Evaluation of a single-tube real-time PCR for detection and identification of 11 dermatophyte species in clinical material

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Abstract

We developed a dermatophyte-specific single-tube real-time PCR assay based on internal transcribed sequences. This assay allows the rapid detection and identification of 11 clinically relevant species within the three dermatophyte genera *Trichophyton*, *Microsporum* and *Epidermophyton* in nail, skin and hair samples within a few hours. Analysis of 145 clinical samples (107 nail, 36 skin scale, and two hair) by both real-time PCR and a PCR–reverse line blot (PCR-RLB) assay described earlier revealed that 133 of the 145 samples had concordant real-time PCR and PCR-RLB detection results (83 positive, 49 negative, and one inhibited). Six samples were positive by real-time PCR and negative by PCR-RLB, and two were negative by real-time PCR and positive by PCR-RLB. Four samples demonstrated inhibition in one of the two PCR assays. Only one of 83 positive samples had discordant identification results between both assays (*Trichophyton verrucosum* and *Trichophyton erinacei* by real-time PCR and *Trichophyton erinacei* by PCR-RLB). Dermatophytes present in seven positive samples that were incompletely identified as *Trichophyton* sp. by PCR-RLB were identified to the species level by real-time PCR as *Trichophyton interdigitale* and *Trichophyton rubrum* in six cases and one case, respectively. One hundred and twenty of 145 samples were also analysed by conventional dermatophyte culture and by direct microscopy. Our single-tube real-time PCR assay proved to be suitable for direct detection and identification of dermatophytes in nail, skin and hair samples with minimal total assay time (4 h after overnight lysis) and hands-on time, without the need for post-PCR analysis, and with good sensitivity and specificity.

Keywords: Dermatophytes, diagnosis, real-time PCR
Original Submission: 24 March 2009; **Revised Submission:** 30 June 2009; **Accepted:** 1 July 2009
Editor: M. Cavling Arendrup
Article published online: 23 September 2009
Clin Microbiol Infect 2010; **16**: 704–710
10.1111/j.1469-0691.2009.02991.x

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Introduction

Dermatophytes (*Trichophyton*, *Microsporum*, and *Epidermophyton*) cause superficial infections such as ringworm, favus, or onychomycosis. Although several PCR-based assays for the detection of dermatophytes have been published, laboratory diagnosis still relies on microscopic examination of clinical samples, microscopic and macroscopic observation of *in vitro* cultures, and metabolism tests. Culture has a low sensitivity (*c.*~70%), is time-consuming (2–4 weeks), and can be difficult [1].

Recently, we reported the characteristics of a PCR–reverse line blot (PCR-RLB) assay for the detection and identification of nine dermatophyte species in clinical material [1]. Li *et al.* [2] published a similar oligonucleotide array assay that could potentially identify 17 dermatophyte species, but it was not validated for detection in clinical specimens. Furthermore, PCR hybridization assays are time-consuming. In 2007, a real-time PCR assay was described for the detection and identification of dermatophytes in clinical specimens [3]. However, that assay cannot identify all clinically relevant species to species level, and it requires two PCR reactions to detect all species.

Recent work has shown that internal transcribed sequences (ITSs) between rRNA genes are sufficiently polymorphic for identification of dermatophytes to species level [1,4–13]. We developed a single-tube dermatophyte-specific real-time PCR assay based on ITS1 sequences, using species-specific probes to detect and identify 11

species in nail, skin and hair samples. With this method, up to 25 samples can be analysed simultaneously within 4 h after overnight lysis, with minimal hands-on time (2.30 h).

Materials and Methods

Strains and clinical samples

Eighteen dermatophyte strains from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), one clinical *Trichophyton erinacei* isolate and 11 non-dermatophyte fungal isolates (Table 1) [14] were used to develop this PCR assay. First, specificity of the assay was tested in two groups of dermatophytes: the 11 clinically most relevant dermatophyte species (Table 1; group A dermatophytes) and more rare dermatophytes (Table 1; group B dermatophytes). For a subgroup, the limit of detection (LOD) was determined, covering all fluorescence channels. Second, the performance of the real-time PCR assay was examined in 145 clinical samples (107 nail, 36 skin scales, and two hair) of patients suspected of having dermatophytosis. They were selected for this study on the basis of PCR-RLB results. From each sample, DNA was extracted using a MagNA Pure Compact (MPC) nucleic acid (NA) extraction robot (Roche). The DNA was first analysed by PCR-RLB, and subsequently by real-time PCR. Additionally, 120 of the 145 samples were analysed by culture and by direct microscopy [1].

TABLE 1. Strains used for real-time PCR analytical specificity testing, with real-time PCR results

Species	Group	Reference	Real-time PCR
Trichophyton			
rubrum	A	CBS 289.86	*T. rubrum*
interdigitale	A	CBS 558.66	*T. interdigitale*
mentagrophytes	A	CBS 572.75	*T. mentagrophytes*
violaceum	A	CBS 319.31	*T. violaceum*
tonsurans	A	CBS 219.32	*T. tonsurans*
verrucosum	A	CBS 134.66	*T. verrucosum*
erinacei	A	CBS 511.73	*T. erinacei* 1[a]
erinacei	B	Clinical isolate	*T. erinacei* 2[b]/*concentricum*
concentricum	A	CBS 109405	*T. erinacei* 2[b]/*concentricum*
schoenleinii	B	CBS 335.32	*T. mentagrophytes*
simii	B	CBS 150.66	*T. mentagrophytes*
gloriae	B	CBS 663.77	–
terrestre	B	CBS 464.62	–
Microsporum			
canis	A	CBS 132.88	*M. canis* complex
audouinii	A	CBS 404.61	*M. audouinii*
ferrugineum	A	CBS 457.80	*M. canis* complex
duboisii	B	CBS 349.49	–
gypseum	B	SKML 2006-II-A	–
Epidermophyton floccosum	A	CBS 358.93	*E. floccosum*
Acremonium sp.		Clinical isolate	–
Aspergillus fumigatus		Clinical isolate	–
Candida albicans		CBS 1893	–
Chaetomium sp.		Clinical isolate	–
Cladosporium herbarum		Clinical isolate	–
Malassezia furfur		Clinical isolate	–
Scopulariopsis brevicaulis		SKMM 98-IV-1A	–
Scytalidium japonicum		Clinical isolate	–
Trichosporon mucoides		CBS 7616	–
Corynebacterium jeikeium		RIVM strain	–
Staphylococcus epidermidis		ATCC 12228	–

–, negative.
[a]Genetic variant 1.
[b]Genetic variant 2.

DNA isolation and real-time PCR amplification

Clinical samples for both PCR methods were cut into small pieces and incubated overnight at 55°C in 400 μL of lysis buffer: MagNA Pure LC Lysis Binding Buffer (Roche, Mannheim, Germany), water (3 : 2) with 32 μL of dithiothreitol (1 M), and 20 μL of proteinase K (20 mg/mL). Then, 5 μL of 1/1000 diluted phocine herpes virus 1 (PhHV-1) [15] was added as an internal control target, and total NA was isolated using the Total NA Isolation Kit (Roche) on an MPC NA isolation robot. DNA was eluted in 100 μL of buffer. Dermatophyte-specific PCR primers and species-specific LC hybridization probe sets for real-time PCR (each consisting of an anchor probe and a sensor probe) were targeted on dermatophyte ITS1 and 5.8S rRNA gene sequences present in the GenBank database, using Multalin (http://bioinfo.genotoul.fr/multalin/multalin.html) and LightCycler Probe Design software (Roche). Probes were labelled with 3′-fluorescein, 5′-LC610, 5′-LC640, 5′-LC670, or 5′-LC705 labels. Anchor probes were designed to match ITS1 sequences of two or three dermatophyte species, whereas sensor probes were designed to perfectly match only one of those species (Fig. 1). In this way, up to three different species can be detected and identified with one probe, using melting curve analysis. Internal control (PhHV-1) primers and probes described by Niesters were adapted to hybridization probe format; reverse primer PhHVR1-705 was internally labelled to also act as a probe in combination with probe PhHVP1-FL [15] (Table 2).

Five microlitres of DNA isolated from clinical samples were used in 20-μL real-time PCR reactions containing 4 μL of LC Multiplex Master Hyb Probes (Roche), 400 nM primer DERMF3 and 1300 nM primer DERMR2, 330 nM each of LC hybridization probe (Table 2), 1.3 mM $MgCl_2$, 0.5 μL dimethylsulphoxide, and 0.2 units of uracil DNA glycosylase (Roche). Positive and negative extraction and PCR controls were included in each run. The following PCR program was performed on a LightCycler 2.0 (Roche): initial denaturation for 10 min at 95°C, and 50 cycles of 20 s at 95°C, 20 s at 55°C, and 20 s at 72°C. This was followed by a melting curve analysis (1 min at 95°C, cooling for 1 min at 50°C, and ramp to 85°C, at a ramp rate of 0.1°C/s). Colour compensation was performed according to the manufacturer's instructions, and fluorescence signals were corrected by the 'back 530' signal.