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Diagnosis of common dermatophyte infections by a novel multiplex real-time polymerase chain reaction detection/identification scheme

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Conflicts of interest

None declared.

Summary

Background In the absence of a functional dermatophyte-specific polymerase chain reaction (PCR), current diagnosis of dermatophytoses, which constitute the commonest communicable diseases worldwide, relies on microscopy and culture. This combination of techniques is time-consuming and notoriously low in sensitivity.

Objectives Recent dermatophyte gene sequence records were used to design a real-time PCR assay for detection and identification of dermatophytes in clinical specimens in less than 24 h.

Patients and methods Two assays based on amplification of ribosomal internal transcribed spacer regions and on the use of probes specific to relevant species and species-complexes were designed, optimised and clinically evaluated. One assay was for detecting the *Trichophyton mentagrophytes* species complex plus *T. tonsurans* and *T. violaceum*. The second assayed for the *T. rubrum* species complex, *Microsporum canis* and *M. audouinii*.

Results The analytical sensitivity of both assays was 0·1 pg DNA per reaction, corresponding to 2·5–3·3 genomes per sample. The protocol was clinically evaluated over 6 months by testing 92 skin, nail and hair specimens from 67 patients with suspected dermatophytosis. Real-time PCR detected and correctly identified the causal agent in specimens from which *T. rubrum*, *T. interdigitale*, *M. audouinii* or *T. violaceum* grew in culture, and also identified a dermatophyte species in an additional seven specimens that were negative in microscopy and culture.

Conclusions This highly sensitive assay also proved to have high positive and negative predictive values (95·7% and 100%), facilitating the accurate, rapid diagnosis conducive to targeted rather than empirical therapy for dermatophytoses.

Dermatophytic skin infection remains one of the most common communicable diseases worldwide, entailing annual diagnosis and treatment costs of over $400 million.[1] Currently, the diagnosis of these diseases is based on the demonstration of fungal structures (hyphae and arthroconidia) in direct microscopy of clinical specimens plus culturing and identification of the causative species.[2] This combination is referred to as the diagnostic gold standard.[3] However, these standard methods, which have been in use for over 80 years, have a number of disadvantages. Direct microscopy lacks specificity as it generally does not allow distinction of different fungal species. Culturing and subsequent species identification is slow, usually requiring 2–4 weeks. The sensitivity of both methods in routine clinical laboratory practice is low, especially for hair and nail infections. For example, the sensitivity of direct microscopy, as reported in a classical review on tinea

capitis, ranges from 67% to 91%.[4] Recently, in an extensive onychomycosis study in which serial specimens from 473 patients were analysed by highly experienced dermatological mycology reference laboratory staff, direct microscopy of potassium hydroxide (KOH)-treated specimens had a sensitivity of just 73·8%, while culture of the primary specimen had a sensitivity of only 74·6%.[5] Even to achieve this level of success, both microscopy and culture identification require considerable training of personnel and also considerable supervisory expertise.

Conventional polymerase chain reaction (PCR)-based diagnostic methodologies[6–8] have not offered a better alternative to the standard methods, mainly because the extraordinary genomic homogeneity characteristic of the dermatophyte species, especially the anthropophilic species, has not permitted successful design of specific primers for genera or species. As a result, the published assays have of necessity been based on the use of general fungal primers. Performance testing showed that these assays were not able to confirm dermatophytic disease nor to reveal the identity of the causative species. A potentially more sensitive approach is used in real-time PCR techniques that are based on detection with cyber green or with specific fluorophore-labelled oligonucleotide probes. This series of techniques has revolutionized the way human pathogens are diagnosed,[9] and in our view could prove to be a powerful practical tool in dermatophyte diagnostics, provided that specific probes can be successfully designed for dermatophyte species.

The primary aim of this study was to design and develop a multiplex real-time PCR assay, based on specific fluorophore-labelled probes, for rapid direct detection and identification of the major pathogenic dermatophytes in clinical specimens. *Trichophyton rubrum*, *Microsporum canis*, *T. tonsurans* and members of the *T. mentagrophytes* complex were selected as target organisms because they are the most important indigenous European dermatophytes, while *T. violaceum* and *M. audouiniii* were selected as the most common imported anthropophilic species in Europe. It should be noted that the *T. mentagrophytes* complex is a group of related species formerly identified under this familiar name but now distinguishable in sequence-based studies as three distinct phylogenetic species complexes, *Arthroderma vanbreuseghemii/T. interdigitale*, *A. benhamiae/T. erinacei* and *T. mentagrophytes* per se, now strictly taxonomically defined as the former *T. mentagrophytes* var. *quinckeanum*, the agent of murine favus. A second aim of the study was to clinically evaluate the novel assay in comparison with current gold standard diagnostic methods in tests of skin, nail and hair specimens from patients with suspected dermatophytosis.

Patients and methods

Patients and clinical specimens: conventional diagnostics

All patients attended the department of dermatology in the Leiden University Medical Centre and gave informed consent. From August to December 2005, the 92 clinical specimens analysed included 53 standard skin scale specimens from suspected tinea pedis, nine specimens from tinea corporis, two from tinea manuum, and two from tinea cruris, along with 21 nail clipping specimens from tinea unguium and five specimens of plucked hairs and/or scalp scrapings from tinea capitis. As a control, 40 specimens were collected from patients known or strongly suspected not to have dermatophytosis: 20 skin scale specimens came from psoriasis or eczema patients and 20 nail specimens came from clinically healthy patients. All specimens were examined by direct microscopy and culture, while a portion of each was stored at −20 °C for use in the real-time PCR assay. Culture was performed with Sabouraud dextrose agar supplemented with chloramphenicol (0·05 g L^{-1}) and cycloheximide (0·5 g L^{-1}), at 27 °C for up to 2 weeks. The isolates were identified according to Kane *et al.*[2]

Nucleic acid extraction

DNA from the clinical specimens was extracted according to the nail-specific protocol of Tahir and Watson,[10] modified by inserting a purification step with the QiaAmp DNA extraction kit (Qiagen, Hilden, Germany) after enzymatic digestion with Proteinase K (Qiagen). Briefly, finely cut (1–3 mm) nail, skin and hair specimens were incubated overnight at 56 °C in 200 μL buffer [10 mmol L^{-1} Tris–HCl pH 8·0, 10 mmol L^{-1} Na_2-EDTA (ethylenediamine tetraacetic acid), 100 mmol L^{-1} NaCl, 2% sodium dodecyl sulfate, all from Sigma, St Louis, MO, U.S.A.] supplemented with 15 μL proteinase K (20 mg mL^{-1}, Qiagen) and 20 μL 1 mol L^{-1} dithiothreitol (Sigma). An additional 10 μL Proteinase K was added and the samples were incubated for a further 3 h. Subsequently, nucleic acid extraction was performed from each specimen with the QiaAmp DNA extraction kit (Qiagen) according to the manufacturer's instructions. At the end of the procedure, DNA was eluted in 60 μL of AE buffer (10 mmol L^{-1} Tris–Cl; 0·5 mmol L^{-1} EDTA; pH 9·0) provided by the manufacturer. Negative controls (sterile distilled water) were included in each extraction run.

Experimental design for polymerase chain reaction

The proposed real-time PCR procedure is based on a two-tube system. The first tube is used to detect the *Trichophyton mentagrophytes* species complex, as well as *T. tonsurans* and *T. violaceum*, based on amplification of the rDNA internal transcribed spacer 1 (ITS1) region. The second tube is designed to detect the *T. rubrum* species complex, *Microsporum canis* and *M. audouinii*, based on the ITS2 amplification. Both assays use general fungal primers and discrimination of targets is achieved by using species-specific and species-complex-specific Taqman® and minor groove binder (MGB) probes carrying discrete fluorophores. Taqman probes had the nonfluorescent quencher BHQ3 (black hole quencher 3) at the 3′ end. MGB probes were used for their purportedly refined specificity, as they have been stated to allow identification based on only a single nucleotide mismatch in the probe sequence.[11] Primers were selected from

the conserved 18S, 5·8S and 28S genes flanking the ITS1 and ITS2 regions while probes were selected from the more variable ITS1 and ITS2 regions. It is usually recommended that clinical real-time PCR assays be designed to produce amplicons smaller than 200 bp, ideally around 150 bp. In this case, the general fungal primers were designed to facilitate positioning on the conserved flanking genes. As a result, longer products (229–301 bp) that amplify the complete ITS regions were to be evaluated for consistency in detecting and identifying the aforementioned dermatophytes in clinical material.

Primers and probes for dermatophyte real-time polymerase chain reaction assays

Primer and probe sequences were selected from an alignment of ITS nucleotide sequences of the six target species as well as other common skin pathogens and common contaminant dermatophytoids (nonpathogenic *Trichophyton* and *Microsporum* species), molds and yeasts. All the relevant ITS sequences available (n = 49) were used in the alignment; they were derived from ex-type or other reference strains (Table 1). The ITS region was selected as a PCR target for several reasons. Firstly, it is a multicopy region and therefore, a few dermatophyte cells in a sample can present hundreds of PCR target molecules. Secondly, it is the only region that has been sequenced for all dermatophyte species, and is also the locus that has most commonly been sequenced for other fungi. Thirdly, it usually contains sufficient interspecies variation to allow the design of specific probes targeted not just at species but also at morphologically defined sibling-species complexes and other infrageneric clades consisting of closely interrelated species. The reference sequences used in the Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA, U.S.A.) were: *T. rubrum*, accession no. Z97993; the *T. mentagrophytes* complex member currently correctly known as the *Trichophyton* anamorph of *Arthroderma benhamiae*, accession no. Z97997; the *T. mentagrophytes* complex member correctly known as *T. interdigitale*, closely related to the teleomorph *A. vanbreuseghemii*, accession no. Z98001; the *T. mentagrophytes* complex member correctly known as *T. mentagrophytes* (the former *T. mentagrophytes* var. *quinckeanum*), accession no. Z97995; *T. violaceum*, accession no. AJ270811; *T. tonsurans*, accession no. Z98008; *M. canis*, accession no. AJ000618; and *M. audouinii*, accession no. AJ000622. The PCR primers and probes were optimised using Beacon Designer 4.0 (Premier Biosoft International) at annealing temperature of 55 °C to minimize primer–primer, primer–probe and probe–probe interactions.

The selected primers and probes are shown in Table 2. The predicted specificity of the primers and probes was evaluated by BLAST [GenBank sequence database, http://www.ncbi.nih.gov/gebank/index.html (accessed 29 May 2007)].

Multiplex real-time polymerase chain reaction assays

Amplification, detection and data analysis were performed using the iCycler IQ4™ real-time detection system (BioRad, Veenendaal, the Netherlands). Before the multiplex assays were set up, PCR conditions were optimized for individual reactions containing only primers and one probe. Subsequently, PCR conditions were optimized for the multiplex configuration. Optimization included adjustments to primer and probe concentrations, thermal cycling temperatures and cycle duration. Each multiplex reaction (50 μL) contained 10 μL DNA extract, 25 μL of 2 × Taqman Universal Master Mix [without uracil-N-glycosylase (UNG), Applied Biosystems, Nieuwerkerk a/d, IJsselstein, the Netherlands], 0·4 μmol L^{-1} of each primer and 0·1 of each probe. PCR consisted of an initial step for polymerase activation of 15 min at 95 °C, followed by 50 cycles of 30 s at 95 °C, 60 s at 55 °C and 30 s at 72 °C, followed by a final extension step of 7 min at 72 °C. The Taqman probes and primers were prepared by Biolegio (Malden, the Netherlands) and the MGB probes by Applied Biosystems. Appropriate positive and negative controls were included in every run.

Internal controls for polymerase chain reaction

To monitor extraction quality and PCR inhibition, an assay for the nonhuman virus *Phocid herpesvirus 1* (*PhoHV-1*) was used. Primers and the Taqman probe used were previously described,[12] while the PCR conditions were optimized so as to be the same as in the main assays to facilitate future multiplexing.

Sensitivity of the real-time polymerase chain reaction assay

Analytical sensitivity was determined using eight serial dilutions (ranging from 100 ng per reaction to 10 fg per reaction) of purified and quantified (Nanodrop ND-1000 Spectrophotometer, Isogen, IJsselstein, the Netherlands) DNA extracted from all six target reference dermatophytes. Reference isolates used (Table 1) included *T. rubrum* strains CBS 392.58 (ex-neotype strain) and CBS 303.38, *T. violaceum* strain CBS 319.31, *T. tonsurans* strain CBS 483.76, *M. canis* strains CBS 132.88 and CBS 282.63, *M. audouinii* strain CBS 280.63, and *T. mentagrophytes* complex strains CBS 318.56 (*T. mentagrophytes* in the strict sense = the former *T. mentagrophytes* var. *quinckeanum*), CBS 558.66 (ex-neotype strain of *T. interdigitale*) and CBS 511.73 (*T. erinacei*). DNA was extracted from pure cultures as described previously.[13]

Specificity of the real-time polymerase chain reaction assay

Common dermatophytes and other fungal dermatological pathogens were used, along with dermatophytoids, nonpathogenic molds, and skin-associated yeasts and bacteria, to determine the specificity of the multiplex PCR assay (Table 1). Human DNA was also tested. Nucleic acids from reference cultures were extracted as described previously[13] and were tested with the multiplex assays.

Table 1 GenBank accession numbers of internal transcribed spacer (ITS) sequences used in alignment and strains included in specificity panel

	GenBank accession number	Sensitivity panel strains
Pathogenic dermatophytes		
Trichophyton rubrum	Z97993	CBS 392.58
		CBS 303.38
T. violaceum (including synonym *T. glabrum*)	AJ270811, AJ270796	CBS 319.31
T. yaoundei	AJ270813	CBS 305.60
T. soudanense	AJ270809	CBS 452.61
T. megninii	Z97994	CBS 735.88
T. mentagrophytes complex (*Arthroderma vanbreuseghemii*/*Trichophyton interdigitale*, including synonym *T. krajdenii*)	Z98001, AF170462	CBS 558.66
T. mentagrophytes complex (*Arthroderma benhamiae*/*Trichophyton erinacei*)	Z97997	CBS 511.73
T. mentagrophytes complex (*T. mentagrophytes* per se/the former *T. mentagrophytes* var. *quinckeanum*)	Z97995	CBS 318.56
T. kanei	AJ270798	–[b]
T. concentricum	Z98012	CBS 196.26
T. schoenleinii	Z98011	CBS 855.71
T. tonsurans	Z98008	CBS 483.76
T. verrucosum	Z98003	CBS 134.66
A. otae (*Microsporum canis*)	AJ000617	CBS 132.88
	AJ000618	CBS 282.63
M. audouinii	AJ000622	CBS 280.63
M. ferrugineum	AJ252335	CBS 457.80
M. gallinae	AJ000620	–[b]
A. fulvum (*M. fulvum*, including synonym *T. longifusum*)	AJ000616	–[b]
A. gypsea (*M. gypseum*)	AJ000621	CBS 161.69
A. obtusum (*M. nanum*)	AJ970149	CBS 314.54
M. racemosum	AJ970146	–[b]
M. boulardii	AJ970143	–[b]
M. praecox	AJ970148	CBS 288.55
A. persicolor (*M. persicolor*)	AJ000614	CBS 871.70
T. equinum	Z98009	CBS 634.82
Epidermophyton floccosum	AJ000629	–[b]
Nonpathogenic dermatophytes (dermatophytoids)		
Arthroderma borellii (*M. amazonicum*)	AJ877220	–[b]
A. quadrifidum (*T. terrestre*)	AJ877214	CBS 567.94
A. uncinatum (*T. ajelloi*)	AJ000607	CBS 120.61
A. cajetani (*M. cookei*)	AJ970145	CBS 228.58
A. gloriae	AJ877209	–[b]
T. phaseoliforme	AJ970152	–[b]
T. vanbreuseghemii	Z98013	–[b]
Other filamentous fungi		
Aspergillus fumigatus	AF109330	CBS 287.95
Eurotium amstelodami	AY373885.1	CBS 518.65
Scopulariopsis brevicaulis	AY625065.1	CBS 467.48
Emmonsia crescens	AF038347	CBS 177.60
Auxarthron kuehnii	AJ271417	CBS 539.72
Uncinocarpus reesii	AJ271566	CBS 121.77
Aphanoascus fulvescens	AF038357	CBS 100.211
Lasiodiplodia theobromae	AF027760.1	CBS 339.90
Yeasts		
Candida albicans	AB018037	CBS 562
Malassezia furfur	AY743634	CBS 1878
Bacteria		
Staphylococcus aureus	AF317719.1	Clinical strain
Streptococcus pyogenes	AY347560.1	Clinical strain
Pseudomonas aeruginosa	AY684792.1	Clinical strain
Mycobacterium absessus	–[a]	Clinical strain
Mycobacterium avium	–[a]	Clinical strain
Human DNA	–[a]	Skin biopsy

[a]Not included in the alignment; [b]not included in the sensitivity panel.

Table 2 Primers and probes for real-time polymerase chain reaction (PCR) assays

Target sequence	Species amplified	Primer or probe sequences	5′-label	PCR product size (bp)
ITS1		5′-CTGCGGAAGGATCATTAAC-3′		
		5′-AAGAGATCCGTTGTTGAAAG-3′		
	T. mentagrophytes complex, *T. schoenleinii*, *T. verrucosum*	5′-GAGGCAACCGAGTAA-3′ (MGB)	FAM	287
	T. violaceum	5′-CAAGGAAAATTCTCTGAAGGGCTG-3′ (TQ)	CY5	301
	T. tonsurans, *T. equinum*	5′-TTGAGCCGCTATAAAG-3′ (MGB)	VIC	299
ITS2		5′-CTGTTCGAGCGTCATTTC-3′		
		5′-GGGTATCCCTACCTGATCC-3′		
	M. canis, *M. audouinii*, *M. ferrugineum*	5′-GGTGGGTGGTTACTG-3′ (MGB)	FAM	245
	M. audouinii, *M. canis*, *M. ferrugineum*	5′-GGTGGGTGGTTATTG-3′ (MGB)	VIC	245
	T. rubrum, *T. violaceum*, *T. soudanense*	5′-GCCCTGGCCCCAATCTTT-3′ (TQ)	CY5	229

ITS, internal transcribed spacer; reporter dyes: FAM, 6-carboxy-fluorescein; CY5, indodicarbocyanine; VIC, a proprietary fluorophore, Applera Corp.; MGB, 3′-minor groove binder probe; TQ, Taqman probe.

Results

Conventional identification of fungi from patient specimens

Forty out of 92 patient specimens analysed by conventional 'gold standard' diagnostics (microscopy and culture) yielded positive results. Overall, direct microscopy was more sensitive than culture, as fungal filaments were observed in KOH preparations in 40 specimens (43%), while culture was positive for an aetiological agent in only 30 specimens (33%). All culture-positive specimens were also positive in microscopy. The isolates obtained in culture included 19 *T. rubrum* isolates, seven representatives of the *T. mentagrophytes* complex, one *T. violaceum* isolate, and three *M. audouinii* isolates. All control specimens from patients with psoriasis or dermatitis as well as from all specimens from healthy nails were negative in conventional diagnostics.

Sensitivity and specificity of the real-time polymerase chain reaction assay

Preliminary analysis of control cultures showed that our procedure satisfied the clinical requirement of simultaneously detecting as many pathogens as possible in a single specimen. This detection of multiple organisms was made possible by the close ITS sequence similarities found among closely related dermatophyte species.[14] For example, the designed *T. rubrum*-complex specific probe could also detect the closely related species *T. soudanense* and *T. violaceum*. Similarly, the probe for members of the *T. mentagrophytes* complex could also detect the species with highly similar ITS sequences such as *T. schoenleinii* and *T. tonsurans*.

Real-time PCR curves generated by the species tested in this study are shown in Figure 1. The multiplex assay identified DNA of all target dermatophytes at a concentration of 100 fg in a PCR reaction volume of 50 μL. Given that the average dermatophyte genome is estimated to be between 30 and 40 Mb[15] and the average DNA molecular weight is 635 Da bp^{-1}[16] this analytical sensitivity corresponds to 2·5–3·3 dermatophyte genomes per sample. The cycle threshold values of the multiplex assays were the same as in the individual assays. Specificity testing showed that no other skin pathogen or commensal was amplified by this assay.

MGB probes were used in our design because they are known to be able to distinguish sequences with only one nucleotide mismatch in the probe area.[11] Despite this, the *M. canis* and *M. audouinii* MGB probes we developed, although differing by exactly one such mismatch, showed a degree of cross-reactivity, indicating that a single such mismatch may not necessarily be sufficient for target discrimination. However, distinction between these species was accomplished by comparing the relative strengths of their reactions: *M. audouinii* produced a weak signal with the *M. canis* probe and a strong signal with the *M. audouinii* probe while the reverse was true for the *M. canis* probe.

Analysis of clinical specimens using multiplex polymerase chain reaction

The real-time PCR detected 47 positive specimens out of 92 (51%) and identified *T. rubrum* in 28 specimens, members of the *T. mentagrophytes* complex in 15, *T. violaceum* in one and *M. audouinii* in three. *T. tonsurans* and *M. canis*, which were included in the assay, were not detected in any of the clinical specimens tested. Although no mixed dermatophyte infections were detected in culture tests, two mixed infections were detected by PCR, both caused by *T. rubrum* and a member of the *T. mentagrophytes* complex (Fig. 2). In the control specimens, one, derived from a patient with upper thigh eczema, was found to be weakly positive for *T. mentagrophytes* complex in PCR. Inhibition, as gauged by failure of the control *PhoHV*-1 PCR test, was observed in only one specimen, which was negative by microscopy and culture.

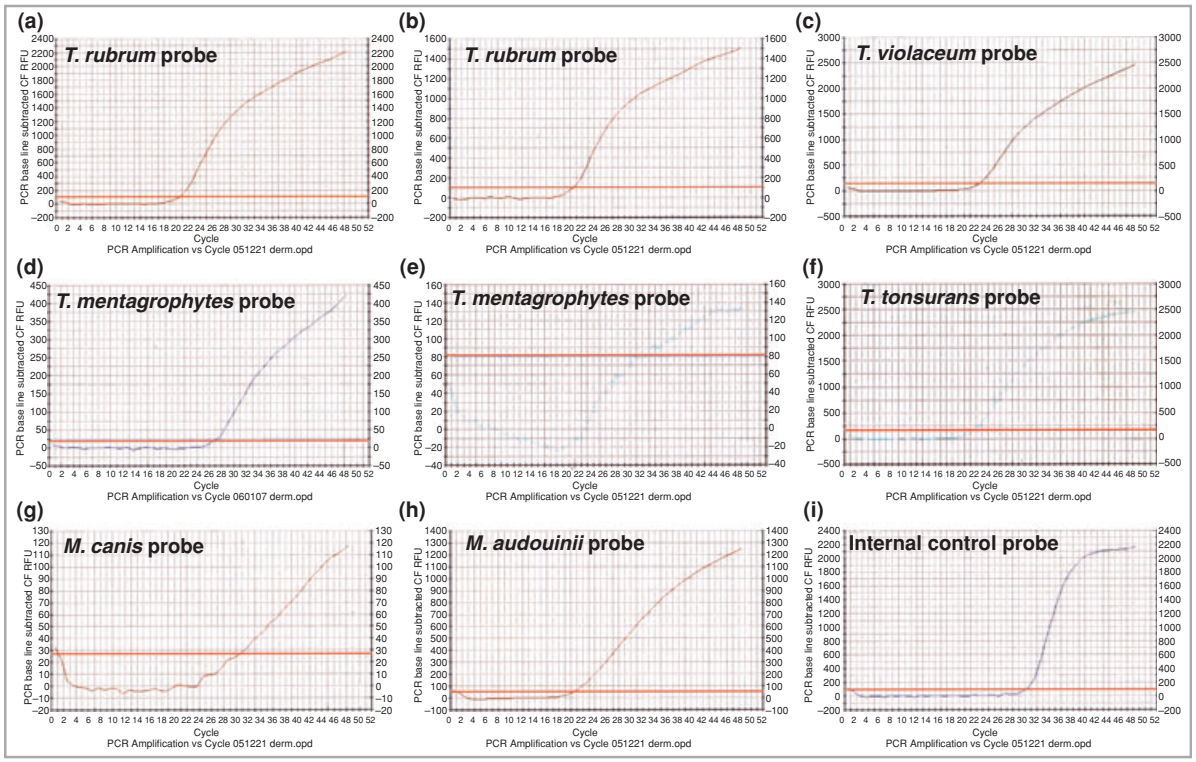

Fig 1. Clinical interpretation of multiplex real-time polymerase chain reaction data. (a) *Trichophyton rubrum* (one signal) with the *T. rubrum* probe; (b, c) *T. violaceum* (double signal, with *T. rubrum* and *T. violaceum* probes); (d) *T. mentagrophytes* complex (one signal, with the *T. mentagrophytes* probe); (e, f) *T. tonsurans* (double signal, with *T. mentagrophytes* and *T. tonsurans* probes); (g, h) *M. audouinii* (weak signal with the *M. canis* probe and a strong signal with the *M. audouinii* probe); *M. canis* displays the inverse; (i) Internal control.

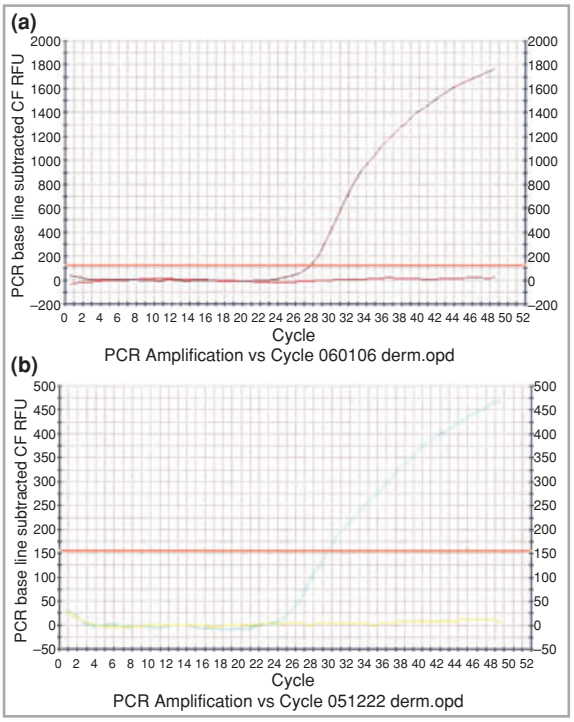

Fig 2. Clinical sample positive for (a) *Trichophyton rubrum* and (b) *T. mentagrophytes* complex by polymerase chain reaction.

Conventional vs. molecular detection and identification

PCR detected fungal material in all 40 specimens shown to be positive in microscopy or culture, and it gave correct identifications for all the species grown in culture. It also detected seven additional positive specimens (four *T. rubrum*, three *T. mentagrophytes* complex) that were negative in microscopy and culture (Fig. 3). Five of the seven additional positive specimens detected by PCR in our study were from skin scale lesions while the remaining two additional positive specimens were from nails. An attempt was made to contact the seven patients involved, who were considered to require repeat examination to clarify the discrepancy between conventional and PCR results. This was ultimately accomplished for five of the patients. Microscopy and culture confirmed dermatophytosis in three of the recalled patients. One of the two patients who were not available for re-sampling had had foot eczema that was only partially relieved by local corticosteroid application. This failure to resolve may signal the presence of an undetected and untreated dermatophytosis. Records for the other unavailable patient showed that cultures of lesional material had twice been positive for the yeast *Candida albicans*, which is known to inhibit dermatophyte outgrowth in culture from dermatological specimens.[2]

The only nail specimen that was negative by PCR but positive by microscopy yielded an *Aspergillus* species in culture. Based on the premise that the PCR would detect any

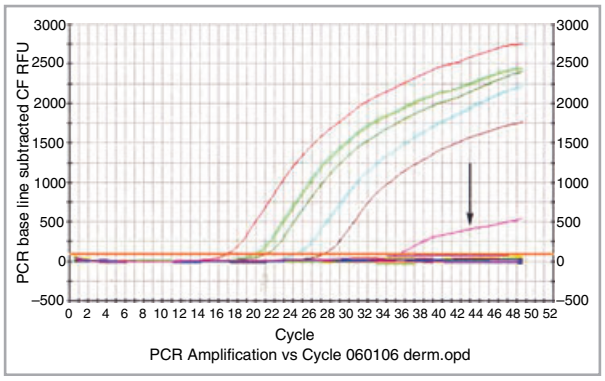

Fig 3. Clinical samples positive for *Trichophyton rubrum* by real-time polymerase chain reaction (PCR). The arrow shows a specimen that was positive only by PCR and negative by microscopy/culture, clearly due to low fungal load.

co-occurring dermatophyte in the lesion, the infection was classified as most probably nondermatophytic and hence beyond the detection spectrum of the PCR assay as designed.

For comparison with the conventional 'gold standard' procedures in dermatophyte diagnostics, positive predictive value of the proposed assay was calculated as 95·7%, while the negative predictive value was reckoned as 100%.

Discussion

Real-time PCR designed specifically for detection of multiple dermatophytes, here described for the first time, is highly sensitive and specific, with high positive and negative predictive values. Because treatment choice in probable skin mycoses can be guided by rapid and accurate diagnosis of the dermatophyte species involved, the assay offers the significant clinical benefit of producing direct detection and identification results in less than 24 h.

The new assay's analytical sensitivity of 100 fg DNA per 50 μL reaction volume is comparable to the sensitivities of published assays for the detection of *Candida* and *Aspergillus* in systemic infections.[17,18] It is superior to the sensitivity of 10–35 pg DNA per reaction found in PCR assays for detection of dermatophytes.[6,19] The combination of direct microscopy and culture, considered the gold standard in dermatophyte diagnostics, has an unsatisfactory sensitivity level, especially in onychomycosis, where studies show that 15–50% of microscopy positive nail specimens fail to yield positive cultures.[5] The superior sensitivity of real-time PCR is evidenced by the finding that this technique detected 14·6% more positive specimens than conventional testing.

The closely related *M. canis* and *M. audouinii* differed by their relative signal intensity in this PCR assay when pure cultures were tested. Also, the three *M. audouinii* positive clinical specimens were correctly identified by PCR. However, absence of clinical specimens infected with *M. canis* in the study population warrants further investigation of distinguishing these two species in a larger patient population. The degree of overlap seen between the pattern for the common *M. canis* and the very uncommon but very closely related *M. audouinii* mean that both these identifications should be considered preliminary, even though under most circumstances probabilities overwhelmingly favour *M. canis*. Epidemiology is useful as an aid to rapid interpretation: in Europe, the Americas and Asia, *M. audouinii* is almost always specifically associated with scalp infection of children who have recently lived in or travelled to Africa, or much less commonly with a school or daycare-related outbreak where such children, or a single child, are index patients. *M. canis* is common from cases clearly inconsistent with this pattern.[20] Where there is ambiguity, conventional examination or sequencing of cultures may be advisable. Recently, discrimination of *M. canis* from *M. audouinii* with minisatellite-derived primers was accomplished using a single PCR detecting and differentiating these two species,[21] while our multiplex PCR assay, which simultaneously identifies six dermatophytes, could be more clinically relevant.

The limited existing genomic data on dermatophytes, the close ITS sequence similarity found among closely related dermatophyte species (e.g. *T. tonsurans* and *T. equinum* differ by only one base in the whole ITS region) and the demands of multiplexing primers and probes in a real-time setting restricted the possibilities of designing probes specific for all traditional dermatophyte species. In practical terms though, the association between *T. verrucosum* and rural life and between *T. schoenleinii* and impoverished areas of the Sahel and rural central Asia is so strong that any background information at all on the patient is usually sufficient to determine whether one of these two is plausible. *M. ferrugineum* is likewise extirpated except in rural central Africa and a few parts of rural China. As the present dermatophyte PCR is not designed to immediately solve all identification problems but instead to greatly speed up the vast majority of identifications, it could be supplemented by conventional testing in unusual cases by anyone trying to use it as a sole test, or it could be sent as a preliminary result and then checked in light of later conventional results where ordinary tests have been done in parallel.

Four conventional PCR assays for direct amplification of dermatophyte DNA from clinical specimens have been reported in the literature so far. In general, nonspecific fungal primers were used in these assays and the dermatophyte species implicated were not distinguished at species level.[6–8] Recently, a *T. rubrum*-specific PCR assay, based on amplification of the microsatellite marker T1, was used for detecting this species in specimens derived from onychomycosis.[19] This assay had an analytical sensitivity of 35 pg per reaction and could distinguish between *T.rubrum*/*T.soudanense* and *T. violaceum* only when a high-resolving acrylamide gel was used.[19] A survey of the GenBank dermatophyte sequences available at the time this PCR scheme was designed showed that another promising area for designing dermatophyte probes could be the multicopy ribosomal nontranscribed spacer (NTS) region. However, so far this region has been completely sequenced only for *T. rubrum* and *T. tonsurans*. Its applicability to species identification is uncertain, in that it is known to show consid-

erable intraspecific variability in some species.[1,22] In-depth studies are required before an attempt is made to use it as a diagnostic PCR target.

Until recently, the only published real-time PCR protocol for dermatophytes[3] suggested use of a LightCycler system and a PCR-RFLP (restriction fragment length polymorphism) assay and employed combinations of seven published fungus-specific (but not dermatophyte-specific) primer pairs. This assay was mainly proposed to distinguish filamentous fungi (dermatophytes and nondermatophytes) from yeasts, and it does not distinguish dermatophytes from other filamentous fungi or distinguish different dermatophyte species. Recently, a real-time Taqman assay, based on amplification of the rRNA intergenic spacer (IGS) area, was used for successfully detecting *T. tonsurans* in patients with suspected tinea capitis.[23] As this assay detects only one of several common causal agents of tinea capitis, it cannot be used diagnostically and is mainly of value in outbreak investigations. Finally, systems that utilize RFLPs are generally of limited clinical value.[19,24]

Because of its high sensitivity, the proposed dermatophyte PCR technique could prove especially valuable in suspected cases of nondermatophyte onychomycosis. One factor limiting the ability to assess the significance of a nondermatophytic fungus isolated from an abnormal nail can be uncertainty about whether or not a dermatophyte, present in the nail but failing by chance to grow in culture, might actually be the principal or only cause of the nail abnormality. A technique capable of detecting dermatophytes in all or nearly all cases where they are present clearly tends to solve this problem, although it does not help with the ancillary problem of recognizing which nondermatophytes growing out from confirmed dermatophyte-infected nails are unimportant contaminants and which are co-occurring agents of mixed infection.[5] The PCR assay could also markedly improve the investigation of scalp dermatophytoses, where conventional mycology often suffers from low sensitivity due to a combination of low-level or patchy fungal load, heavy encrustation of debris blocking microscopy, and the contamination of samples by clinically ineffective (in tinea capitis) topical antifungals such as ketoconazole from shampoos.[2,4,25] In addition, tinea capitis of the elderly, an entity often remaining unrecognised,[26] could be further elucidated by PCR, as conventional methodology is occasionally hampered by the low fungal load. Highly sensitive PCR assays could also contribute to elucidating whether subclinical dermatophyte infections are implicated in some eczematous, psoriatic and icthyosis–hyperkeratotic skin lesions, or whether positive PCR assays from nonlesional clinical specimens would substantiate asymptomatic transient dermatophyte colonization as a realistic clinical entity.

In keeping with the general trends seen in PCR-based diagnostics, real-time PCR could be envisioned as an emerging replacement for both direct microscopy and culture, combining the speed of microscopy with the specific information generated by culture. The higher cost of PCR is presently a limiting factor but this could change by the decrease of reactant market prices and the savings entailed by the replacement of conventional diagnostics. This assay could be incorporated into clinical laboratory diagnostic procedures after being subjected to a detailed technical validation study, although ideally this should be preceded by further simplification and by standardization of the extraction procedure.

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References

1 Gaedigk A, Gaedigk R, Abdel-Rahman SM. Genetic heterogeneity in the rRNA gene locus of *Trichophyton tonsurans*. *J Clin Microbiol* 2003; **41**:5478–87.
2 Kane J, Summerbell R, Sigler S *et al. Laboratory Handbook of Dermatophytes*. Belmont, CA: Star Publishing Co., 1997.
3 Gutzmer R, Mommert S, Küttler U, Werfel TH. Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR. *J Med Microbiol* 2004; **53**:1207–14.
4 Onsberg P. Dermatophyte species, microscopic and cultural examination. *Mycopathologia* 1979; **67**:153–5.
5 Summerbell RC, Cooper E, Bunn U *et al.* Onychomycosis: a critical study of techniques and criteria for confirming the etiologic significance of nondermatophytes. *Med Mycol* 2005; **43**:39–59.
6 Turin L, Riva F, Galbiati G, Cainelli T. Fast, simple and highly sensitive double-rounded polymerase chain reaction assay to detect medically relevant fungi in dermatological specimens. *Eur J Clin Invest* 2000; **30**:511–18.
7 Baek SC, Chae HJ, Houh D *et al.* Detection and differentiation of causative fungi of onychomycosis using PCR amplification and restriction enzyme analysis. *Int J Dermatol* 1998; **37**:682–6.
8 Kano R, Hirai A, Muramatsu M *et al.* Direct detection of dermatophytes in skin samples based on sequences of the chitin synthase 1 (*CHS1*) gene. *J Vet Med Sci* 2003; **65**:267–70.
9 Espy MJ, Uhl JR, Sloan LM *et al.* Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* 2006; **19**:165–256. Erratum in: *Clin Microbiol Rev* 2006; **19**:595.
10 Tahir M, Watson N. Typing of DNA HLA-DQ alpha alleles extracted from human nail material using polymerase chain reaction. *J Forensic Sci* 1995; **40**:634–6.
11 Kutyavin IV, Afonina IA, Mills A *et al.* 3′-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res* 2000; **28**:655–61.
12 Niesters HGM. Clinical virology in real time. *J Clin Virol* 2002; **25**:S3–12.
13 Sansinforiano ME, Padilla JA, Hermoso de Mendoza J *et al.* Rapid and easy method to extract and preserve DNA from *Cryptococcus neoformans* and other pathogenic yeasts. *Mycoses* 1998; **41**:195–8.
14 Gräser Y, El Fari M, Vilgalys R *et al.* Phylogeny and taxonomy of the family *Arthrodermataceae* (dermatophytes) using sequence analysis of the ITS region. *Med Mycol* 1999; **37**:105–14.
15 Fungal Genome Initiative, *White Paper Developed by the Fungal Research Community*, 8 February 2002, available at http://www.broad.mit.edu/annotation/fungi/fgi/FGI_01_whitepaper_2002.pdf (accessed 29 May 2007).
16 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*, vol. 3. ColdSpring Harbor: ColdSpring Harbor Laboratory Press, 1989.

17 Loeffler J, Henke N, Hebart H *et al.* Quantification of fungal DNA by using fluorescence resonance energy transfer and the light cycler system. *J Clin Microbiol* 2000; **38**:586–90.
18 White PL, Shetty A, Barnes RA. Detection of seven *Candida* species using the Light-Cycler system. *J Med Microbiol* 2003; **52** (Pt 3):229–38.
19 Kardjeva V, Summerbell R, Kantardjiev T *et al.* Forty-eight-hour diagnosis of onychomycosis with subtyping of *Trichophyton rubrum* strains. *J Clin Microbiol* 2006; **44**:1419–27.
20 Hay RJ, Robles W, Midgley G *et al.* Tinea capitis in Europe: new perspective on an old problem. *J Eur Acad Dermatol Venereol* 2001; **15**:229–33.
21 Roque HD, Vieira R, Rato S, Luz-Martins M. Specific primers for rapid detection of *Microsporum audouinii* by PCR in clinical samples. *J Clin Microbiol* 2006; **44**:4336–41. Epub 27 Sep 2006.
22 Jackson CJ, Barton RC, Kelly S, Evans EGV. Strain identification of *Trichophyton rubrum* by specific amplification of subrepeat elements in the ribosomal DNA nontranscribed spacer. *J Clin Microbiol* 2000; **38**:4527–34.
23 Sugita T, Shiraki Y, Hiruma M. Real-time Taqman aassay for detecting *Trichophyton tonsurans*, a causative agent of tinea capitis, from hairbrushes. *Med Mycol* 2006; **44**:579–81.
24 Machouart-Dubach M, Lacroix C, de Chauvin MF *et al.* Rapid discrimination among dermatophytes, *Scytalidium* spp., and other fungi with a PCR-restriction fragment length polymorphism ribotyping method. *J Clin Microbiol* 2001; **39**:685–90.
25 Zolberman-Saltiel E. Patchy alopecia in a young girl. *Arch Dermatol* 1989; **125**:113–18.
26 Terragni L, Lasagni A, Oriani A. Tinea capitis in adults. *Mycoses* 1989; **32**:482–6.

