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Comparison of real-time PCR with conventional methods to detect dermatophytes in samples from patients with suspected dermatophytosis ☆

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ABSTRACT

A PCR detecting dermatophytes within a short turnaround time would significantly enhance the management of patients with suspected dermatophytosis. This study aimed at comparing the results of a real-time PCR assay with those of the conventional diagnostic (direct microscopy and culture) performed by a dermatologist working in a medical mycology laboratory for the detection of dermatophytes in nail and skin samples.
A total of 112 specimens (54 nail and 58 skin) were collected from 52 patients with one to four suspected dermatophytosis lesions. The PCR diagnostic indices were calculated for either sample- or patient-based dermatophytosis diagnosis. The sample-based diagnostic efficacy yielded 79% sensitivity and 73% specificity. The patient-based diagnostic efficacy was higher with 100% sensitivity and 82% specificity. Interestingly, PCR yielded significantly ($p < 0.004$) lesser false negative results and performed overall better (diagnostic odds ratio = 24.0 vs. 5.5) in nail than in skin samples. In conclusion, this real-time PCR assay performance was consistent with those of the conventional methods in the hands of a skilled expert and particularly efficacious in diagnosing dermatophyte onychomycosis. This PCR is suited to high throughput batch processing; if used instead of direct microscopy, it could reduce hands-on time in the routine clinical laboratory workflow.

1. Introduction

Dermatophytes are a common cause of nail and skin infections but the biological diagnosis of dermatophytosis is rarely done in the clinical practice. A study by the European Onychomycosis Observatory on 44,972 patients, who consulted either a general physician (n = 13,681) or a dermatologist (n = 31,291), showed that 96.6% of the general physicians and 60.4% of the dermatologists do not perform any sampling to diagnose dermatophytosis (Effendy et al., 2005). Consequently, many patients, although not infected, are treated, sometimes for months, with systemic antidermatophyte agents and are thus unduly exposed to drug related adverse-events. Practitioners cope to treat their patients based on clinical findings only for various reasons but mainly because collecting nail or skin specimens for an efficient biological diagnosis of dermatophyte infection requires skill. Yet even when the sampling is adequate, the test results show the relatively poor sensitivity and long turnaround time of the conventional diagnostic methods (dermatophytes grow slowly and culture may take up to 4 weeks) (Robert and Pihet, 2008). Therefore, a highly sensitive diagnostic test detecting dermatophytes in a very short turnaround time would meet the practitioners' requirements and thus significantly enhance the management of patients with suspected dermatophytosis. Therefore, since the late 1990s numerous attempts to set up a PCR-based assay for the diagnosis of dermatophytosis have been done. These methods, which can be divided into conventional PCR, real-time PCR and PCR–RFLP (restriction fragment length polymorphism) techniques, have been recently reviewed (Jensen and Arendrup, 2012).

We developed a MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments)-compliant real-time PCR to detect dermatophyte DNA in clinical samples (Bustin et al., 2009). This study aimed at comparing the results of this PCR with those of the conventional methods performed by a dermato-mycologist.

2. Materials and methods

2.1. Study design

All patients who consulted a dermato-mycologist (CV) at the Cochin University Hospital in Paris from October 2010 to August 2011 and who

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were suspected of dermatophytosis were prospectively included in the study. For each patient, the clinical specimens included nail and/or skin samples collected by scraping and/or clipping. Each sample was split in two parts. One part was used for the conventional diagnosis at the Parasitology–Mycology laboratory in the Cochin University Hospital (Paris, France). The other part was sent to the Parasitology–Mycology laboratory at the Timone University Hospital (Marseille, France) and used for PCR diagnostic. Those who performed the PCR diagnostic were blinded to the conventional diagnosis' results and vice versa.

2.2. Conventional diagnosis

Direct microscopy examination of the sample was performed by the conventional method with black chlorazol solution (1 g/l CBE + 5 g/l KOH) (Sigma-Aldrich, St Louis, MO, USA). Sample culture was performed on Sabouraud dextrose agar slants with and without cycloheximide (0.5 g/l) (Bio-Rad, Marnes-la-Coquette, France). The culture was maintained at 30 °C in aerobic atmosphere for 4 weeks. All isolates were identified by examination of their macro- and microscopic features. We would like to stress that a highly skilled dermatologist, who is specialized in the diagnosis of superficial fungal infections for more than twenty years performed both the collection of the clinical samples during the consultation and the mycological diagnosis in the laboratory.

2.3. Dermatophytosis case definition

A dermatophytosis was defined by at least one positive dermatophyte culture and/or presence of fungal hyphae at the direct microscopic examination of the sample. This last criterion was considered as positive only if no non-dermatophyte filamentous fungus was cultured from the sample.

2.4. PCR reaction design

Thirty-three 18S rDNA sequences of 27 dermatophyte species were selected and aligned using the ClustalX software (Conway Institute UCD, Dublin, Ireland) to identify conserved regions among the various dermatophyte species. Primers and probe were designed using the primer-BLAST™ (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer3™ (http://frodo.wi.mit.edu/primer3/) softwares, respectively; they are detailed in Table 1. The PCR amplicon length was 105 bp sequence (position 1280 to 1385 from *Trichophyton rubrum* GenBank accession number X58570.1). As internal amplification control, we used the M13 (pGEM™ -3Zf(+), Applied Biosystems, USA) plasmid (Table 1). This exogenous DNA was included in the reaction mixture and coamplified with the target gene to detect PCR inhibition.

The real-time PCR was optimized and run on a LightCycler 480™ (Roche Diagnostics, Meylan, France) machine with the following reaction profile: 10 min, 95 °C, 45 cycles of 10 s, 95 °C; 30 s, 54 °C; 10 s, 72 °C. The real-time PCR reaction was carried out in a 26 µl volume with 2 µl DNA template, 0.5 µM of each DMP primer, 0.2 µM of a TaqMan™ hydrolysis "DMP" probe (Table 1), 0.4 µg of M13 plasmid, 0.36 µM of M13 probe, 0.27 µM of each M13 primer and 13 µl of LightCycler™ 480 Probes Master (Roche Diagnostics).

Table 1
Details of the primers and probe used in the real-time PCR assay.

DMP refers to primers and probe that hybridize dermatophyte DNA. M13 refers to primers and probe that hybridize the internal amplification control (pGEM 3Zf, Applied Biosystems). Alb refers to primers and probe that hybridize human albumin gene, used for DNA extraction control in a distinct PCR reaction for dermatophyte PCR negative samples.

Names	Sequence 5′ → 3′
Dermatophyte PCR	
DMP-F	TTATTGCCTCAAACTTCCAT
DMP-R	TAACGAACGAGACCTTAACC
DMP	FAM-CTAAATAGCCCGGTCGGCGT-TAMRA
Internal amplification control	
M13-F	TGTAAAACGACGGCCAGT
M13-R	GAGCGGATAACAATTTCACACA
M13	VIC-TTGCATGCCTGCAGGTCGAC-TAMRA
DNA extraction control PCR	
Alb-F	GCTGTCATCTCTTGTGGGCTGT
Alb-R	AAACTCATGGGAGCTGCTGGTTC
Alb	VIC-CTAAATAGCCCGGTCGGCGT-TAMRA

2.5. Efficiency, analytical sensitivity and specificity

The real-time PCR reaction efficiency was evaluated using a triplicate serial dilution from 10^{-1} to 10^{-7} of a *T. rubrum* DNA extract. According to the Ct standard curve, the real-time PCR had 93% efficiency. In addition, a stock DNA solution obtained by extraction of a *T. rubrum* and *Trichophyton mentagrophytes* clinical isolate was used to evaluate the analytical sensitivity (limit of detection) of the PCR. This 38 µg/ml stock solution was diluted from 10^{-1} to 10^{-7} in triplicate. The PCR assay identified dermatophyte DNA at a concentration of 80 fg in a PCR reaction volume of 26 µl and this analytical sensitivity corresponds from 2 to 3 equivalent dermatophyte genomes per reaction/sample (Arabatzis et al., 2007).

Moreover, in agreement with the in silico analysis performed before, the primers and probe combination proved highly specific to dermatophyte DNA, as evidenced by the negative PCR results with the DNA of various microbial strains (31 filamentous fungi; 3 bacteria and 2 yeast strains, detailed in Supplemental Table 1) as reaction template. Human DNA was also tested with a negative result. A blank (water) control was used in all reaction series, and confirmed that no contaminating PCR product was amplified.

2.6. DNA extraction

Skin or nail samples were homogenized for 40 s in 2 ml tubes containing 1.4 mm ceramic spheres using a FastPrep™-24 Instrument. DNA was extracted using the NucliSENS™ easyMAG™ (bioMérieux).

2.7. DNA extraction control

To validate the DNA extraction yield, the human albumin gene was amplified in a separate real-time PCR reaction. The primers and probe are detailed in Table 1. This PCR reaction was carried out in a 26 µl volume with 2 µl template DNA, 0.1 µM of Alb-F primer, 0.03 µM of Alb-R primer, 0.1 µM of the TaqMan™ hydrolysis "Alb" probe (Table 1) and 13 µl of LightCycler™ 480 Probes Master (Roche Diagnostics). This reaction was run on the same real-time PCR machine with the following profile: 10 min at 95 °C followed by 45 cycles of 10 s at 95 °C; 30 s at 58 °C and 10 s at 72 °C.

2.8. Dermatophyte PCR interpretation

The PCR results were interpreted as follows:

1. If the dermatophyte PCR was positive, as evidenced by a significant increase in FAM fluorescence, the result was: presence of dermatophyte DNA.
2. If the dermatophyte PCR was negative, we first checked for the presence of PCR inhibitors:
   a. If the internal amplification control (VIC fluorescence) was not amplified, the DNA template was diluted to 1/20 and the PCR was run again.
      i. If PCR inhibition was detected in this second run, the PCR result was: uninterpretable because of the presence of PCR inhibitors in the sample.

ii. Otherwise, the results were interpreted as in the absence of PCR inhibitor.

b. If no PCR inhibitor was detected (the internal amplification control was amplified at the expected Ct), we checked the presence of a sufficient amount of DNA used as template (the DNA extraction yield) with the human albumin PCR.

i. If the human albumin PCR was negative, the PCR result was: uninterpretable, because of insufficient amount of DNA template.

ii. If the albumin PCR was positive, the PCR result was: absence of dermatophyte DNA.

2.9. Statistical analysis

Variables were presented as percentages with their exact binomial 95% confidence intervals (95%CI). Because multiple samples were usually collected from one patient, the diagnostic indices of the PCR assay were analyzed separately for a given patient or for a given sample. The diagnostic indices were calculated as a function of the proportion of patients or samples with true positive, false positive and negative tests. In the patient-based analysis, a positive PCR result was defined as the detection of dermatophyte DNA in at least one of the patients' samples. In the sample-based analysis, a positive PCR result was defined as the detection of dermatophyte DNA in the sample. As detailed above, the PCR was considered uninterpretable in samples that failed to validate the extraction and/or amplification controls; these samples were thus excluded from the diagnostic indices' calculation. The sensitivity, specificity, diagnostic odds-ratio (DOR) and number needed to diagnose (NND = 1 / [sensitivity − (1-specificity)]) were computed with their 95% confidence interval (95%CI). Analyses were performed with the SAS 9.2 (Cary, NC, USA) statistical software. All statistical tests were two-sided with a $p \leq 0.05$ significance level.

3. Results

We included 52 patients and analyzed 112 (54 nails, 58 skins) specimens. For each patient, specimens were collected from either 1 (n = 14), 2 (n = 19), 3 (n = 16) to a maximum of 4 (n = 3) distinct lesions. A dermatophyte grew in at least one sample in 34 (65.4%) of the 52 patients analyzed. The isolated species were *T. rubrum* (n = 27) and *Trichophyton interdigitale* (n = 8). In one sample, *T. rubrum* was associated with *T. interdigitale*. The results of dermatophyte and non-dermatophyte filamentous fungi (NDF) obtained from culture are detailed in Table 2. Among the 12 samples with NDF isolates five had a positive dermatophyte PCR result: the genera were *Fusarium* (n = 3), *Acremonium* (n = 1) and *Cladosporium* (n = 1). PCR inhibition did not occur in this study. The PCR results were uninterpretable in 11 samples (skin = 9, nail = 2) of nine patients because the DNA control was not validated. Of these 11 samples, three had a positive direct microscopy examination and five a positive dermatophyte culture.

The diagnostic indices (sensitivity, specificity, DOR, NND) of this PCR assay for either patient- or a sample-based dermatophytosis analysis are tabulated in Table 3, and are further detailed for nail or skin samples. The performances of PCR were excellent for patient-based dermatophytosis diagnosis, with 100% sensitivity, 82% specificity and 1.2 NND. As expected, the diagnostic indices were lower (79% sensitivity; 1.9 NND) in the sample-based analysis. However, PCR yielded significantly ($p = 0.0033$) lesser false negative results and performed overall better in nail (DOR = 24.0) than in skin (DOR = 5.5) samples (Table 3). In nine patients, culture positive results with PCR negative results were observed (Table 4). Taking into account all patients, the sensitivity of the PCR was at least equal to the sensitivity of the direct microscopic examination (Table 4).

Table 2
Detail of the dermatophytes and non-dermatophyte isolates recovered in culture from the 112 specimens collected from the 52 patients included in the survey.

Species	Specimens (n = 112)	Patients (n = 52)
Dermatophytes		
Trichophyton rubrum	54	26
Trichophyton interdigitale	18	7
T. rubrum + *T. interdigitale*	1	1
Non-dermatophytes		
Scytalidium species	1	1
Fusarium species	5	4
Acremonium species	2	2
Cladosporium species	2	1
Candida albicans	2	2
Total	83	43

4. Discussion

This study's main result is that the efficacy of PCR for the diagnosis of dermatophytosis is very similar to the efficacy of conventional methods, based upon microscopic identification of hyphae directly from lesion materials followed by culture. In contrast with the usually reported ~30% of false negative culture rate for dermatophyte detections (Hay, 2005), culture performed particularly well in this study probably because of the high quality clinical samples collected by a dermatologist. By contrast to conventional methods, real-time PCR assays are suited to high throughput batch processing and could thus dramatically reduce hands-on time for the diagnosis of dermatophytosis in the routine clinical laboratory. With respect to dermatophytosis diagnosis in patients, our real-time PCR assay displayed excellent diagnostic indices, with 100% sensitivity and 82% specificity. The specificity was reduced by the positive PCR results in specimens where a non-dermatophytei (NDF) were grown. But, at least in some cases, this fast-growing NDF might have overgrown or impaired the culture of a dermatophyte that was present in the sample. Unlike dermatophytes, NDF utilize previous keratin destruction by dermatophytes, trauma, or another nail disease to cause onychomycosis. Therefore, some of the NDF isolated from nail specimens were probably involved in co-infections with dermatophytes. NDF causing onychomycosis include *Scopulariopsis brevicaulis*, *Aspergillus* sp., *Fusarium* sp., *Scytalidium* sp. and *Onychocola canadensis* (Hwang et al., 2012). Especially, *Fusarium* sp. was isolated from three out of the five samples with both a positive dermatophyte PCR result and a positive non-dermatophyte culture. As expected, the PCR diagnostic indices were lower when considering dermatophyte detection in a given sample, but PCR proved particularly effective in nail samples, with 88% sensitivity and 77% specificity. Other studies showed a higher performance of the PCR assay, based on various target genomic regions, in the dermatophytosis diagnosis compared to conventional methods. Luk et al. (2012), who compared the detection rates of a PCR targeting the Topoisomerase II gene with those of direct examination and culture in nail samples from patients with suspected onychomycosis, demonstrated the good performances of PCR with 29.2%, 10% and 40% dermatophyte detection rates for direct examination, culture and PCR respectively. But this study's limitations were mainly that the authors used a conventional PCR assay, which is less suited than real-time PCR for the routine clinical laboratory, and that NDF were cultured in 23 of the 120 nail samples for which the authors did not detail the microscopy, culture and PCR results. The study by Brillowska-Dabrowska et al. (2010), who developed a multiplex PCR assay targeting the chitin synthase 1 region for the diagnosis of *tinea unguium*, dermatophytes were detected in 33.9%, 20.2% and 33.9% of the samples by direct examination, culture and PCR, respectively. The main limitation of this multiplex PCR that amplifies the DNA of any dermatophyte species and specifically *T. rubrum* DNA is that it could not detect mixed infection if any other dermatophyte species was associated with *T. rubrum*. Bergmans et al. (2010) developed a dermatophyte

Table 3
Real-time dermatophyte PCR assay diagnostic indices, either in a patient or a sample and within nail or skin sample subgroups.

	Sensitivity	95%CI[a]	Specificity	95%CI	DOR[b]	95%CI	NND[c]	95%CI
Patient	100	[90.5–100]	82.4	[56.6–96.2]	+∞	[21.0–+ ∞]	1.2	[1.2–1.8]
Sample (all)	79.1	[69.0–87.1]	73.1	[52.2–88.4]	10.3	[3.7–28.2]	1.9	[1.4–3.5]
Nails	87.8	[73.8–95.9]	76.9	[46.2–95.0]	24.0	[4.9–118.1]	1.5	[1.2–3.3]
Skin	71.1	[55.7–84.7]	69.2	[38.6–90.9]	5.5	[1.4–21.2]	2.5	[1.5–19.6]

[a] 95%CI: 95% confidence interval.
[b] DOR: diagnostic odds ratio.
[c] NND: number needed to diagnose.

specific real-time PCR targeting the internal transcribed spacers of the rRNA genes that proved also more sensitive than conventional methods (61.7% vs. 47.5%) in nail, skin and hair samples. Yet this PCR requires using 10 different probes aiming at hybridizing dermatophyte species specific DNA. The interest of this assay was that it could theoretically identify the dermatophyte species involved in the patient's lesion but this PCR has a rather high (20 genome equivalent) limit of detection and its costs were increased proportionally to the number of probes used and could thus be estimated as 10 fold higher than the herein described PCR assay.

Over the last decade, all PCR assays that have been developed to detect dermatophytes in nail or skin specimens have demonstrated a higher sensitivity and/or specificity than conventional microscopy and culture methods (Wisselink et al., 2011; Luk et al., 2012). Our PCR detected dermatophytes in seven samples that were negative for both microscopy and culture. Although a false positive PCR result cannot be excluded, it is more likely that these were false negative results of the conventional diagnostic methods (Effendy et al., 2005).

In keeping with previous studies (Brillowska-Dabrowska et al., 2010; Alexander et al., 2011; Wisselink et al., 2011; Jensen and Arendrup, 2012) we observed false negative PCR results in a few samples from which a dermatophyte had been cultured (n = 9). The most plausible explanation lies in the heterogeneous distribution of the dermatophyte within the sample. The fungus might be absent in the part used for PCR and present only in the one used for culture. However those samples were collected in nine patients from whom two to three samples had been collected, and at least one sample was positive by PCR in each of these patients. Thus a diagnosis of dermatophytosis could have been made in all patients by using only PCR.

We found that PCR performed better in nails than in skin samples (Table 3). It is known that tissues infected by dermatophytes may contain substantial areas of dead mycelium. Indeed, up to 20% of the samples from patients with *tinea unguium* contain only dead, i.e. non-cultivable, dermatophytes material, but may grow contaminant fungi from dormant propagules or surface colonization (Weitzman and Summerbell, 1995). Previous antidermatophyte treatments may also explain false negative dermatophyte culture results despite the presence of fungal hyphae when performing a direct microscopic examination (Hay and Jones, 2010). As discussed before, mixed dermatophyte and non-dermatophyte infections do sometimes occur; there a rapidly growing mold will hamper the dermatophyte culture (Summerbell, 1997).

In contrast to PCR that only detects the specific target it has been designed for, culture detects any cultivable rare and/or unexpected pathogens, such as *Scytalidium*, *Fusarium*, or *Scopulariopsis* in our patients. Because our PCR was designed to specifically detect clinical dermatophytes but not to identify their genus or species, it could neither separate anthropophilic from zoophilic dermatophytes nor diagnose non-dermatophytes mycoses. Therefore this PCR assay should remain associated to, and not replace, culture for the diagnosis of superficial mycoses because identifying the dermatophyte species has both epidemiological and therapeutic implications.

We envision our dermatophyte PCR assay as a replacement for direct microscopic examination in the routine clinical laboratory. Indeed, in our hands, PCR proved as sensitive as direct microscopic examination but was more specific, since non-dermatophyte fungi were not detected. Direct microscopic examination is fast and inexpensive, but not specific and relatively insensitive, showing false negative results in up to 15% of cases (Liu et al., 2000). In our study, if we consider the direct microscopic examination as gold standard method, the PCR had 81.8% (63/77) sensitivity. For 14 samples, hyphae were present when performing a direct examination but PCR was negative. In contrast, microscopic examination was negative and PCR positive in 12 samples. Among these samples 5 grew a dermatophyte and the 7 remaining samples were culture negative. In a recent study that compared dermatophyte PCR with culture of microscopy-positive samples, 12 grew dermatophytes and 23 non-dermatophytes, whereas the dermatophyte PCR was positive in 19 of the microscopy-positive samples (Luk et al., 2012). This is in line with the higher sensitivity of the PCR compared to direct microscopic examination to detect dermatophytes. It has been reported that up to 10% of nail infections may be microscopically positive and culture negative (Brillowska-Dabrowska et al., 2010) calling for a second visit to perform another sampling. We must also stress that self-treatment with over-the-counter antidermatophyte drugs would significantly alter culture but probably not PCR results (Jensen and Arendrup, 2012).

Overall, we would like to stress that, to date, regardless of the assay used, the mycological diagnosis of dermatophytosis remains limited by the poor quality of samples. We would therefore recommend collecting and testing at least three samples per patient to avoid false-negative PCR or culture results. This PCR would miss rare infections caused by non-dermatophyte fungi thus practitioners should use their best endeavors to obtain good-quality samples from their patients with suspected onychomycosis that could be caused by a NDF even though the PCR is negative. To avoid poor quality clinical samples, the sampling should be performed by a dermatologist or by an, unfortunately increasingly rare, skilled staff.

In conclusion, the short hands-on time makes this PCR assay very suitable as a routine diagnostic assay for dermatophytosis. It could advantageously replace the direct microscopic examination, but not the culture, of the samples in the clinical laboratory routine. The specific results and short response time of this PCR are well adapted to health care-practitioner needs. It might stimulate them to request a mycological

Table 4
Combinations of detection of hyphae by direct microscopic examination, dermatophyte culture and dermatophyte PCR results in the 112 skin or nail samples included in the study.

Direct microscopic examination N positive: 77 (68.7%)	Dermatophyte culture N positive: 70 (62.5%)	PCR N positive: 75 (66.9%)	Frequency n (%)
Positive	Positive	Positive	56 (50%)
Negative	Negative	Positive	7 (6.2%)
Positive	Negative	Positive	7 (6.2%)
Positive	Negative	Negative	9 (8.0%)
Negative	Positive	Positive	5 (4.4%)
Negative	Positive	Negative	4 (3.6%)
Positive	Positive	Negative	5 (4.4%)
Negative	Negative	Negative	19 (16.9%)

diagnosis in patients with suspected dermatophytosis, thus preventing them to prescribe unnecessary antifungal regimens.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2013.08.015.

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