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Optimized 5-hour multiplex PCR test for the detection of tinea unguium: performance in a routine PCR laboratory

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We recently reported the development of a 5-hour multiplex PCR test for the detection of tinea unguium and the optimization of this test by the inclusion of an inhibition control. Here we report the performance of this procedure as used in a routine clinical laboratory as compared to conventional microscopy and culture-based techniques performed in a mycology reference laboratory. We found in processing 109 samples that 22 (20.2%) yielded fungi in culture while the suspected etiologic agents were noted microscopically in 15 (13.8%) that were negative in culture. Fungi were detected by PCR in 37 (33.9%) samples, of which only three were positive in culture. Since the majority of PCR positive but culture negative samples were positive in microscopic examinations, the increased sensitivity was not due to contamination. PCR inhibitors were present in 5% of the samples, but this was overcome by re-running the samples with a 50% reduction of sample DNA. In conclusion, the PCR test performance in the routine setting was excellent and provided a markedly reduced time to diagnosis with a higher sensitivity.

Keywords dermatophytosis, PCR, diagnostics, internal control

Introduction

The dermatophyte *Trichophyton rubrum* is the main etiological agent of onychomycosis [1–3]. The conventional diagnosis of dermatophytosis is based on the recovery of the etiologic agent in culture and the subsequent micro- and macro-morphologic features of the colonies. However, the latter can take up to 4 weeks to develop [4]. Introduction of molecular-based diagnosis techniques enables a reduction of time needed for diagnosis to one working day [5,6]. The multiplex PCR reaction described previously detects any dermatophyte DNA in the specimen and specifically *T. rubrum* DNA allowing its species-identification [5].

We subsequently developed an internal control in order to avoid false-negative results due to PCR inhibitors present in the specimens. We here report the results of the performance of this PCR assay implemented in a high throughput PCR routine laboratory running 15 different diagnostic PCR tests and approximately 6,000 samples per technician per year. We compare this data to that obtained by conventional culture based diagnostics performed in a mycology reference lab.

Material and methods

Clinical samples

A total of 109 clinical samples obtained for routine examination at the Mycology Reference Laboratory at Statens Serum Institute (SSI) (Denmark) (Table 1) were included in this investigation. The only inclusion criterion was the presence of a sufficient amount of material for PCR analysis, as well as for use for microscopic studies and culture. The samples were divided equally, with half of the material mounted for direct microscopic analysis and culture for 4 weeks on Sabouraud agar containing cycloheximide (0.5 g/l). All clinical isolates were identified by examination of their macro- and micro-morphologic characters.

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Table 1 Comparison of microscopy & culture versus PCR for the detection of tinea unguium in a total of 109 cases.

Microscopy (M) and Culture (C) results and interpretation		PCR pos		PCR neg	PCR inhibition	In total	Culture positive
		panDerm[a]	*T. rubrum*				
M+C+	Dermatophyte with ID	4[b] (3.7%)	0	0	0	4 (3.7%)	
	T. rubrum	0	11 (10.1%)	2 (1.8%)	0	13 (11.9%)	22 (20.2%)
M−C+	Dermatophyte with ID	2[c] (1.8%)	0	1[d] (0.9%)	0	3 (2.8%)	
	T. rubrum	0	2 (1.8%)	0	0	2 (1.8%)	
M+C−	Fungus	2 (1.8%)	9 (8.3)	2 (1.8%)	2[e] (1.8%)	15 (13.8%)	–
M−C−	Negative	0	5 (4.6%)	63 (57.8%)	4 (3.7%)	72 (66%)	–
In total		8 (7.3%)	27 (24.8%)	68 (62.4%)	6 (5.5%)	109	–
PCR positive		35 (32.1%)		–	2[e] (1.8%)	–	–

[a]Samples pan-Derm positive by PCR were subsequently cultured in order to perform species identification.
[b]Three *T. mentagrophytes,* one *T. tonsurans.*
[c]One *T. mentagrophytes,* one *T. violaceum.*
[d]One *T. mentagrophytes.*
[e]Two samples were inhibited when using 4 µl DNA containing solution but panDerm positive when repeated using 2 µl.

Sample preparation and multiplex PCR (patent application WO/2006/133701)

DNA from nail specimens was released by a 10-min incubation of the sample in 100 µl of extraction buffer (60 mM sodium bicarbonate [$NaHCO_3$], 250 mM potassium chloride [KCl] and 50 mM Tris, pH 9.5) at 95°C and subsequent addition of 100 µl anti-PCR inhibition buffer (2% bovine serum albumin). After vortex mixing, 4 µl of this DNA-containing solution was used for identification by multiplex PCR with 1 µM of the following primers: panDerm1 (5′ GAAGAAGATTGTCGTTTGCATCGTCTC 3′), panDerm2 (5′ CTCGAGGTCAAAAGCACGCCAGAG 3′) detecting a DNA fragment encoding chitin synthase 1, Trubrum-for (5′ TCTTTGAACGCACATTGCGCC 3′) and Trubrum-rev (5′ CGGTCCTGAGGGCGCTGAA 3′) detecting internal transcribed spacer 2 from *T. rubrum*, 1 µl of internal control (see below), 10 µl of PCR Ready Mix (Sigma, Germany) in a volume of 20 µl. The time-temperature profile for PCR was 45 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, preceded by initial denaturation for 10 min at 95°C. The presence of specific PCR products of approximately 366 bp (dermatophyte band), 203 bp (*T. rubrum* band) or 668 bp (internal control band) was examined using electrophoresis on a 1% agarose gel and staining with ethidium bromide. In case of the lack of any PCR products, the multiplex reaction was repeated as described above, but using 2 µl instead of 4 µl DNA-containing solution.

PCR internal control

In order to detect the presence of *Taq* DNA polymerase inhibitors or sub-optimal reaction conditions an internal process control (IPC) was constructed as previously described [7]. In brief, primers amplifying parts of the phage lambda genome were constructed including the sequence of each of the panDERM primers added to the 5′ end of the corresponding lambda specific primer (sequence in bold corresponds to the phage lambda sequence): IPCpanDERM366-for (5′ GAAGAAGATTGTCGTTTGCATCGTCTC**CTGACGGTTTCTAAC** 3′) and IPCpanDERM366-rev (5′ CTCGAGGTCAAAAGCACGCCAGAG**GACATACGGAAATAG** 3′). PCR products thus containing the binding sites of the panDERM primers on each side of the lambda phage sequence were obtained by amplification of 1 ng purifed lambda DNA with an annealing temperature of 40°C. After gel-purification of the amplicons, a 10-fold titration was performed and the dilution of the IPC, producing no increase in the detection-limit of purified dermatophyte DNA, was used in the reaction.

Results

Dermatophytes were recovered from a total 22 (20.2%) of the specimens (15 or 68% of which were *T. rubrum* (Table 1). An additional 15 samples (13.8%) were microscopically positive but culture negative. This may represent culture negative dermatophyte infections, infection/colonization with non-dermatophytes, and/or false positive microscopic results due to artifacts misinterpreted as fungal elements. Thus, 46.8% of the microscopy positive samples were negative by culture. Considering microscopy positive samples as true positives a total of 37/109 samples were positive (33.9%).

Using 4-µl DNA extracts from the samples for analysis by PCR, we found that (a) 35 (32.1%) of the samples were

positive for either *T. rubrum* or a dermatophyte other than *T. rubrum*, (b) 68 (62.3%) samples were negative and (c) six were inhibited (5.5%, Table 1). Repeating these six samples with a smaller amount of template (2 µl, instead of 4 µl DNA) allowed for detection of dermatophyte DNA in two samples (both microscopy positive) and confirmed negative results obtained by conventional diagnostic methods for the remaining four specimens. Thus, in total, 37 samples (33.9%) were found to be positive by PCR analysis. *T. rubrum* was detected in 27 (73.0% of the PCR positive samples, 25% of all samples) and a dermatophyte other than *T. rubrum* in 10 samples (27.0% of the PCR positive samples, 9% of all samples). In 5 (4.6%) cases the PCR was positive for *T. rubrum* despite negative microscopy and culture, and in 2 (1.8%) cases the PCR was negative despite positive culture. In the majority of samples that were positive by PCR, but negative by culture, microscopic studies were positive (11/16, 68.8%). Furthermore only 5/72 culture and microscopy negative samples were positive by PCR (6.7%) while the PCR positivity rate was much higher among microscopic positive but culture negative samples 11/15 (73.3%).

Discussion

The results of these studies demonstrate that in a routine setting the PCR test is as sensitive as traditional diagnostic methods performed in a mycology reference laboratory if microscopic positive but culture negative samples are considered as true positives (33.9% positive by both tests). However, PCR is far more sensitive if only culture positive samples are considered true positives as is the practice in Nordic countries. If we consider samples that were microscopically positive but culture negative as true positives as is the practice in the UK and US, then PCR increased the proportion of samples for which the presence of a dermatophyte was confirmed from 20.1% to 32.1% and the proportion of samples with species identification from 20.1% to 24.8%. The introduction of an internal control demonstrated that 5% of the samples contained PCR inhibitors but this was overcome by re-running these samples with a 50% reduction of DNA-containing solution.

Another significant advantage of the dermatophyte multiplex PCR test is the possibility of obtaining the results during 1 working day, with a significant reduction of technician time per sample. Although onychomycosis is not a life threatening infection, rapid diagnosis may contribute to greater favourable outcomes. In countries where antifungal treatment is initiated on the basis of microscopic and/or culture positive results, treatment may be delayed in cases that are culture positive but microscopically negative (8% of positive cases, 3% of all patients). In countries where antifungal treatment is not recommended before the infection is culture-verified, a greater number of patient samples (5 or 15%) [8–10] need re-examination due to false negative culture data. This leads to additional costs of sampling and inconvenience for the patient associated with the extra visit at the doctor. Moreover, a rapid diagnosis allows earlier initiation of treatment, which at least in theory might shorten the necessary duration of treatment.

The limitations of this multiplex PCR test include the fact that geophilic dermatophytes like *T. terrestre* which are rarely true pathogens but may occur as contaminants/colonizers will not be separated from other dermatophyte positive samples. However, according to our experience these are infrequently encountered in clinical samples. For example *T. terrestre* was found in only 36/24,752 samples at Statens Serum Institute in 2003 [11]. In addition, this test does not detect infections caused by other moulds such as *Neoscytalidium, Scopularopsis, Fusarium*, which are responsible for 3% of fungal feet and nail infections [1,12]. Therefore, patients with clinical signs of onychomycosis but with negative PCR test on relevant specimens should subsequently be tested by microscopy and culture. Furthermore, this PCR test does not provide species identification of dermatophytes other than *T. rubrum*. In most countries, however, *T. rubrum* is the predominant species and *Microsporum* spp. are very rarely encountered in tinea unguium and athlete's foot. Thus appropriate treatment, which varies by genus, can be initiated even for dermatophyte positive but *T. rubrum* negative samples. However, for epidemiological purposes we routinely culture these samples (approximately 9% of all samples) in order to provide the species identification.

As shown in Table 1, PCR was negative for two samples that were culture positive for *T. rubrum* and one that was positive for *T. mentagrophytes*. A likely reason for these false negative results is that they represent an artifact of the nail specimens being divided between conventional and molecular testing, and where, by chance alone, positive material was not included in the subsample set aside for molecular testing. The sampling is destructive and this idea cannot be directly tested, but such problems with sample division have long been a known factor in dermatologic mycology testing. Especially as on the other hand five microscopy and culture negative samples were PCR positive (all *T. rubrum*), insufficient sensitivity of the molecular testing is not likely the reason.

In conclusion, despite some limitations this routine evaluation of the 5-hour multiplex PCR test, including inhibition control, demonstrates that the test is robust and can be easily run in a routine laboratory with markedly reduced time to diagnosis and higher sensitivity as obvious advantages.

Declaration of interest: Anna Brillowska-Dabrowska, Sanne Søgaard Nielsen, Henrik Vedel Nielsen: No conflict of interest.

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