A rapid two-step DNA extraction method and a multiplex PCR for the detection of dermatophytes in general and *Trichophyton rubrum* specifically were developed and evaluated with DNA extracted from pure cultures and from clinically diseased nails. DNA from the following dermatophytes was used: *Epidermophyton floccosum*, *Microsporum audouinii*, *Microsporum canis*, *Microsporum gypseum*, *Microsporum nanum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton schoenleini*, *Trichophyton soudanense*, *Trichophyton terrestre*, *Trichophyton tonsurans*, *Trichophyton verrucosum*, and *Trichophyton violaceum*. Human DNA and DNA from the following nondermatophyte fungi were included as controls: *Alternaria*, *Aspergillus niger*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Malassezia furfur*, *Saccharomyces cerevisiae*, and *Scopulariopsis brevicaulis*. A total of 118 nail samples received for routine microscopy and culture for dermatophytes were subsequently tested by the two PCRs separately and in a multiplex format. Using DNA extracted from pure cultures and the pan-dermatophyte PCR, the *T. rubrum*-specific PCR sequentially and in a multiplex format correctly detected all dermatophytes and additionally correctly identified *T. rubrum*. Comparison of the traditional diagnostic evaluation (microscopy and culture) of nail samples with PCR on DNA directly extracted from the nails showed excellent agreement between PCR and microscopy, but the number of samples with dermatophyte species identification was increased considerably from 22.9% to 41.5%, mainly due to the identification of *T. rubrum* by PCR in microscopy-positive but culture-negative samples. In conclusion, this 5-hour diagnostic test was shown to increase not only the speed but also the sensitivity of investigation for nail dermatophytes.
implement in a routine laboratory receiving large numbers of nail specimens.

In this paper we present an alternative multiplex PCR-based method especially developed for the detection of dermatophyte nail infections. By a two-step extraction procedure followed by a single multiplex PCR and electrophoresis, the method enables the diagnosis of infection caused by any one of the dermatophytes (pan-dermatophyte) and in the case of *T. rubrum* even a genus and species identification. A two-step, 15-minute method for extraction of DNA directly from patient samples allows application of this method in routine diagnostic laboratories.

**MATERIALS AND METHODS**

**Strains and clinical isolates.** Twelve fungal strains were purchased from the National Collection of Pathogenic Fungi (United Kingdom). Clinical isolates were obtained from the Mycology Laboratory of the Statens Serum Institute (SSI) (Denmark) (Table 1). All clinical isolates were identified by observation of macro- and micromorphology.

**Clinical nail samples.** One hundred eighteen nail samples received for routine examination at the Laboratory of Mycology at SSI were prospectively included. The only inclusion criterion was the presence of a sufficient amount of material for investigation by direct microscopy and culture as well as PCR analysis.

**DNA preparation from dermatophyte cultures.** The strains and clinical isolates were cultured in 2 ml of Sabouraud liquid medium with cycloheximide and chloramphenicol (SSI Diagnostika, Denmark) and incubated with shaking for up to 5 days at 27°C. After harvest, the pellet was resuspended in 500 μl of lysin buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% chloramphenicol (SSI Diagnostika, Denmark) and incubated with shaking for up to 30 min at 4°C, followed by a single centrifugation (1 min, 12,000 g). The supernatant was transferred to a new tube, and an equal volume of isopropyl alcohol was added. The DNA pellet was washed in 70% ethanol. The dried DNA pellet was dissolved in 50 μl of TE (10 mM Tris, 1 mM EDTA) buffer. Two microliters of the DNA was used in 20 to 50 μl of the PCR mixture. Reagents were, unless otherwise stated, purchased from Sigma (Germany).

**DNA preparation from nail samples.** For DNA preparation (1), DNA from nail samples was extracted by a 10-min incubation of the nail sample in 100 μl of extraction buffer (60 mM sodium bicarbonate [NaHCO₃], 250 mM potassium chloride [KCl] and 50 mM Tris, pH 9.5) in 95°C and subsequent addition of 100 μl anti-inhibition buffer (2% bovine serum albumin). After vortex mixing, this DNA-containing solution was used for PCR.

**Pan-dermatophyte PCR.** Pan-dermatophyte PCR (1) was as follows. Based on the comparison (VectorNTI; InforMax, Inc.) of nucleotide sequences of different dermatophytes in the NCBI nucleotide database, a set of primers detecting a DNA fragment encoding chitin synthase 1, panDerml (5′GAAAGAGATTGTCGTTGATCGTCTC3′) and panDerm2 (5′CTCGAGTGCAAAGCAGCGCAGACG3′), was designed. Twelve dermatophyte reference strains, 89 clinical dermatophyte isolates, 22 non-dermatophyte fungal isolates, and purified human DNA (Table 1) were tested. PCR mixtures consisted of 10 μl of PCR Ready Mix (Sigma, Germany), 0.2 μl of each primer (panDerm1 and panDerm2) at 100 μM, and 4 μl of DNA in a volume of 20 μl. PCR was performed in a MWG-Biotech thermal cycler. The time-temperature profile for PCR was 45 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s of extension at 72°C, preceded by initial denaturation for 10 min at 95°C and subsequent addition of 100 μl anti-inhibition buffer (2% bovine serum albumin). After vortex mixing, this DNA-containing solution was used for PCR.

**Trichophyton rubrum-specific PCR.** On the basis of alignment (VectorNTI; InforMax, Inc.) of sequences of internal transcribed spacer 2 in the NCBI nucleotide database, universal (uni, 5′CTCTGTAGCACTGGATGGCC3′) and Trichophyton rubrum-specific (Trubrum-rev, 5′CGTCTTGAGGCGCTGAAT3′) primers were designed. Each reaction was performed in a volume of 20 μl by the addition of 4 μl of DNA from microorganisms listed above, 0.2 μl of each primer at 100 μM, and 10 μl of PCR ReadyMix (Sigma, Germany). The amplification was performed in a thermal cycler (MWG-Biotech, Germany) and consisted of one initial cycle of denaturation for 5 min at 94°C and 45 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s of extension at 72°C. After the thermal cycles, the amplicons were electrophoresed in a 2% agarose gel and stained with ethidium bromide. To standardize the procedure, different DNA concentrations and thermal cycles were tested (data not shown).

**Multiplex PCR.** The multiplex PCR was performed using the two specific sets of primers described above (panDerm1 and panDerm2 primers and uni- and Trubrum-rev primers). The reaction was performed under different conditions; 0.2 mM of each primer was used. The following time-temperature profile was chosen: one initial cycle of denaturation for 5 min at 94°C and 45 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s of extension at 72°C. After the thermal cycles, the amplicons were electrophoresed in a 2% agarose gel and stained with ethidium bromide. Specificity of the multiplex PCR was tested with DNAs from all of the strains listed in Table 1 and with human DNA. To standardize the procedure, different DNA concentrations and thermal cycles were tested (data not shown). The multiplex PCR and (respectively the pan-dermatophyte and the *T. rubrum*-specific PCRs) were subsequently evaluated using 97 nail specimens received for routine analysis.

**RESULTS**

Evaluation of pan-dermatophyte and *T. rubrum* PCRs using DNA extracted from fungal cultures. Extracted DNA from cultures of 12 reference dermatophyte strains, 89 clinical dermatophyte isolates, and 21 other fungi (Table 1) was used for evaluation of the pan-dermatophyte primers and the *T. rubrum*-specific primers separately as well as in a multiplex PCR format. A 203-bp PCR product corresponding to *T. rubrum* was observed for 13/13 *T. rubrum* DNA samples with the *T. rubrum*-specific PCR separately and in multiplex format, and specific 366-bp PCR products were obtained for 101/101 dermatophyte DNA samples with the pan-dermatophyte PCR alone and in multiplex format (examples of results of the pan-dermatophyte and *T. rubrum*-specific PCRs are presented in Fig. 1). No PCR products were detected by the pan-dermatophyte PCR, the *T. rubrum*-specific PCR, or the multiplex PCR for the 21 non-dermatophyte fungal isolates or for three sam-
by PCR (examples of results of the pan-dermatophyte and *T. rubrum*-specific PCRs are presented in Fig. 2). One specimen showed none of the three dermatophyte specific PCRs positive, and 15 (23.4%) were PCR positive but negative by microscopic examination (5.6%) (unspecific PCR products were synthesized).

(38.9%), and the result for one sample was not possible to interpret (5.6%) (unspecific PCR products were synthesized). Two specimens which were negative by microscopic examination of the nail but *T. rubrum* positive in culture were negative by PCR (examples of results of the pan-dermatophyte and *T. rubrum*-specific PCRs are presented in Fig. 2). One specimen diagnosed by conventional examination as *T. tonsurans* was pan-dermatophyte and *T. rubrum* PCR positive. Finally, PCR results for nine specimens diagnosed by conventional examination as non-dermatophyte species are presented in Table 2.

To investigate whether the lack of PCR products in PCR-negative samples could be due to the presence of PCR-inhibitory substances in the samples, all PCR-negative specimens were spiked with *T. rubrum* DNA and subsequently retested in the multiplex pan-dermatophyte–*T. rubrum* PCR. A PCR product was produced in all cases (data not shown). All the *T. rubrum*-specific PCRs obtained from specimens not diagnosed as *T. rubrum*-positive samples by traditional methodology were sequenced (MWG Biotech, Germany), and the sequences of all of them matched that of *T. rubrum* reference strain NCPF 113. The pan-dermatophyte PCR product obtained from the DNA of the specimen diagnosed conventionally as *T. rubrum* and *T. mentagrophytes* was sequenced, and the sequence matched that of *T. mentagrophytes* reference strain NCPF 224.

A comparison of results obtained by conventional diagnostics and PCR is shown in Table 3. Overall, the number of positive samples was increased by 11% (45 [38.1%] versus 50 [42.2%]) of 118 specimens were positive by the conventional and PCR methodologies, respectively). Furthermore, due to the presence of a considerable number of microscopy-positive but culture-negative samples, the percentage of samples with a species identification was almost doubled by use of the PCR (49 out of 118 specimens were found to be *T. rubrum* positive by the PCR-based method, while only 27 out of 118 specimens were dermatophyte positive by culture).

![Fig. 1](Image)

**FIG. 1.** Example of *Trichophyton rubrum*-specific and pan-dermatophyte PCR product analysis. Lanes: 1 and 12, molecular size marker (fragment sizes, 501, 489, 404, 321, 240, 147, 111, and 110 bp); 2 and 3, results of *T. rubrum*-specific PCR performed for *T. mentagrophytes* DNA (lane 2) and *T. rubrum* DNA (lane 3); 4 to 11, results of pan-dermatophyte PCR performed for *Microsporum audouinii* (lane 4), *T. mentagrophytes* var. *mentagrophytes* (lane 5), *Trichophyton schoenleinii* (lane 6), *Trichophyton terrestre* (lane 7), *T. rubrum* (lane 8), *T. tonsurans* (lane 9), *Trichophyton soudanense* (lane 10), and *Epidermophyton floccosum* (lane 11).

![Fig. 2](Image)

**FIG. 2.** Example of *Trichophyton rubrum*-specific and/or pan-dermatophyte multiplex PCR product analysis. Lanes: 1, molecular size marker (100 bp DNA ladder); 2 to 6, results of multiplex PCR performed for DNA extracted directly from nail specimens diagnosed by conventional methods as negative (lane 2), *M. audouinii* (lane 3), *T. rubrum* (lane 4), *T. mentagrophytes* (lane 5), and *Aspergillus* sp. (lane 6).
TABLE 3. Comparison of the results of conventional and PCR-based examinations of nail specimens

<table>
<thead>
<tr>
<th>Method</th>
<th>% (no./total)</th>
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<tbody>
<tr>
<td></td>
<td>Dermatophyte</td>
<td>Species-specific</td>
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<tr>
<td></td>
<td></td>
<td>identification</td>
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<tr>
<td>Conventional</td>
<td>38.1 (45/118)</td>
<td>22.9 (27/118)</td>
</tr>
<tr>
<td>PCR based</td>
<td>42.4 (50/118)</td>
<td>41.5 (49/118)</td>
</tr>
<tr>
<td>Difference in detection by PCR-based method</td>
<td>4.3</td>
<td>18.6</td>
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</tbody>
</table>

DISCUSSION

Day-to-day detection of dermatophyte infection in nails is obviously a major improvement in the diagnosis of tineaunguim, allowing antifungal treatment to be instituted promptly upon correct diagnosis and at the same time restricted to those with dermatophyte infections. Although the species distributions vary between different parts of the world, *T. rubrum* is in most surveys reported to be the major pathogen in tinea unguim, accounting for 63 to 89% of the infections (1a, 9, 10, 19, 23, 27). At the same time, dermatophytes belonging to the less terbinafine-susceptible genus *Microsporum* are unanimously reported to be very rare agents of onychomycosis, and the detection of dermatophyte DNA in a nail specimen will therefore represent infection with a terbinafine-susceptible dermatophyte in the vast majority of cases and thus provide sufficient information to guide the clinician despite a lack of species identification.

The clinical evaluation of single and multiplex PCR detection of any dermatophyte and of *T. rubrum* specifically in nail specimens showed increased sensitivity compared to conventional diagnosis (Table 3). In this comparison, samples positive by microscopy but negative by culture were regarded as dermatophyte positive by traditional diagnostics, though we cannot rule out the possibility that some of these cases may represent nondermatophyte infections. It is not uncommon, however, to obtain negative culture results from patients with dermatophytosis, due to difficulties associated with sampling (insufficient material or use of nail clippings instead of subungual material) or to prior medical treatment, etc., and cases with positive microscopy but negative culture should therefore always be investigated further (5). On one occasion the species identifications obtained by conventional culture and PCR were conflicting (*T. tonsurans* by culture and *T. rubrum* by PCR). The fact that the *T. rubrum* PCR was negative when applied to the *T. tonsurans* reference strain as well as to all the clinical control *T. tonsurans* isolates tested initially raises the question of whether this was a case of misidentification by conventional identification or a double infection with *T. tonsurans* and *T. rubrum*. As nail infections in Denmark caused by *T. tonsurans* are extremely rare, especially among Danes (as in this case), the former explanation is the more likely in our opinion; however, the isolate was not stored and thus no further examinations were possible. One specimen was by conventional methodology diagnosed as a mixed infection with *T. rubrum* and *T. mentagrophytes*, but the PCR yielded solely a pan-dermatophyte PCR product. This was sequenced and the sequence matched that of *T. mentagrophytes*, in agreement with the culture result. Several explanations for this apparent lack of detection of the *T. mentagrophytes* isolate in this case exist. (i) Although the specimens used for conventional and PCR testing derived from the same patient, they are not exactly the same material and the *T. rubrum* may not have been present in the specimen used for PCR. (ii) This may be a case of contamination of the culture plates by *T. rubrum*. (iii) The sensitivity of the *T. rubrum* PCR may be insufficient in cases of mixed infections. The facts, however, that the *T. rubrum*-specific primers target a multicopy gene, in contrast to the pan-dermatophyte primers, and that the *T. rubrum* PCR was also negative when the sample was run in a single-PCR setup suggest that the sensitivity of the *T. rubrum* PCR should not be inferior to that of the pan-dermatophyte PCR. However, examination of additional samples from cases of documented mixed infections is necessary to evaluate this further.

The interpretation of the detection of nondermatophyte molds in nail specimens is controversial. Such findings may reflect the presence of mold elements in the nail specimen due to contamination, transient colonization or infection of a traumatized or otherwise diseased nail, or contamination in the laboratory. Therefore, at least repeated recovery of identical mold species is typically required before a pathogenic role is considered, and even in these cases the recovery may represent an infection which is secondary to an underlying pathological nail condition. The finding in this study that two nails yielded molds by culture but *T. rubrum* by PCR may reflect overgrowth of the rapidly growing contaminating or colonizing mold or true double infection.

Although dermatophyte and/or *T. rubrum* identification in the nail specimens has been attempted using a range of molecular methods, only one recently published study involved DNA extraction directly from nail specimens without prior culture (15). The extraction method described, however, was a multistep procedure involving 14 steps and thus was labor-intensive and per se associated with an increased risk of contamination. The application of a two-step, 15-min procedure for extraction of DNA directly from nail specimens and a multiplex PCR-based diagnosis of any dermatophyte and/or *T. rubrum* with increased sensitivity compared to conventional diagnostic procedures allow for the first time integration of a molecular biology-based method into the routine examination of nail dermatophytosis also for diagnostic laboratories receiving specimens on a larger scale. This brings hope that rapid, specific, and low-cost diagnoses of onychomycosis may become broadly available in the near future.

REFERENCES