-temperature profile for PCR was 5 min initial denaturation at 95ºC, 40 cycles consist of: 15 sec denaturation at 95ºC, 15 sec annealing at 55ºC, acquisition of signal after 25 sec elongation at 72ºC. The reaction was followed by melting curve analysis (15 sec at 95ºC, 20 sec at 40ºC at ramp of 4ºC/s and 95ºC for 20 sec at ramp of 0.1ºC/s). The melting temperature of the generetad PCR products in the range of 87 to 92ºC indicates the presence of dermatophytes DNA in the examined sample.

Trichophyton rubrum real-time PCR. *Trichophyton rubrum* real-time PCR was performed as follows. The specific primers detecting internal transcriber spacer 2, universal, uni (5' TCT TTG AAC GCA CAT TGC GCC 3') and *T. rubrum* specific, Trubrum-rev (5' CGG TCC TGA GGG CGC TGA A 3') (Brillowska-Dąbrowska *et al.,* 2007) were used. Each reaction was performed in a volume of 20 µl by the addition of 2 µl of DNA from

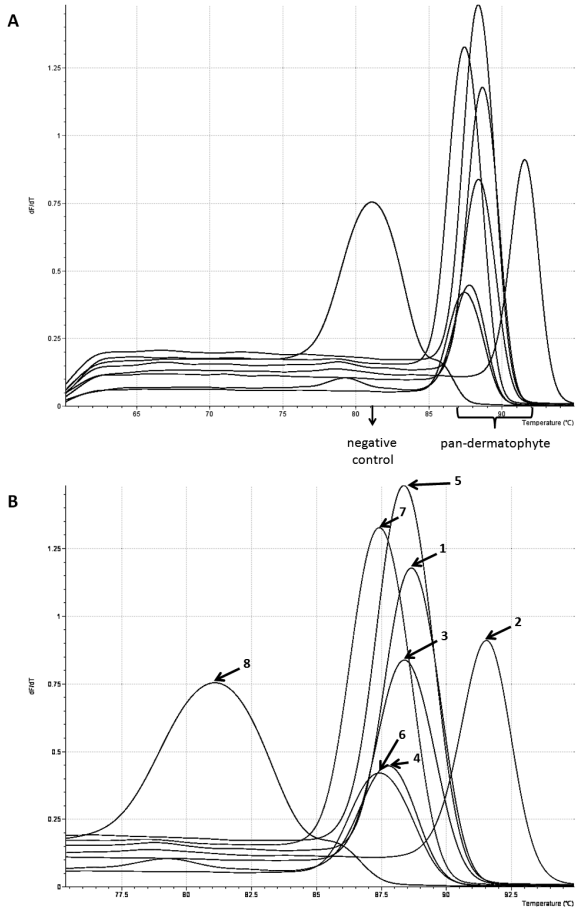

Figure 1. Pan-dermatophyte melting-curve analysis which enables detection of all dermatophytes spesies.
The change in fluorescence/change in temperature is plotted against temperature. (**A**) Representative melting-point analysis of pan-dermatophyte PCR products at annealing temperature 55°C. (**B**) Dermatophytes were represented by: 1, *Epidermophyton floccosum;* 2, *Microsporum audouiniii;* 3, *Trichophyton mentagrophytes;* 4, *Trichophyton rubrum;* 5, *Trichophyton terrestre;* 6, *Trichophyton verrucosum;* 7, *Trichophyton violaceum* and 8, negative control.

microorganisms listed above, 0.2 μl of primers (at 100 μM), 10 μl of SybrA. The real-time PCR was performed in LightCycler® Nano (Roche). The time-temperature profile for PCR was 5 min initial denaturation at 95ºC, 40 cycles consist of: 15 sec denaturation at 95ºC, 15 sec annealing at 62ºC, acquisition of signal after 25 sec elongation at 72°C. The reaction was followed by melting curve analysis (15 sec at 95ºC, 20 sec at 40ºC at 4ºC/s ramp and 95ºC for 20 sec at ramp of 0.1ºC/s). The samples, which melting curve indicated temperature in the range of 92 to 93ºC were considered as positive.

RESULTS

The real-time PCR results using pure culture DNA and controls extracted with rapid procedure (Brillowska-Dabrowska, 2007), and the dermatohyte specific primers (panDerm1 and panDerm2), and *T. rubrum* specific primers (uni and Trubrum-rev) are shown in Table 1. The pan-dermatophyte specific product melts in the temperature range of 87 to 92ºC, the primer-dimer melting temperature is 81ºC (Fig. 1). As seen in Table 1 a 100% specificity was found for pan-dermatophyte PCR

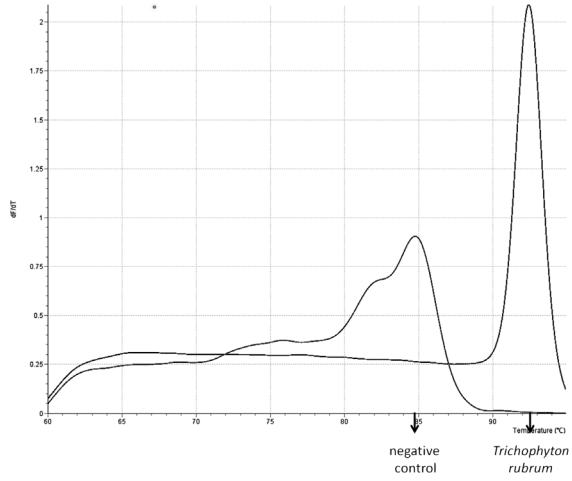

Figure 2. Melting-curve analysis which enables detection of *Trichophyton rubrum* species.
Representative melting-point analysis of *Trichophyton rubrum* specific real-time PCR products at annealing temperature 62°C. Change in fluorescence/change in temperature is plotted against temperature.

as positive results were found for all 64/64 samples containing target DNA and negative for the 18/18 control fungal and bacterial samples. The same 100% specificity was achieved for *T. rubrum* PCR as all 14 *T. rubrum* isolates were positive, all other 34 dermatophytes, and 16 non-dermatophyte molds were negative as expected (Table 1). The *T. rubrum*-specific product melts in the temperature range of 92 to 93ºC and the primer-dimer melting temperature is 84.8ºC (Fig. 2). Additionally, the test was performed with negative results on DNA of 4 healthy individuals.

DISCUSSION

There are several advantages of real-time PCR over conventional PCR, that are especially important in routine clinical settings working 8 hours a day. One is shortening of the hands-on time of analysis. Real-time PCR based assay takes around 1 h, whereas conventional PCR takes around 2 h. Furthermore, the final unequivocal results of real-time PCR are available immediately after completion of the analysis, that allows completing the diagnosis within one working day. Besides, the electrophoresis that is necessary for the analysis of PCR products should be prepared and performed in the separate room or at least as a last task in the laboratory work. Moreover, real-time PCR-based methods provide not only specific and simple diagnosis, but also reduce risk of contamination by PCR products. On the other hand, a majority of the described dermatophyte identification methods implementing conventional and real-time PCR requires multistep DNA extraction methods (Miyajima *et al.*, 2013; Bergman *et al.,* 2013) increasing risk of contamination as well as elongate procedure.

In the paper, we have been the first to present the possibility of using DNA extracted by fast procedure described previously (Brillowska-Dąbrowska, 2007) as a template for the real-time PCR technique. The use of pan-dermatophyte primers in real-time PCR allowed a 100% detection of dermatophyte DNA isolated from pure culture. In case of *T. rubrum*-specific real-time PCR there was 100% specificity and 100% sensitivity. Analysis of melting temperatures of the PCR products revealed presence of non-specific products in non-template con-

trols, which are produced by primers binding to each other ('primers-dimers') due to abundance of primer and lack of template. There is no formation of 'primer-dimer' structure in the samples containing template. In both cases, they exhibit a lower melting temperature than the amplicon. Moreover, this method allowed reduction of detection time, as conventional PCR and electrophoretic detection of the PCR products takes 3 hours, whereas real-time PCR takes only 1 hour.

However, the introduction of the described test to the routine praxis requires detailed examination on the large number of patient specimens.

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