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INVESTIGATIVE REPORT

18S rDNA Polymerase Chain Reaction and Sequencing in Onychomycosis Diagnostics

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Diagnostic approaches to onychomycosis have traditionally been based on a combination of culture and microscopy. In the present study clinical specimens from 346 patients with suspected onychomycosis were analysed by 18S polymerase chain reaction (detection) followed by sequencing and subsequent database search (identification) in parallel with routine culture on agar (detection and identification). In 49 samples *Trichophyton rubrum* was identified by culture and sequencing. In 67 additional culture negative samples, a positive dermatophyte sequence was obtained (*T. rubrum* in 54, *T. mentagrophytes* in 5, and *T. species* in 8 samples). Fifteen samples cultured positive while no sequence was obtained. Two hundred and seven samples were negative by culture as well as by sequencing. Nails from 10 healthy controls were negative by culture and sequencing. In conclusion, the number of specimens that were positive by polymerase chain reaction was more than double the number that were positive by culture alone. *Key words: onychomycosis; Trichophyton rubrum; nails.*

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Onychomycosis is a common and increasing problem. The increase is believed to reflect the expanding number of elderly persons and immunocompromised patients as well as increasing number of people participating in physical activities (1, 2). Dermatophyte nail infections are associated with past or current fungal infection of the feet. Numerous nail conditions may, however, mimic onychomycosis. Long-term systemic antifungal treatment has potential side-effects and is expensive. The need for accurate diagnosis is essential, and laboratory confirmation is important in the diagnostics. *Trichophyton rubrum* is the most common of these species, accounting for approximately 80% of the infections (3).

Diagnosis of onychomycosis traditionally relies on direct microscopic examination and culture of nail samples. The specimen must be obtained from the nail bed as close as possible to the advancing infected edge of the lesion, as this area is most likely to contain viable hyphae. If the sample is not obtained under the correct conditions, both direct microscopy and culture give a high rate of false negative results. With correct sampling, direct microscopy gives rapid evidence of fungal infection, however identification of fungi still remains. Culture results are traditionally delayed by 2–4 weeks because of the slow growth of dermato phytes. Although techniques based on molecular genetics are promising tools in diagnostics of mycoses (4), culture still remains the standard procedure. Since DNA is extremely resistant and can persist even in the absence of viable hyphae, DNA amplification techniques, such as polymerase chain reaction (PCR), may represent a useful addition to standard procedure. Most genetic studies on dermatophytes have focused on identification of cultured isolates.

Various targets have proven useful for PCR-based assays for identification of dermatophytes, e.g. the topoisomerase II gene (5–7), the chitin synthase 1 gene, or ribosomal DNA (rDNA) internal transcribed spacer regions have been chosen (8–13). In some studies rDNA genes have been used, for example in 1994 Bock et al. (14) as well as other authors (15–18) targeted the 18S rDNA gene, while Ninet et al. (19) used the 28S rDNA part of the genome in a commercially available kit. Articles describing several other PCR-based assays are available (20–24). The authors have illuminated various genetic aspects, e.g. taxonomy, specificity and sensitivity as well as time-saving aspects.

Only a few studies have addressed genetic detection of dermatophytes directly in clinical samples (18, 25–26). However, such articles have focused mainly on therapeutic efficacy and species identification of cultured dermatophytes, although Arca et al. (18) focused on dermatophyte detection directly in clinical samples.

The aim of the present study was to compare 18S rDNA PCR-based sequencing with standard agar cultivation. The present molecular genetic analyses were performed directly on clinical nail samples from patients with suspected onychomycosis.

MATERIALS AND METHODS

Patients

Patients were recruited from dermatological departments at university hospitals, private dermatologists and general prac-

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titioners from March 2002 through August 2004. Nail samples were collected prospectively from patients with clinically suspected onychomycosis (median age 48 years, range 4–86 years). Nails from 10 healthy controls were included in the analyses (median age 36 years, range 2–62 years).

Methods

Specimens were processed for agar cultivation and molecular genetic analyses in parallel, i.e. each specimen was cut into two equal parts and analysed by culture and PCR/sequencing.

All samples were cultured on the following dermatophyte agars in parallel as part of the routine diagnostics: (*i*) Sabouraud-Dextrose agar (Difco) including penicillin 12 mg/l and streptomycin 40 mg/l; and (*ii*) Mycosel agar (BBL). Identification included further culture as well as microscopy according to standard procedures (27).

Nail samples were cut into small pieces by scalpel. For DNA extraction, samples were incubated at room temperature for 1 h in 400 µl of lysis buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl pH 7.5, 1% mercaptoethanol). DNA was extracted by adding 200 µl phenol/chloroform/isoamyl alcohol (25:24:1) (28). A quick centrifugation (13000 rpm, 1 min) was done to separate the phases, and the aqueous phase was pipetted into a 1.5 ml tube. DNA was precipitated with 1.2 volumes of 88% isopropanol/0.2 M potassium acetate. After repeated centrifugation (12000 rpm 10 min) and washing (70% ethanol), pellets were air-dried, 20 µl Tris-EDTA buffer (28) was added, and 1 µl was used for PCR. MJ Research PT200 was used for PCR, while sequencing was performed with a Beckman CEQ2000. ClustalW was used for alignment of the sequences, while Blast was used to match the sequences with those in Genebank database (NCBI). *T. tonsurans* and *T. mentagrophytes* differed by two base pairs only, as did *T. mentagrophytes* and *T. rubrum,* while *T. tonsurans* and *T. rubrum* differed by four base pairs.

The detection level for the PCR protocol was 100 copies of 18S DNA per µl (equalling about one cell per µl).

RESULTS

Nail samples from 346 patients were analysed in parallel by molecular genetics and culture. In 49 of these samples, culture and sequencing yielded similar results (*T. rubrum*) (Table I). In 67 additional samples, which were culture negative, a positive sequence was obtained: *T. rubrum* (54 samples), *T. mentagrophytes* (5 samples) and *T.* species (8 samples). Fifteen sequence negative samples were found to be culture positive: *T. rubrum* was cultured in 12 of these samples, while the remaining 3 were *T. mentagrophytes*. However, in 2 of these 15 specimens, which were categorized as sequence negative, a PCR product was obtained. This PCR product proved to be from a non-dermatophyte.

In 5 specimens *T. rubrum* was identified by culture, the corresponding sequencing did not allow identification further than to genus level. In three more samples identification was discordant: culture gave *T. mentagrophytes* while sequencing gave *T. rubrum*.

Two hundred and seven samples were negative by culture as well as by sequencing (Table I). Nail specimens from 10 healthy controls were negative by culture and PCR (data not shown).

DISCUSSION

In the present study positive results obtained by molecular genetics more than doubled the positive results obtained by culture alone. That is, in 49 samples *T. rubrum* was co-identified by culture and sequencing, while 67 additional samples which were culture negative, were positive by sequencing. Molecular genetics has rarely been used to detect dermatophytes directly in clinical samples from patients with suspected onychomycosis. Hence, we have not been able to find any literature combining PCR with subsequent sequencing of product directly in nail samples from such patients. However, in an article based on agarose gel detection of PCR products, Arca et al. (18) showed increased sensitivity of PCR compared with culture. Their analyses were performed directly on nails from patients with suspected onychomycosis, but sequencing and species identification were not performed. The present results are in accordance with those of these authors. It may be speculated that contamination and colonization contribute to false positive results compared with culture, also sampling technique may be less critical for molecular diagnostics than for culture, since DNA from dermatophytes could easily yield a positive PCR result, while positive culture will have to rely on viable hyphal elements.

The present work shows that sequencing of this 18S DNA target enabled species differentiation in the majority of positive samples. However, there were a few challenges in species identification: in 8 culture negative and 5 culture positive samples sequencing reached genus level only. This challenge most probably reflects the high level of similarity in target sequences, a challenge not reduced by blurry base sequences. In contrast to Turin et al. (17), the present work shows that 18S DNA PCR sequencing makes it possible to identify closely related species. In their agarose gel-based assay, species

Table I. *Comparison between agar cultivation and 18S polymerase chain reaction (PCR) sequencing (number of samples)*

Culture	18S PCR sequencing				
	T. rubrum	*T. mentagrophytes*	*T.* species	Negative	Total
T. rubrum	49	–	5	12	66
T. mentagrophytes	3	–	–	3	6
Negative	54	5	8	207	274
Total	106	5	13	222	346

identification was not possible due to a high level of similarity in target sequence between *T. rubrum* and *T. mentagrophytes*. This challenge was also addressed by Kano et al. (29) in another assay based on detection of PCR product in agarose gel. The discrepant identification in three specimens in the present work (culture gave *T. mentagrophytes* while sequencing gave *T. rubrum*) was also regarded as a result of the high level of base sequence similarity between these two species in the target sequence, only two base pairs differed between *T. rubrum* and *T. mentagrophytes*.

Among the 207 samples reported as negative by sequence and culture, no dermatophyte sequence was obtained. However, among these 207 samples were two minor groups of 52 samples that gave a PCR product: (*i*) one group including non-dermatophyte fungal species by sequence, and (*ii*) another group of unsuccessfully sequenced specimens. In the first group (*i*) we found 18 specimens of various non-dermatophyte fungal species: 10 *Candida parapsilosis*, 5 *C. spp*., 1 *C. albicans*, 1 *Aureobasidium pullulans*, and 1 *A. verrucosum*. In the other group (*ii*) were 34 unsuccessfully sequenced specimens. The sequence of these samples was not possible to match with any of the sequences found in Blast. We chose to report all these 52 specimens as dual negative ones, although they could have been alternatively reported as false positive.

The lack of positive PCR in the 15 culture positive samples was striking (Table I). This lack of PCR positivity may reflect presence of inhibitors, sample quality variation, or simply too low a test volume. The present results are in agreement with those of Arca et al. (18), who found negative PCR product in several specimens that cultured positive.

In development of our assay, the work of Turin et al. (17) was used as a reference. Since the *18S* gene is much conserved amongst various fungal species, universal primers were expected to allow amplification of all fungal 18S DNA, nevertheless interspecies genetic variation in the target region should still allow identification of the species by DNA sequencing. The assay with primers TR1 and TR2 (17) sometimes gave human as well as fungal 18S DNA after DNA sequencing and Blast search. In order to optimize the assay several other primers containing sequence differences from human 18S were tried. These primers also amplified fungal regions with more genetic variations. We ended up choosing primers TR3 and Rev3 (5'-GCGGTAATTCCAGCTCCA-3' and 5'-CCGATCCCTAGTCGGCATA-3'), which matched the ends of a 600 bp sequence. These primers gave the best PCR result and the target also contained more differences between the species than did TR1 and TR2. After changing to these new sets of primers human 18S DNA was not amplified.

An obvious weakness of the present work is the lack of microscopy results. It is well known that sample microscopy improves positive findings from patients with suspected onychomycosis (30). As the present samples were collected from a variety of specialists and general practitioners, it was assumed that standardization of microscopy at the clinical level would be impossible. However, standardization could have been obtained by leaving this responsibility to the laboratory. Microscopy could have improved interpretation of the results, especially for the PCR positive but culture negative results. Future studies should take care of this important aspect.

The present results demonstrate the obvious power of molecular techniques compared with culture, as demonstrated by the higher number of positive results compared with culture alone. However, obvious challenges still exist, among these challenges are culture positive samples being PCR negative. This point should be addressed in future work, the aspect may be at least partly solved by increasing the sample volume. Microscopy of samples should also be included in such studies.

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