Fast and reliable PCR/sequencing/RFLP assay for identification of fungi in onychomycoses

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Fusarium spp. and other non-dermatophyte fungi are repeatedly isolated from abnormal nails. To investigate whether these fungi are the aetiological agents of infection or simply transient contaminants, a PCR/sequencing/RFLP assay was developed for direct and routine identification of the infecting fungi in onychomycosis. Fungal DNA was readily extracted using a commercial kit after dissolving nail fragments in a Na₂S solution. Amplification of part of the 28S rDNA by PCR was performed with universal primers and the fungal species were identified by sequencing. The PCR/sequencing results were comparable with microbiological identification from the same nail sample. In addition to dermatophytes, *Fusarium* spp. and other less frequently isolated non-dermatophyte fungi were identified as single fungal agents in onychomycosis. Moreover, mixed infections were clearly demonstrated in 10 % of cases by RFLP analysis of PCR products. Identification of infectious agents could be obtained in 2 days, whilst results from fungal cultures take 1–3 weeks. Rapid and reliable molecular identification of the infectious fungus expedites the choice of appropriate antifungal therapy, thereby improving the cure rate of onychomycosis.

Received 15 May 2006

Accepted 30 May 2006

INTRODUCTION

Onychomycosis is the most prevalent nail disease (Chabasse, 2004). Direct microscopic examination of infected nail material should confirm a clinical diagnosis of fungal infection whenever possible before oral treatment is commenced. Subsequent fungal culture from nail sampling permits the species involved in the infection to be determined. Onychomycoses are mainly caused by dermatophytes, but various non-dermatophyte filamentous fungi (NDF) are often isolated from abnormal nails (Summerbell *et al.*, 2005). Repeated isolations of the same NDF permit us to conclude with some certainty their involvement in nail infection (English, 1976; Gupta *et al.*, 2001a; Summerbell *et al.*, 2005). However, whether NDF are really the aetiological agents of onychomycosis or whether they should be considered as casual and transient contaminants often remains an open question. In addition, it is common to obtain negative results from fungal cultures of nail specimens where direct microscopic examination showed fungal elements. This problem frequently occurs with nails of patients already being treated with antifungal therapy. In all cases, correct identification of the aetiological agent of nail infections is necessary to identify the appropriate treatment, as NDF are weakly responsive or unresponsive to

certain antifungal drugs (Tosti *et al.*, 2000; Gupta *et al.*, 2001b; Garcia-Effron *et al.*, 2004).

Direct identification of the infecting agent in the nail has been shown to be feasible using PCR and RFLP. Different investigations have focused mainly on the identification of dermatophytes and *Scytalidium* spp. (Baek *et al.*, 1998; Turin *et al.*, 2000; Machouart-Dubach *et al.*, 2001; Menotti *et al.*, 2004; Kardjeva *et al.*, 2006). Here, we report the development of a PCR/sequencing/RFLP assay that was able to identify directly the large number of *Fusarium* spp. and other NDF that we routinely isolate in culture from abnormal nail samples. The assay was found to be reliable by comparing the results obtained from the sequences of PCR products of extracted fungal DNA with the results of cultures from the same nail sample. We showed that *Fusarium* spp. and NDF are the aetiological agents of an appreciable percentage of onychomycosis in Switzerland. In addition, mixed infections could be clearly demonstrated by RFLP.

METHODS

Clinical samples. Nail scrapings and fragments were collected from patients with suspected onychomycosis. Routinely, one part of each sample was examined in nail-dissolving solution containing a fluorochrome (Monod *et al.*, 1989). The reactive solution was prepared by dissolving 1 g Na₂S in 7.5 ml distilled water and subsequently adding 2.5 ml ethanol. Thereafter, 10 µl Blankophor P

Abbreviations: LSU, large subunit; NDF, non-dermatophyte filamentous fungi.

flüssig (Bayer) or 20 µl 1% aqueous solution of Tinopal UNPA-GX (Fluorescent brightener 28, F3543; Sigma) was added to this mixture. In parallel, another portion of nail sample was seeded in test tubes containing Sabouraud's agar medium with chloramphenicol (50 µg ml⁻¹) or with chloramphenicol plus cycloheximide (400 µg ml⁻¹). Cultures were incubated at 30 °C. Dermatophytes and moulds were identified after 14–21 days of growth by macroscopic and microscopic examination (Mock *et al.*, 1998; de Hoog *et al.*, 2000). When enough material had been collected, a one-third portion of the nail sample was conserved at room temperature in a dry box for subsequent fungal DNA extraction. Fragments of normal nails from healthy volunteers were used as negative controls.

DNA extraction. Nail scrapings and fragments (2–30 mg) were incubated in a 1.5 ml microcentrifuge tube overnight in 500 µl nail-dissolving solution without fluorochrome. After centrifugation at 5000 g for 2 min, the sample precipitate containing fungal elements dissociated from keratinocytes was washed twice with distilled water. Subsequently, fungal DNA was extracted using the EZNA Fungal DNA Mini kit (Peqlab Biotechnologie) using the protocol provided by the manufacturer. The DNA was eluted from the disposable spin column with 50 µl distilled water.

PCR and DNA sequencing. PCR was performed under standard conditions using large subunit (LSU) primers (Table 1) to amplify a portion of the 28S rDNA as follows. Extracted DNA (3–20 µl), 2.5 µl of each sense and antisense oligonucleotide (42 mM), 4 µl dNTP mix (10 mM each dNTP) and 5 µl 10 × PCR buffer [100 mM Tris/HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂] were mixed with deionized water to give a total reaction volume of 50 µl. To each reaction, 2.5 U AmpliTaq DNA polymerase (Perkin Elmer) was added. The reaction mixture was incubated for 1 min at 94 °C, followed by 40 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C and 0.5 min at 72 °C, with extension for 10 min at 72 °C.

PCR amplicons and restriction enzyme digestion products ranging from 100 to 350 bp were loaded on 1.5% (w/v) agarose gels (TBE buffer) in the presence of ethidium bromide (0.5 µg ml⁻¹). DNA molecular mass marker XIV (100 bp ladder; Roche) was added. After running for 1 h at 6 V cm⁻¹, DNA fragments were visualized with UV light (300 nm) and recorded photographically.

For sequencing, amplified DNA was treated with exonuclease I (Fermentas Life Sciences) to digest the original primers and with alkaline phosphatase (Fermentas Life Sciences) to dephosphorylate free nucleotides for 15 min at 37 °C. Enzymes were heat-inactivated (15 min at 80 °C). DNA sequencing was performed by Synergene Biotech (Switzerland) on an automated ABI PRISM 3100 DNA Sequencer (Applied Biosystems). The rDNA sequences were aligned using CLUSTAL_X version 1.8 and annotated by BLAST on a non-redundant database.

Table 1. Primers used in this study

LSU2 was used as the antisense primer for all PCRs.

Primer	Sequence (5'→3')	Target	Fragment size (bp)
LSU1	GATAGCGMACAAGTAGAGTG	Dermatophyte and NDF 28S rDNA	310–314
LSU2	GTCCGTGTTTCAAGACGGG	Dermatophyte and NDF 28S rDNA	–
FUS	TGACCAGACTTGGGCTTGG	<i>Fusarium</i> spp. 28S rDNA	221
CAND	GGGCTTGAGATCAGACTTGG	<i>Candida</i> spp. 28S rDNA	230

Table 2. Prevalence of different fungi in nail specimens analysed by direct mycological examination in Lausanne University Hospital from 2003 to 2005

	Positive	Negative	Total
Dermatophytes	879	12	891
<i>Fusarium</i> spp.	128	13	141
<i>Aspergillus</i> spp.	119	24	143
<i>S. bevicaulis</i>	51	6	57
<i>Candida</i> spp.	362	178	540
Other NDFs	760	541	1301
Negative fungal culture	1515	2260	3775
Total	3814	3034	6848

RESULTS AND DISCUSSION

Fungi isolated from nail specimens

During a 3 year period (2003–2005), the total number of abnormal nail samples sent for mycological analysis was 6848 (Table 2). When fungal elements were detected by direct mycological examination (positive samples), only 23% of nail cultures revealed dermatophyte infection. *Trichophyton rubrum* was the most frequently isolated dermatophyte with a prevalence of 81%. *Trichophyton mentagrophytes* was identified in 18% of the cases. Rare dermatophytes were *Trichophyton soudanense* (four cases), *Epidermophyton floccosum* (one case) and *Microsporum audouinii* (one case). *Fusarium* spp. cultures were obtained with a significant frequency from positive nail samples (4%). The numbers of dermatophytes and other fungi, such as *Scopulariopsis brevicaulis* and *Fusarium* spp., obtained from samples that did not show any fungal elements (negative samples) were low in accordance with previous studies (Baudraz-Rosselet *et al.*, 1992). The use of fluorescence microscopy reduced the risk of not observing fungi in direct mycological examinations. Fungal spores and hyphae produced bright fluorescence, which is easily detected even in specimens with few fungi (Monod *et al.*, 1989).

Identification of infecting fungi in nail samples by PCR

DNA was extracted from nail samples that showed fungal hyphae or spores by direct mycological examination. After 2–6 h incubation in Na₂S nail-dissolving solution, fungal

Table 3. Results of PCR (fragment sequencing and RFLP) analysis versus morphological identification of fungi isolated from nail specimens

All tested samples were positive for fungal elements after direct mycological examination. M, mixed infection; NI, not identified.

ID number of sorted samples	Culture results	PCR identification	GenBank accession nos
1–31	Dermatophytes (<i>Trichophyton</i> spp.)		
1–21	<i>T. rubrum</i>	<i>T. rubrum</i>	AY213629
22	<i>T. rubrum</i> *	<i>T. krajdienii</i> = <i>T. mentagrophytes</i> type I	AY176740/AF378738
23–25	<i>T. rubrum</i>	M: <i>Trichophyton</i> sp.†/ <i>Candida</i> sp.‡	–
26–28	<i>T. rubrum</i>	M: <i>Trichophyton</i> sp.†/NI	–
29	<i>T. rubrum</i>	No 28S rDNA amplification	–
30–31	<i>T. mentagrophytes</i>	<i>T. krajdienii</i> = <i>T. mentagrophytes</i> type I	AY176740/AF378738
32–52	<i>Fusarium</i> spp.		
32–48	<i>Fusarium</i> spp.	<i>Fusarium</i> spp.	AY188919, AY097316, AF291060, AY097325, AY213704
49	<i>Fusarium</i> sp.*	<i>Verticillium</i> sp.	AY312608
50	<i>Fusarium</i> sp.§	<i>T. rubrum</i>	AY213629
51	<i>Fusarium</i> sp.§ + <i>Alternaria</i> sp.	<i>Alternaria daucill</i>	AY154701
52	<i>Fusarium</i> sp.¶	NI#	–
53–57	<i>Scopulariopsis brevicaulis</i>		
53–56	<i>S. brevicaulis</i>	<i>S. brevicaulis</i>	AF378737
57	<i>S. brevicaulis</i> §	<i>Aspergillus versicolor</i>	AY235001
58–68	<i>Aspergillus</i> spp.		
58–59	<i>A. flavus</i>	<i>A. flavus</i>	AY216669
60	<i>Aspergillus</i> sp.	<i>A. versicolor</i>	AY235001
61	<i>Aspergillus</i> sp.	M: <i>Aspergillus</i> sp.†/ <i>Candida</i> sp.‡	–
62	<i>A. niger</i> §	<i>A. flavipes</i>	AY216668
63	<i>A. niger</i> §	<i>T. rubrum</i>	AY213629
64	<i>A. flavus</i> §	<i>T. krajdienii</i> = <i>T. mentagrophytes</i> type I	AY176740/AF378738
65–68	<i>Aspergillus</i> sp.¶	NI#	–
69	<i>Acremonium</i> sp.	<i>Acremonium alternatum</i>	U57349
70–80	<i>Penicillium</i> spp.		
70–71	<i>Penicillium</i> sp.	<i>Penicillium citrinum</i>	AF033422
72	<i>Penicillium</i> sp.	M: <i>Trichophyton</i> sp.†/ <i>Penicillium</i> sp. or <i>Aspergillus</i> sp.†	–
73	<i>Penicillium</i> sp.§	<i>T. rubrum</i>	AY213629
74	<i>Penicillium</i> sp.§	<i>T. krajdienii</i> = <i>T. mentagrophytes</i> type I	AY176740/AF378738
75–76	<i>Penicillium</i> sp.§	<i>Fusarium solani</i>	AY097317
77	<i>Penicillium</i> sp.§	<i>Aspergillus versicolor</i>	AY235001
78	<i>Penicillium</i> sp.§	<i>Trichosphaeria pilosa</i> or <i>Cladosporium</i> ll	AY590297, AY342132
79	<i>Penicillium</i> sp.§	M: <i>Trichophyton</i> sp.†/ <i>Candida</i> sp.‡	–
80	<i>Penicillium</i> sp.¶	NI#	–
81–91	<i>Alternaria</i> spp.		
81	<i>Alternaria</i> sp.	<i>Alternaria</i> sp.	AY234951
82	<i>Alternaria</i> sp.*	<i>Curvularia senegalensis</i>	AB161074
83–87	<i>Alternaria</i> sp.§	<i>T. rubrum</i>	AY213629
88	<i>Alternaria</i> sp.§	<i>T. mentagrophytes</i> type II	AF378739
89	<i>Alternaria</i> sp.§	<i>S. brevicaulis</i>	AF378737
90	<i>Alternaria</i> sp.§	<i>Candida</i> sp.‡	AF156537
91	<i>Alternaria</i> sp.§	M: <i>Trichophyton</i> sp.†/ <i>Candida</i> sp.‡	–
92–93	<i>Mucor</i> spp.		
92	<i>Mucor</i> sp.§	<i>T. rubrum</i>	AY213629

Table 3. cont.

ID number of sorted samples	Culture results	PCR identification	GenBank accession nos
93	<i>Mucor</i> sp.	<i>M. circinelloides</i>	AY213710
94–109	Negative fungal culture		
94–97		<i>T. rubrum</i>	AY213629
98		<i>T. mentagrophytes</i> type II	AF378739
99–100		<i>Fusarium acutatum</i>	AY213704
101		<i>Acremonium strictum</i>	AY138482
102		<i>Aspergillus flavus</i>	AY235016
103		<i>Aspergillus versicolor</i>	AY235001
104		<i>Emericella quadrilineata</i>	AY213696
105		<i>Pleospora papaveracea</i> or <i>Alternaria</i> spp.	AY152615
106		M: <i>T. rubrum</i> /NI	–
107		M: <i>Candida</i> sp.‡/NI	–
108		NI#	–
109		No 28S rDNA amplification	
110–111	<i>Candida</i> spp.		
110	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>	AY497686
111	<i>C. albicans</i>	M: <i>C. albicans</i> /NI	AY233747

*Probable mis-identification.

†Identification by RFLP only.

‡PCR positive using CAND primers (Table 2).

§Probable contaminant.

||Uncertain identification due to poor-quality sequence.

¶Uncertain aetiological agent.

#Weak amplification and uninterpretable sequencing results (seven samples).

Fig. 1. *RsaI* and *HinfI* restriction maps of amplified DNA for the most prevalent fungi in onychomycosis. The primers in Table 1 are indicated by horizontal arrows.

elements were totally dissociated from keratinocytes. A quantity of 50–550 ng DNA in a volume of 50 µl could be extracted from nail samples of 2–30 mg. PCR was performed as described in Methods using primers LSU1 and LSU2 (Table 1). Each PCR included *Saccharomyces cerevisiae* DNA as a positive control and no DNA as a negative control. PCR results were visualized on agarose gels and no PCR products were obtained from normal, uninfected nails. The DNA extraction method used was simple and presented several advantages: (i) nail samples were processed in a single test tube before extraction of the DNA on a spin column; (ii) mechanical homogenization or shearing of the nail fragment was not required; and (iii) treatment with large amounts of lyticase and proteinase K was not required. DNA from 20 samples could be extracted simultaneously using a bench-top microcentrifuge.

Positive PCR products (15 ng) were sent for sequencing. First, we identified the infecting fungi in samples from which *Fusarium* spp. and dermatophytes were obtained in culture (samples 1–52; Table 3). The PCR results agreed with those of classical mycological identification in 17/21 cases for *Fusarium* spp. Three PCR products (samples 49–51; Table 3) revealed *Verticillium* sp., *T. rubrum* and *Alternaria dauci* after sequencing. *T. rubrum* was found in

Fig. 2. Amplification and RFLP gels of DNA extracted from human onychomycosis samples. M, 100 bp ladder; U, undigested PCR product; R, *RsaI* digestion; H, *HinfI* digestion. T, F, C, A, P and NI represent the species *Trichophyton*, *Fusarium*, *Candida*, *Aspergillus*, *Penicillium* and non-identified species, respectively. Numbers in parentheses are the sample numbers in Table 3. Samples 23, 26, 61 and 72 are mixed infections. Weak *Candida* spp. DNA fragments in sample 61 are indicated by an asterisk.

21/29 cases where this fungus grew in culture. In one case, *T. mentagrophytes* type I (Ninet *et al.*, 2003), or *T. krajdennii* (Kane *et al.*, 1992; Summerbell *et al.*, 1999), was identified by sequencing (sample 22). In samples 23–28 it was not possible to interpret the sequencing results and examination of the trace files led to a suspicion of mixed infections.

The 330 bp *Trichophyton* spp. PCR amplicon contained a unique *HinfI* restriction site, whilst that of *Fusarium* spp. contained a unique *RsaI* site (Fig. 1). Therefore, separate *HinfI* and *RsaI* restriction digestions of amplified DNA allowed a quick differentiation of *Fusarium* spp. from *Trichophyton* spp. (Fig. 2). In the six cases where sequencing results were not interpretable (samples 23–28), RFLPs confirmed the presence of two different 330 bp fragments in the PCR product and allowed us to identify *Trichophyton* spp. (Fig. 2). In conclusion, the results obtained from RFLPs and sequencing of PCR products correlated with the results of cultures from the same nail samples when *Fusarium* spp. or *Trichophyton* spp. were isolated. In two cases (samples 50 and 51), *Fusarium* sp. was considered to be a contaminant, rather than the aetiological agent.

In a second step, the PCR/sequencing/RFLP assay was used for tentative identification of the infecting fungi in 59 nail samples (samples 53–111; Table 3) from which neither dermatophytes nor *Fusarium* spp. were obtained in culture. Dermatophytes and *Fusarium* spp. were revealed by sequencing in 16 and 4 cases, respectively. In addition, *Scopulariopsis brevicaulis* and species of *Acremonium*, *Aspergillus*, *Penicillium*, *Alternaria*, *Curvularia*, *Mucor* and *Candida* were identified apparently as single infectious agents of onychomycosis (Table 3). In particular, *Aspergillus flavus* and *Aspergillus versicolor* were identified three and four times, respectively. When *Penicillium* spp. and *Alternaria* spp. were identified in culture, PCR sequencing most often revealed the presence of a different fungus. In these cases, *Penicillium* spp. and *Alternaria* spp. were considered to be contaminants.

RsaI and *HinfI* RFLPs were performed with all PCR products in an attempt to circumvent sequencing in further analysis (Fig. 2). RFLP allowed a quick distinction of *Trichophyton* spp. from NDF and *Candida* spp. However, *Fusarium* spp. and *Candida* spp. as well as *Aspergillus* spp. and *Penicillium* spp. showed identical band patterns (Figs 1 and 2). Therefore, sequencing remains the gold standard for identification of the species involved in onychomycosis. In addition, rare infecting moulds and possible new aetiological agents of onychomycosis can only be determined with certainty after sequencing. *RsaI* and *HinfI* RFLPs were useful to show clearly the presence of mixed infections when non-interpretable sequencing trace files were obtained in 13 of the 111 cases examined (12 %) (Fig. 2). We distinguished *Candida* spp. from *Fusarium* spp. in mixed infections using specific primers. DNA amplification using the primers CAND and LSU2 amplified a 230 bp 28S rDNA fragment specific for *Candida* spp. (data not shown).

PCR identification failed in nine cases among the 111 investigated (8 %). Retrospective investigation revealed that these results were from samples where very little nail material was available and where few fungal elements were observed.

Prevalence of NDF in onychomycosis

Direct identification of fungi in nail samples showed that the prevalence of NDF in onychomycosis is high. The majority of *Fusarium* spp. and *Scopulariopsis brevicaulis* and ~50 % of *Aspergillus* spp. identified in culture were shown to be the aetiological agent. Furthermore, in 43 samples (samples 69–111; Table 3) where moulds were identified other than the aforesaid fungi (60 % of positive samples; Table 2), NDF were found 18 times as the sole fungus (42 %). Therefore, in our laboratory, we estimate that the frequency of onychomycosis with NDF is ~30 %, not including mixed infections. Statistical analysis, based on results from cultures, varies significantly (Summerbell *et al.*, 2005). For example, 3.3 %

of NDF onychomycoses have been reported in Canada (Summerbell *et al.*, 1989); in contrast, NDF appeared to be more prevalent than dermatophytes in onychomycoses in a geriatric population in the USA (Scherer *et al.*, 2001). During the preparation of this manuscript, an article was published presenting PCR identification of *T. rubrum* and other moulds in onychomycosis from Bulgaria and Greece (Kardjeva *et al.*, 2006). NDF were detected in only 4% of infections among 181 positive nail specimens. *Scopulariopsis brevicaulis*, *Fusarium* spp. and *Aspergillus nidulans* were only detected six times, twice and once, respectively.

Conclusions

PCR methods may provide significant benefits in the rapid diagnosis of onychomycosis. First, NDF can be identified with certainty as the aetiological agents of onychomycosis and discriminated from dermatophytes as well as from transient contaminants. This is particularly relevant as moulds causing onychomycosis are not responsive to certain antifungal drugs in patients (Tosti *et al.*, 2000; Gupta *et al.*, 2001b). Secondly, identification of the infecting agent can be obtained in 24 h with RFLP or 48 h with sequencing, whereas results from fungal cultures can take 1–3 weeks; thus this allows selective treatment to be initiated sooner. The costs of a DNA extraction, a PCR and a sequencing reaction are approximately €2, €1 and €5, respectively. For comparison, the cost of a culture is approximately €2, but there is a frequency of negative fungal results of 40% when direct nail mycological examination is positive (Table 2). The simplicity, reliability and high NDF frequency support the use of the PCR/sequencing/RFLP assay reported in the present study, provided that enough nail material is collected by the clinician. Increased pricing of laboratory analyses should always be considered against the high cost of treatment of onychomycoses (more than €1000).

ACKNOWLEDGEMENTS

We thank Dr Bertrand Favre, Dr Lee Applegate and Dr Derry K. Mercer for critical review of the manuscript and assistance with the English and Giancarlo Tomio and Michael Spörri (Synergene Biotech GmbH) for helpful discussion and technical assistance. This work was supported by the Swiss National Foundation for Scientific Research, grant 3100-105313/1.

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