Evaluation of a commercial PCR test for the diagnosis of dermatophyte nail infections

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INTRODUCTION

Tinea unguium, known as onychomycosis, is a dermatophyte infection of nails with worldwide distribution. Conventional methods for detecting fungi in nail specimens are either non-specific (microscopy) or insensitive (culture). PCR has been used to improve sensitivity in detecting the causative fungi in nail specimens from patients with suspected onychomycosis. Results of a commercial multiplex PCR for the detection of dermatophytes, especially Trichophyton rubrum (the main dermatophyte implicated), as compared to conventional methods are presented. A total of 418 nail scrapings obtained from dermatological outpatients were handled in the Laboratory of Microbiology between May 2010 and May 2013. Among them, multiplex PCR detected 126 (30.1 %) dermatophyte-positive samples, whereas culture revealed 44 (10.5 %). Direct microscopy revealed 63 (15.1 %) positive specimens. T. rubrum was identified in 116 out of 126 (92 %) positive PCR samples and 40 out of 44 (91 %) dermatophyte-positive cultures. Implementation of PCR increased species-specific detection of dermatophytes by 21.1 %, leading to a threefold increase as compared to culture alone. Multiplex PCR offers a time-saving diagnostic tool for tinea unguium and augments laboratory assistance to clinical evaluation for proper treatment.

Methods

Four hundred and eighteen nail scrapings from an equivalent number of elderly (50–80 year old) outpatients with clinically suspected onychomycosis (nail discoloration, thickening and separation from associated species are Epidermophyton floccosum and Trichophyton verrucosum (Moriarty et al., 2012). In addition to dermatophytes, Candida and non-dermatophyte moulds may be recovered from clinically affected nails; however, their clinical significance is controversial (Brillowska-Dabrowska et al., 2007). Conventional diagnosis is based on detection of fungal elements by direct microscopy of clinical specimens, followed by culture and morphological identification of the fungus. The whole procedure is time-consuming, requiring 10 to 15 days, sometimes up to 3 to 4 weeks, and accuracy depends on the expertise of the personnel. Introduction of PCR-based methodology could increase sensitivity, specificity, and speed, and, potentially, even reduce cost in the diagnostic approach associated with additional visits to the clinician and additional sampling and diagnostic tests, and inconvenience for the patient due to the delay in appropriate treatment (Jensen & Arendrup, 2012). Herein, we present the performance of a commercial multiplex PCR as compared to conventional methods (direct microscopy and culture of nail samples) for the diagnosis of onychomycosis.

Abbreviations: 95 % CI, 95 % confidence interval; DOR, diagnostic odds ratio; LR−, negative likelihood ratio; LR+, positive likelihood ratio; NND, number needed to diagnose; NPV, negative predictive value; PPV, positive predictive value.
the nail bed) were processed in our laboratory from May 2010 to May 2013. Patients who had used topical or systemic antifungal drugs within the previous 2 weeks and 6 months, respectively, were excluded from sampling. The only prerequisite was an amount of clinical sample of at least 90–100 mg. Specimens were homogenized and divided into three portions (30–33 mg each). Samples were examined by direct microscopy of 30 % KOH-treated and lactophenol cotton blue mounts. Cultures were performed on Sabouraud glucose agar, Sabouraud agar containing 0.005 % chloramphenicol and 0.5 % cycloheximide (Mycobiotic), and dermatophyte test medium agar. Plates were incubated at 26 °C for 30 days before being assessed as negative due to no growth. Positive cultures were evaluated and identified at the species level based on the gross morphological characteristics of the colonies, followed by microscopic examination. In parallel, the DNA from 30–33 mg nail samples was extracted by a 10 min incubation at 95 °C in 100 µl extraction buffer (60 mM sodium bicarbonate, 250 mM potassium chloride and 50 mM Tris, pH 9.5) and, afterwards, 100 µl anti-inhibition buffer (2 % BSA) was added (Brilliowska-Dabrowska et al., 2007, 2010). A multiplex PCR (Dermatophyte PCR kit; Statens Serum Institut Diagnostica) was performed following the manufacturer’s instructions. The reagents consisted of two pairs of primers; the first (panDerm1 5’-GAGGAGGAATTGTTGTCGTTTGCATGCCTC-3’ and panDerm2 5’-CTGGAGGTCAAAACGACCCAGAG-3’) targets the chitin synthase-encoding gene (chitin synthase 1 – chs1) and served for detection of dermatophytes in general, whereas the second (Trubrum-for 5’-CTTTTGAAGGCAATTGGGCGC-3’ and Trubrum-rev 5’-CGGTCCGTAGGGCGGCTGAA-3’) targets internal transcribed spacer gene 2 (its2) for the specific detection of T. rubrum (Brilliowska-Dabrowska et al., 2007, 2010). Amplification was performed, after initial denaturation for 10 min at 95 °C, by 45 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. Afterwards, amplicons (approx. 366 bp for pan-dermatophyte and 203 bp for T. rubrum) were subjected to electrophoresis in 1 % agarose gel and stained with ethidium bromide. T. rubrum and pan-dermatophyte DNA were used as positive controls, buffer was used as a negative control, and the primer mix included an internal plasmid control that serves as a template for the T. rubrum-specific primers. A 100 bp DNA ladder (Promega) was used as a molecular size marker. A strong band at 203 bp and a weaker or no band at 366 bp indicated the presence of T. rubrum, whereas a strong band at 366 bp indicated the presence of a non-T. rubrum dermatophyte. Multiplex PCR was completed in 5 h and was applied twice a week. Results of direct microscopy were available within 1–2 h of handling the specimen. PCR-negative samples were not considered negative until culture was completed (30 days). Investigators who assigned the PCR results were blinded to results of direct microscopy or culture and vice versa. This is a retrospective study and the Standards for Reporting of Diagnostic Accuracy (STARD) statement were taken into account (Bossuyt et al., 2003).

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 21.0 (released 2012), and MedCalc 2014 (MedCalc Software). The sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), positive likelihood ratio (LR + ), negative likelihood ratio (LR − ), diagnostic odds ratio (DOR) and number needed to diagnose (NND) with their 95 % confidence intervals (95 % CIs) for PCR, culture and direct microscopy were estimated. Statistical significance was achieved if P<0.05. For an appropriate diagnostic test, desirable values for LR + and LR − should be ≥ 10 and ≤ 0.1, respectively (Garg et al., 2007). A DOR >1 is indicative of useful tests and higher DORs are indicative of better test performance. The NND is the number of tests required in order to gain a positive diagnosis. All diagnostic indices were estimated by two approaches. In the first approach, the ‘gold’ reference standard for diagnosis of onychomycosis was defined as the combination of a positive dermatophyte culture and/or the presence of fungal hyphae assessed by direct microscopy. The last criterion was considered as positive only if no non-dermatophyte filamentous fungus was cultured from the sample. In the second approach, the combination of positive culture and PCR results was considered as the gold reference standard for diagnosis of onychomycosis.

RESULTS

A total of 418 specimens obtained from dermatological outpatients with clinically suspected tinea unguium (one sample per patient) were handled in our laboratory during a 3 year period. The majority (72 %) originated from females and 78 % involved toenails. All samples were processed by conventional methods (direct microscopy and culture) and multiplex PCR analysis.

Direct microscopy by two independent personnel members identified 63 (15.1 %) positive samples. Growth of dermatophytes was observed in 44 (10.5 %) specimens. T. rubrum was identified to be the main dermatophyte as it represented 91 % of isolates (40 cases). The remaining four (9 %) specimens were characterized as T. interdigitale. Both direct microscopy and culture were positive in 39 cases. Direct microscopy was positive in 24 dermatophyte-negative cultures. Direct microscopy was negative in five specimens that grew dermatophytes in culture. Altogether, the combination of conventional methods revealed 68 (16.3 %) positive specimens. Finally, both direct microscopy and culture were negative for dermatophytes in 350 cases.

However, multiplex PCR was positive in 126 (30.1 %) of all nail specimens tested. Results of a PCR run are presented in Fig. 1. T. rubrum was identified in 116 (92 %) samples, whereas species other than T. rubrum were recognized in 10 (8 %) samples. In total, the combination of culture and PCR identified 132 (31.6 %) dermatophyte-positive samples, including 122 T. rubrum and 10 dermatophytes other than T. rubrum. The variety of combinations between conventional methods and PCR results are presented in Table 1.

The presented data show that implementation of PCR increases detection of dermatophytes by 21.1 % and leads
to a threefold increase as compared to culture alone (10.5 % by culture vs 30.1 % by PCR, $P<0.001$; and 10.5 % by culture vs 31.6 % by culture and/or PCR, $P<0.001$). Similarly, culture by itself identified 33.3 % (44/132) of laboratory-verified cases (by culture and/or PCR) of tinea unguium, whereas PCR alone identified 95.5 % (126/132).

As shown in Table 2, *T. rubrum* was isolated by culture in six cases where PCR was negative. In these cases, the internal control was positive excluding the presence of PCR inhibitors.

The sensitivity, specificity, NPV, PPV, LR$^+$, LR$^-$, DOR and NND, with their 95 % CIs for PCR, culture and direct microscopy, are shown in Table 3. By using conventional methods as the gold standard, PCR demonstrated sensitivity up to 85.3 %, higher than culture (64.7 %), whereas specificity was 80.6 %. The NPV was excellent (96.6 %) and 1.52 samples were adequate to achieve onychomycosis diagnosis. However, the PPV was low (46 %), as positive PCR results were considered as false positives in cases where dermatophytes did not grow in culture. By using the combination of PCR and culture as the gold standard, PCR was superior exhibiting sensitivity values up to 95.4 %, whereas culture and direct microscopy reached 33.3 and 44.7 %, respectively. Specificity values and PPVs were 100 % for PCR and culture as these two tests were considered as the gold standard, while the respective values were 98.6 and 93.65 % for direct microscopy. PCR performance was excellent in all diagnostic indices tested.

Finally, growth of yeasts and non-dermatophyte moulds was observed in 47 (11.2 %) samples (Table 2). In four (1 %) additional cases, mixed dermatophyte–non-dermatophyte cultures were obtained (in three cases *T. rubrum* and non-albicans Candida and in one case *T. rubrum* and *Penicillium*). *Candida* spp., predominantly non-albicans Candida (81.5 %), were isolated from 27 (6.5 %) samples, whereas non-dermatophyte moulds, such as *Acremonium*, *Alternaria*, *Aspergillus*, *Penicillium* and *Scopulariopsis* spp., were recovered in 24 (5.7 %) of them. PCR was negative in 38 out of 51 (74.5 %) non-dermatophyte mould, yeast and mixed positive cultures (Table 2). Among the remaining 13 (25.5 %)
PCR-positive samples, non-dermatophyte cultures were obtained in 10 (19.6 %), whereas 3 cases concerned mixed *T. rubrum*–non-dermatophyte-positive cultures.

**DISCUSSION**

Results indicate that incorporation of multiplex PCR techniques in routine laboratory processing of nail scrapings not only augments detection of dermatophytes, but also, in the vast majority of cases, identifies the causative agent. In another study, employment of PCR increased detection of *T. rubrum* in nail scrapings by approximately 20 %, whereas positive *T. rubrum* culture but negative PCR was found in 3 % of samples (Brasch et al., 2011). In addition, the specific commercial PCR, used in the present study, was evaluated in four other studies also (Brillowska-Dabrowska et al., 2007, 2010; Chandran et al., 2013; Kondori et al., 2010). These studies outline the importance of the dermatophyte PCR kit use as it increases detection rates by 10 to 19.5 %, as compared to cultures alone. The amount of extracted DNA is also an important parameter determining sensitivity of PCR and has been investigated (Bontems et al., 2009). A technical difficulty of PCR is the determination of the appropriate quantity of sample that will reassure a positive result. Different recommended amounts varying from 1–2 to 30–100 mg are mentioned in the literature (Bontems et al., 2009; Litz & Cavagnolo, 2010). In our study, the use of the dermatophyte PCR kit was evaluated in a larger number of samples (418 specimens), with a fixed large amount of sample (30–33 mg per sample). PCR performed better as compared to culture alone or the combination of conventional methods by detecting 19.6 and 13.8 % additional dermatophyte-positive samples, respectively.

Diagnostic indices for PCR were estimated by two approaches. The rationale for the above was that despite the fact that conventional methods are generally acknowledged as the gold standard for the diagnosis of dermatophytosis, this approach has raised scepticism among researchers (Gräser et al., 2012; Jensen & Arendrup, 2012). PCR assays have demonstrated higher sensitivity and/or specificity than conventional 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; Paugam et al., 2013). In the present study, PCR was found to be far more sensitive for detection of dermatophytes than conventional methods. Thus, an alternative approach by use of a combination of PCR and culture dermatophyte-positive results as the gold standard stands for a more realistic assessment of true onychomycosis cases. Similar approaches have been implemented by other researchers (Garg et al., 2007; Rothmund et al., 2013). Low PCR diagnostic indices such as PPV, likelihood ratios and specificity, at a lesser extent, by the use of conventional methods as the gold standard can be explained by the lower number of onychomycosis cases identified by culture and/or direct microscopy.
as compared to culture. The only way to increase the positivity rate of a low sensitivity test, such as culture, is to increase the number of analysed samples (Paugam et al., 2013).

Despite positive PCR, negative culture results (88 cases or 21% of nail samples) may be explained because of entrapment of fungus in the keratin; the PCR extraction step facilitates overcoming this handicap. In addition, previous treatments, such as local antifungal agents, may lead to non-viable fungi unable to grow on culture media (Brasch et al., 2011; Mehlig et al., 2014). In our study, use of antifungals was an exclusion criterion and cannot explain the low sensitivity of culture. Nevertheless, PCR will detect non-viable cells with intact nucleic acid (Bergman et al., 2013). Finally, overgrowth of non-dermatophyte moulds in the culture medium may prevent or obscure development of a pathogen (Bontems et al., 2009). Negative PCR results despite a positive culture, although rare (6 cases, 1.4% of nail samples), may be explained by an imbalanced distribution of fungal elements within different parts of a sample leading to an insufficient amount of DNA within the material used (Paugam et al., 2013). Alternatively, the presence of PCR-inhibitory substances may be involved (Bergman et al., 2013; Brasch et al., 2011; Mehlig et al., 2014). The presence of PCR-inhibitory substances is excluded in our study since internal control was positive in all cases. A limitation inherent to this multiplex PCR assay is that mixed dermatophyte infections including T. rubrum cannot be differentiated from infections with T. rubrum alone. Mixed infections are relatively rare, but may occur in ~3% of cases (Paugam et al., 2013) and this might have negative consequences on the assessment of dermatophyte epidemiology. The collection of at least 90–100 mg of nail sample from each patient as an inclusion criterion requires an advanced stage onychomycosis and results could be different in patients at an early stage and/or with a limited form of the disease.

Although non-dermatophyte filamentous fungi can be the aetiological agent of onychomycosis, detection in nail specimens may be attributed to contamination, transient colonization and infection of a traumatized or otherwise diseased nail, mixed infection and persistence after cure of the dermatophyte or even contamination in the laboratory; thus, repeated recovery is often required before a pathogenic role is considered (Bontems et al., 2009; Brillowska-Dabrowska et al., 2007; Mehlig et al., 2014). The low number of colonies in cases where Aspergillus, Penicillium, Scopulariopsis, Acremonium and Alternaria were observed, along with the fact that direct microscopy was negative and they were not isolated in a subsequent sample, enhanced their role as contaminants. Our data show that in ten samples where culture yielded growth of non-dermatophyte moulds and yeasts, PCR was positive for T. rubrum (seven) and other dermatophytes (three). In a previous study, the specificity of PCR was assessed by using 21 strains of non-dermatophyte fungi, including Aspergillus niger and yeasts. In all cases, no PCR products were detected (Brillowska-Dabrowska et al., 2007). This is reinforced by our study, where PCR was negative in 38 non-dermatophyte mould and yeast positive cultures.

Indisputably, culture is necessary for identification of dermatophytes other than T. rubrum or even non-dermatophyte moulds that may be involved in nail infections (Paugam et al., 2013). However, the low sensitivity of culture in the diagnosis of onychomycosis is commonly accepted (Litz & Cavagnolo, 2010; Mehlig et al., 2014). However, direct microscopy, especially with the addition of fluorochrome and periodic acid–Schiff staining (Gupta et al., 2008; Idriss et al., 2013; Litz & Cavagnolo, 2010), has higher sensitivity but lacks specificity as it cannot provide identification of species or even at the genus level and does not differentiate unquestionably between dermatophytes and other moulds (Bontems et al., 2009; Brillowska-Dabrowska et al., 2007; Garg et al., 2007; Jensen & Arendrup, 2012; Mehlig et al., 2014). Taking into account the low sensitivity of culture-based diagnosis in tinea unguium in combination with the long turnaround time for growth, as well as the requirement of experienced personnel, we strongly recommend application of complementary methods. PCR technology offers rapid results, as well as high sensitivity and specificity. Hence, a number of PCR-based strategies have been developed to identify dermatophytes directly from clinical specimens. Along with conventional or end-point PCR methods, real-time PCR assays, as well as the implementation of post-PCR-techniques, have become available. Conventional PCRs benefit from simplicity and lower cost, and target the identification of the most common dermatophyte, T. rubrum (Brasch et al., 2011), or pan-dermatophytes (Dhib et al., 2012; Luk et al., 2012). By means of a multiplex PCR, simultaneous identification of T. rubrum and pan-dermatophytes (Brillowska-Dabrowska et al., 2007, 2010; Kondori et al., 2010; Chandran et al., 2013; the present study) or T. rubrum, T. mentagrophytes and pan-dermatophytes (Dhib et al., 2014) is achieved. Moreover, Kim et al. (2011) identified nine different dermatophyte species, and Mehlig et al. (2014) identified an assortment of dermatophytes along with non-dermatophyte moulds (Scopulariopsis brevicaulis, Aspergillus spp.) and yeasts (Candida spp.), whereas Li et al. (2011) distinguished the groups of pathogens implicated in onychomycosis (dermatophytes, yeasts and moulds). In a recent work, the primers adopted by the commercial PCR kit that is evaluated in the present study were used in two separate PCRs for the detection of pan-dermatophytes and specific detection of T. rubrum, supplemented with a third PCR for T. interdigitale (Dubljanin et al., 2014). However, real-time PCR is less laborious, may enable a broader spectrum of simultaneous species detection and as a closed system reduces contamination risk (Alexander et al., 2011; Bergman et al., 2013; Bergmans et al., 2010; Miyajima et al., 2013; Paugam et al., 2013; Wisselink et al., 2011). Finally, post-PCR strategies, e.g. nested PCR (Verrier et al., 2013), PCR-ELISA (Beifuss et al., 2011; Panekwitz et al., 2013), PCR-RFLP (Elavarasi et al., 2013; Ghannoum et al., 2013; Verrier et al., 2012) and DNA microarray assays (Sato et al., 2010), may increase the number of species identified;
however, they prolong the turnaround time (Jensen & Arendrup, 2012). In our settings, specific detection of *T. rubrum* in parallel with pan-dermatophyte primers is sufficient as the vast majority of positive samples were identified as *T. rubrum* [40 out of 44 (91 %)] by culture, and 122 out of 132 (92.4 %) by the combination of culture and PCR. Similar results were obtained in recent studies (Bergman et al., 2013; Verrier et al., 2012). Nevertheless, dermatophytes belonging to the less terbinafine-susceptible genus *Microsporum* are unanimously reported to be very rare agents of onychomycosis. Therefore, detection of DNA of dermatophytes other than *T. rubrum* in a nail specimen by the pan-dermatophyte primers will probably represent infection with a terbinafine-susceptible dermatophyte and will provide sufficient information to guide the clinician, despite a lack of species identification (Brillowska-Dabrowska et al., 2007).

In conclusion, by using a large number of samples, and a fixed amount of specimen, we have demonstrated that application of multiplex PCR in clinical settings provides rapid results with high sensitivity and specificity, augmenting laboratory assistance to clinical evaluation of tinea unguium and saving the patient from delay in initiating an appropriate treatment.

REFERENCES


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