Pathology (June 2020) **52(4)**, pp. 473–477

MICROBIOLOGY

Detection and identification of dermatophyte fungi in clinical samples using a commercial multiplex tandem PCR assay

IAN L. ROSS, GERHARD F. WELDHAGEN, SARAH E. KIDD

National Mycology Reference Centre, Microbiology and Infectious Diseases, SA Pathology, Adelaide, SA, Australia

Summary

We evaluated the performance of a commercial multiplex tandem polymerase chain reaction (PCR) for detection of dermatophytes and other fungi in skin and nail specimens by (1) testing a range of fungal and bacterial reference cultures, (2) retrospectively testing a set of skin and nail specimens with known microscopy and culture results, and (3) prospectively testing skin and nail specimens in parallel to microscopy and culture. The AusDiagnostics Dermatophytes and Other Fungi assay accurately detected and identified a range of common dermatophytes to species, species complex or genus level, as well as *Candida*, *Aspergillus* and *Scopulariopsis* spp. It was unable to detect uncommon dermatophytes such as *Nannizzia fulva* (previously *Microsporum fulvum*), and *Paraphyton cookei* (previously *Microsporum cookei*). PCR identified a dermatophyte in 25.9% of prospective specimens which were culture negative. Sensitivity, specificity, positive predictive value, and negative predictive value were highest where microscopy and PCR results were combined, versus microscopy and culture combined, which highlights the significant contribution of microscopy in the diagnostic pathway. This assay has the potential to reduce the workload and results turnaround time associated with culturing and identification of dermatophytes, although microscopy remains important.

Key words: Dermatophyte PCR; *Trichophyton*; *Microsporum*; *Nannizzia*; *Epidermophyton*.

Received 2 January, revised 5 March, accepted 17 March 2020
Available online 16 April 2020

INTRODUCTION

Dermatophyte fungi are a common cause of skin, nail and hair infections globally, ranging from mild to debilitating in nature. The overall prevalence of dermatophytosis is around 14%;[1] the most common presentations are tinea pedis and onychomycosis, which have increased prevalence in athletes, soldiers, those with diabetes mellitus, and the elderly.[2,3] While the most common aetiological agents are *Trichophyton rubrum* and *T. interdigitale*,[1,2] dermatophytes belonging to the genera *Trichophyton*, *Epidermophyton, Microsporum*, and *Nannizzia*, *Paraphyton* and *Lophophyton* can cause infection,[4] and may be anthropophilic, zoophilic or geophilic in nature. Onychomycosis caused by non-dermatophyte moulds such as *Scopulariopsis* spp, *Aspergillus* spp., *Fusarium* spp., and *Acremonium* spp. may also occur but the diagnosis of these infections is complicated by the causative fungi also being common environmental contaminants.[5,6]

Specimens requiring fungal microscopy and culture for suspected dermatophyte infection make up approximately 40% of the workload in our laboratory. Whilst still considered the gold standard, this traditional method is labour intensive, has poor sensitivity,[7] requires up to 4 weeks for fungal growth, and significant expertise for identification of the fungi. Molecular diagnostics have the potential to improve sensitivity, reduce labour requirements and decrease test result turnaround times.

We compared the performance of a Therapeutic Goods Administration (TGA) approved, commercial, semi-automated real-time multiplex tandem polymerase chain reaction (PCR) (Dermatophytes and Other Fungi, 12-Well; AusDiagnostics, Australia) to that of microscopy and culture for the detection and identification of dermatophytes from skin, nail and hair specimens. This test utilises a multiplex tandem polymerase chain reaction (MT-PCR) for the amplification of DNA from seven dermatophyte targets [*Trichophyton* spp., *Trichophyton rubrum* complex including *T. rubrum* and *T. soudanense*, *Trichophyton mentagrophytes* complex including *T. mentagrophytes* and *T. interdigitale, Microsporum* spp., *M. canis*, *Nannizzia gypsea* (previously *M. gypseum*), and *Epidermophyton floccosum*]; four non-dermatophyte targets [*Scopulariopsis* spp., *Aspergillus* spp. including *A. fumigatus, A. clavatus, A. giganteus, A. niger* and *A. flavus*, *Candida* (differentiates *C. albicans* and *Meyerozyma guilliermondii*, previously *Candida guilliermondii*), and *Candida* 2 (differentiates *C. parapsilosis* complex and *C. glabrata*)]; and finally, an artificial assay control target to detect inhibition.[8]

MATERIALS AND METHODS

Study design

This study comprised three phases, conducted from October 2018 to August 2019:

1. Specificity test using pre-identified dermatophyte cultures including common and rare species, common fungal skin flora or contaminants, and common skin bacteria species.
2. Testing of stored specimens with comparison to microscopy and culture results. These specimens were heavily biased towards positive specimens, with 70% having microscopy results consistent with dermatophyte hyphae, and 13% having microscopy consistent with yeast forms.
3. Prospective clinical specimen testing with comparison to microscopy and culture results, conducted between February and August 2019.

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2020.03.002

Reference cultures

A range of reference or pre-identified dermatophyte species (n=40), other fungal genera (n=11) and bacterial species (n=6) (Supplementary Table 1, Appendix A) stored in the National Mycology Reference Centre culture collection were revived onto Sabouraud's dextrose agar (fungi) or blood agar (bacteria) as appropriate, and sub-cultured again prior to use. Sequencing of the ITS1-5.8S-ITS2 ribosomal DNA was performed as required to confirm the identification of fungal isolates.[9]

Clinical specimens

Skin scrapings, nails and hair specimens that were submitted to SA Pathology for fungal microscopy and culture were tested with the Dermatophyte PCR either retrospectively (n=127) or prospectively (n=272) in parallel. Those specimens that were tested retrospectively were selected largely on the basis of positive direct microscopies indicating the specimen might be expected to yield positive cultures and/or PCR results. These were stored at 4°C for up to 4 weeks following microscopy and culture, before being used for dermatophyte PCR.

Fungal microscopy

Specimens were digested in 10% potassium hydroxide (KOH) for at least 6 hours or overnight, and visualised with calcofluor white stain using a UV microscope to identify fungal elements, which were classified as fungal hyphae resembling a dermatophyte (thin septate hyphae breaking up into arthroconidia), fungal hyphae (septate or non-septate branching hyphae), budding yeast cells with or without pseudohyphae, or broad-based budding yeast cells with pseudohyphae resembling *Malassezia furfur*.

Fungal culture

Each specimen was cultured onto lactritmel 'Honey Milk' agar containing chloramphenicol, gentamicin and cycloheximide (Edwards Group, Australia), and Sabouraud's dextrose agar containing chloramphenicol and gentamicin (Edwards Group). Nails were manually scraped and pared to expose the infected portions of the specimen prior to inoculation. Cultures were incubated at 26°C for up to 4 weeks and checked weekly for fungal growth. Identification of dermatophytes was performed by microscopic and colony morphologies.[10] ITS sequencing was also performed as required, resolving any uncertainty in species identification.

Dermatophyte PCR

The AusDiagnostics Dermatophytes and Other Fungi MT-PCR was performed according to the manufacturer's protocols.[8] This included a pre-extraction step where a small portion of specimen (ca. 2 mm × 2 mm, as recommended) was placed in lysis solution [300 µL sterile phosphate buffered saline, 180 µL, MagNA Pure 96 Bacterial Lysis buffer (Roche Diagnostics, Australia), 20 µL recombinant grade Proteinase K (19 mg/mL; Sigma-Aldrich, USA)] and incubated at 65°C for 30 min. After centrifugation, 200 µL of the supernatant was used for DNA extraction. Automated DNA extraction was performed using the MagNA Pure 96 DNA and Viral RNA Small Volume kit (Roche), following the Universal Pathogens Small Volume 200 protocol (Roche).

Extracted DNA, and Step 1 PCR kit components including master mixes were assembled on the High Plex 24 PCR processor (AusDiagnostics) and run according to the AusDiagnostics defined 'REF 24115 Ver:01 Dermatophytes and Other Fungi' proprietary step 1 program.[8] This program specifies 10 µL DNA, robotic sampling, and 15 cycles of PCR. The step 2 PCR plate was run according to the AusDiagnostics defined 'Ver:01 Dermatophytes and Other Fungi' step 2 program on a LightCycler 480 (Roche) instrument.

PCR results were analysed using AusDiagnostics MT Analysis software, which displays the cycling curves and the melt curves of each run. Sensitivity was set to normal (required for diagnostic use) and on the basis of these vendor-defined parameters, targets were interpreted as 'Present', or 'Not Detected'.

A negative control of lysis solution extracted in the absence of specimen was included in each dermatophyte PCR run. In addition, a full run of 24 negative samples was run through the process of pre-extraction, extraction, and PCR to assess for the presence of background fungal DNA in reagents or from environmental sources.

Statistical analysis

The analytical sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the dermatophyte PCR were calculated using the web-based statistical package VassarStats (http://vassarstats.net/), on the basis of results obtained from the prospective phase of the study. These were assessed using three different combinations of tests and standards: PCR versus culture, PCR versus microscopy and culture, and microscopy and culture versus microscopy and PCR.

Ethical approval

This study was approved by the Central Adelaide Local Health Network (CALHN) Research Services (CAHLN reference number 12508).

RESULTS

PCR specificity

Using a panel of pre-identified cultures, the Dermatophyte PCR detected all isolates of a range of common and uncommon dermatophyte species including *T. rubrum* (n=6), *T. soudanense* (n=3), *Trichophyton violaceum* (n=4), *Trichophyton gourvilii* (n=1), *Trichophyton tonsurans* (n=4), *Trichophyton interdigitale* (n=1), *T. mentagrophytes* (n=4), *Trichophyton quinckeanum* (n=1), *Trichophyton verrucosum* (n=1), *Arthroderma vanbreuseghemii* (n=1), *E. floccosum* (n=3), *N. gypsea* (n=2), *Microsporum audouinii* (n=2), and *M. canis* (n=2). *Trichophyton gourvilii*, *T. violaceum*, and *T. soudanense* were identified as *T. rubrum complex*; *T. interdigitale* and *A. vanbreuseghemii* identified as *T. mentagrophytes* complex; and *M. audouinii* identified as *M. canis*. *Trichophyton tonsurans, T. quinckeanum, T. equinum, T. erinacei, T. verrucosum* were identified as *Trichophyton* spp.

The assay did not detect *Trichophyton terrestre, Microsporum racemosum, Paraphyton cookei, Nannizzia persicolor, Nannizzia nana* or *Nannizzia fulva* as the assay contains no targets designed for this purpose. There was no cross-reaction with *Fusarium*, *Penicillium*, or bacterial species (Supplementary Table 1, Appendix A). A complete run of 24 negative samples showed no amplification of fungal targets.

Dermatophyte PCR of stored specimens

A total of 127 stored skin scrapings (n=31), nail scrapings or clippings (n=95) and hair specimens (n=1) were tested retrospectively. Direct microscopies were positive for fungal hyphae in 92 (72.5%) and yeast forms in 13 (10.2%), and negative for 22 (17.3%). Dermatophyte PCR results were in agreement with positive or negative culture results for 89 (70.1%) of specimens tested retrospectively. Thirty-six (28.3%) specimens were microscopy positive but culture negative, and positive by dermatophyte PCR, suggesting superior sensitivity of the PCR compared to culture. For two (1.6%) specimens that grew dermatophytes in culture, the PCR was negative.

Dermatophyte PCR of prospectively tested clinical specimens

A total of 272 samples from 263 patients, comprising skin scrapings (n=81), and nail scrapings or clippings (n=191) were tested prospectively in parallel to routine fungal microscopy and culture. No hair samples were received for testing during the prospective phase of the study. Of these,

143 (52.6%) were positive for hyphal or yeast forms on direct microscopy, 86 (31.6%) were positive in culture for fungi detected by this PCR (dermatophytes, *Candida*, *Aspergillus*, *Scopulariopsis*), where 66 (24.3%) were positive on both microscopy and culture.

A comparison of microscopy, culture and PCR results is summarised in Table 1; yeast and non-dermatophyte mould related results (n=59) were excluded from this comparison as these are common skin flora and/or contaminants, and usually not clinically significant. There was agreement between the dermatophyte culture result and the dermatophyte PCR result (including negatives) in 156 (73.2%) of 213 specimens. The PCR identified a dermatophyte consistent with microscopy results but with negative culture in 44 (20.7%) specimens, indicating superiority of the PCR over culture in these cases, and a further 5.16% PCR positive for a dermatophyte where both culture and microscopy were negative. However, two specimens (0.94%) grew a dermatophyte in culture (*T. rubrum* and *T. interdigitale*) that were not detected by PCR, and 11 (5.16%) were both PCR and culture negative but with fungal hyphae observed on microscopy.

In one specimen, which was microscopy positive (fungal hyphae resembling a dermatophyte) with growth of *T. rubrum* in culture, the *Trichophyton* spp. target amplified, but not the *T. rubrum* complex target.

The sensitivity, specificity, PPV and NPV of the dermatophyte PCR were calculated with three combinations of test positivity and standard (Table 2). PCR versus culture alone had a high sensitivity (0.965) and NPV (0.980), but poor specificity (0.647) and PPV (0.500). PCR versus microscopy and culture improved the specificity and PPV (0.891 and 0.900, respectively), but all four test characteristics were highest for microscopy and PCR versus microscopy and culture.

Fifty specimens, predominantly skin scrapings and fingernails, yielded results consistent with yeast infection or colonisation. Eighteen (45%) of these had budding yeast forms on microscopy, and 23 (46%) grew yeast in culture. The PCR assay was positive for *Candida* species in 22 of the 23 specimens with yeast on culture, and positive for a further 13 that were microscopy positive but culture negative. Nine of the 50 (18%) specimens had microscopy consistent with *Malassezia furfur*, and were negative on culture and PCR, as expected.

Eleven specimens were positive for *Scopulariopsis* or *Aspergillus* spp. on PCR. Of these, seven were consistent with microscopy results (fungal hyphae not typical of a dermatophyte), and eight were consistent with culture.

DISCUSSION

The AusDiagnostics dermatophyte PCR assay detected all dermatophytes that are commonly isolated in Australia (including *T. rubrum, T. interdigitale, T. mentagrophytes, T. violaceum, N. gypsea, M. canis*), and did not cross-react with other fungal or bacterial organisms that may be found as skin flora or saprophytes. The most commonly identified dermatophytes were *T. rubrum* complex and *T. mentagrophytes* complex, which reflects the global epidemiology.[2] The assay did not detect some of the less common dermatophyte species (e.g., *T. terrestre, M. racemosum, P. cookei, N. persicolor, N. nana,* and *N. fulva*). It should be noted that none of these species were grown in culture amongst the stored or prospectively tested specimens during the validation period, and none have been deposited into the National Mycology Reference Centre culture collection during the past 5 years.

A shortcoming of the 12 target assay is that some dermatophyte species are only identifiable to genus or species complex level, whereas with microscopy and culture we typically report dermatophytes to species level, along with comments regarding the anthropophilic, zoophilic or geophilic nature of that species. This level of detail would not be possible using the PCR assay alone (e.g., *T. tonsurans*, commonly isolated from scalp scrapings, is only identifiable as *Trichophyton* spp.), although the consequences of this loss of detail are limited to observational and epidemiology issues and would not affect the clinical management of infection. Presumably, part of the reason for this assay design was to minimise costs for what is intended to be a commercially viable test for routine diagnostic use, as well as an issue of

Table 1 Summary of 213 dermatophyte PCR results obtained from prospectively tested clinical samples, with microscopy and/or culture results consistent with dermatophyte infection

Dermatophyte microscopy	Dermatophyte culture	N	Dermatophyte PCR targets detected							
			Trichophyton spp.	*Trichophyton rubrum* complex	*Trichophyton mentagrophytes* complex	*Nannizzia gypsea*	*Microsporum* spp.	*Microsporum canis*	*Epidermophyton floccosum*	Spike only (negative)
Skin samples (n=57)										
Positive	Positive	17	1[a]	9	5			2		
Positive	Negative	4		2				1		1
Negative	Positive	3	3[a]							
Negative	Negative	33								33
Subtotal		57	4	11	5	0	0	3	0	34
Nail samples (n=156)										
Positive	Positive	33	1[b]	27	3				1	1
Positive	Negative	51		30	10				1	10
Negative	Positive	4	1[a]		2					1
Negative	Negative	68		5	6					57
Subtotal		156	2	62	21	0	0	0	2	69
Total		213	6	73	26	0	0	3	2	103

Specimens with microscopy and culture results suggestive of yeast or non-dermatophyte onychomycosis were excluded.
Dermatophyte cultures corresponded to [a]*T. tonsurans* and [b]*T. rubrum.*

Table 2 Sensitivity, specificity, positive and negative predictive values of dermatophyte PCR using different reference standards

Test	PCR	PCR	Microscopy and PCR
Standard	Culture	Microscopy and culture	Microscopy and culture
Sensitivity	0.965	0.884	0.991
Specificity	0.647	0.891	0.891
PPV	0.500	0.900	0.910
NPV	0.980	0.874	0.989

NPV, negative predictive value; PPV, positive predictive value.

differentiating species which often have very high genetic similarity.[4]

In the testing of both stored specimens and prospective specimens, the PCR was negative for two specimens from each set where a dermatophyte was isolated in culture. In all four cases, specimen quantity was 'scant' and hyphal distribution is rarely homogenous throughout the specimen; therefore, there may have been insufficient fungal DNA, subject to the limit of detection of these targets. There is a further possibility that these PCR results may have been negative due to the fungi representing unusual sequence variants that could not be amplified by the PCR. The PCR was also negative in a further 11 culture negative specimens that had fungal hyphae seen on microscopy; again these results may be partly attributed to sampling artefact.

For one specimen that grew *T. rubrum* in culture, the *Trichophyton* spp. target but not the *T. rubrum* complex target amplified on PCR. However, when the corresponding fungal isolate was put through the assay, the result indicated *T. rubrum*, indicating that this discordance is related to sensitivity rather than specificity. The limit of detection of the *Trichophyton* spp. target is lower than that of the *T. rubrum* complex target (8–12 copies/10 µL versus 33–41 copies/10 µL, respectively);[8] therefore, *T. rubrum* DNA present in low concentrations may be detected as *Trichophyton* spp., rather than *T. rubrum* complex. While we did not observe it during our study, theoretically, the same could occur with the *T. mentagrophytes* complex target (limit of detection 36–44 copies/10 µL). Again, this issue is unlikely to affect the clinical management of infection.

The high sensitivity and negative predictive values make this assay a useful screening tool for dermatophyte infections with typical clinical presentation. The higher sensitivity of the PCR compared to culture was demonstrated by 55 specimens that were PCR positive for a dermatophyte, but culture negative (n=44), or microscopy and culture negative (n=11). This may in part be attributed to detection of dermatophytes that would otherwise have been masked by saprophytes in culture, and detection of non-viable fungal elements. A similar study of the same assay has found comparable results.[11] Sensitivity, specificity, PPV, and NPV were all highest when microscopy and PCR positivity was tested against the standard of microscopy and culture (Table 2). This highlights the important role of microscopy in the diagnosis of dermatophyte infections.

Hands on time for staff is greatly reduced for the PCR compared to culture, since the specimen preparation is reduced (e.g., scraping and paring of nails is not required), there is no longer the need to monitor cultures for fungal growth, and no need for morphological identification of fungi. Overall turnaround time of specimens in the laboratory is reduced from ~4 weeks to approximately 2–5 days, depending on workflow, which would reduce the time to commencement of treatment.

In addition to dermatophyte testing some *Aspergillus* and *Scopulariopsis* species were detected in culture and PCR, demonstrating potential utility of these targets for diagnosis of non-dermatophyte onychomycosis (NDO).[5,12] However, given that they are also common saprophytes and environmental contaminants, we would not consider reporting these fungi without a supporting microscopy result (fungal hyphae not resembling a dermatophyte), and would recommend repeat testing in line with guidelines.[3] Additionally, the AusDiagnostics assay does not detect other causes of NDO such as *Fusarium* spp., *Acremonium* spp., or *Neoscytalidium dimidiatum*, which limits the role of this assay for diagnosis of NDO.

The *Candida* targets that reliably detect *C. albicans, C. parapsilosis* complex, *C. glabrata* and *Meyerozyma (Candida) guilliermondii* are also potentially useful for reporting yeast infections of the skin and fingernails. However, given that *Candida* species are common skin flora, some care in determining their clinical importance would be required to determine whether or not to report, including direct microscopy result, site of infection, and underlying conditions. In this context, and considering the inability of the PCR to detect pityriasis versicolor caused by *Malassezia furfur*, direct microscopy of skin and nails remains relevant to laboratory practice.

Due to current Australian pharmaceutical benefit scheme (PBS) requirements, which require a positive microscopy or a positive culture result to authorise prescribing oral terbinafine for onychomycosis, some reflex testing is required for microscopy negative, PCR positive nail specimens.[13] Based upon our prospective data, this would affect 11/213 (5.16%) specimens. Given that the PBS guidelines for terbinafine prescribing have not been updated since November 2007 and the significant advances in technology for diagnosis of dermatophyte infection since that time, a review of PBS guidelines is warranted.

CONCLUSIONS

The AusDiagnostics Dermatophytes and Other Fungi PCR is a highly sensitive assay with good specificity and high negative predictive value, making it a useful screening tool for dermatophyte infection and a suitable alternative to fungal culture for skin, nail and hair specimens. Non-dermatophyte

targets should be interpreted within the context of microscopy results and the site of infection. Using the PCR, staffing requirements and specimen turnaround time would be significantly reduced as compared to culture methods.

Acknowledgements: The authors thank staff in the mycology and molecular laboratories at SA Pathology for assistance with specimen preparation.

Conflicts of interest and sources of funding: All components of the AusDiagnostics Dermatophytes and Other Fungi PCR assay were supplied free of charge by the manufacturer for the purposes of validating the assay. AusDiagnostics personnel have not reviewed the results of this study or had any role in preparing the manuscript. The authors state that there are no conflicts of interest to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2020.03.002.

Address for correspondence: Dr Sarah E. Kidd, National Mycology Reference Centre, SA Pathology, PO Box 14, Rundle Mall, SA 5000, Australia. E-mail: sarah.kidd@sa.gov.au

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