ORIGINAL ARTICLE

# Comparison of fungal fluorescent staining and ITS rDNA PCR-based sequencing with conventional methods for the diagnosis of onychomycosis

F. Bao,[1,2,3] Y. Fan,[4] L. Sun,[2,3] Y. Yu,[2,3] Z. Wang,[2,3] Q. Pan,[2,3] C. Yu,[1,2,5] H. Liu,[1,2,3] F. Zhang[1,2,3,5,*]

[1]Shandong Provincial Hospital for Skin Diseases, Shandong University, Jinan, Shandong, China
[2]Shandong Provincial Institute of Dermatology and Venereology, Shandong Academy of Medical Sciences, Jinan, Shandong, China
[3]Shandong Provincial Key Lab for Dermatovenereology, Jinan, Shandong, China
[4]Department of Toxicological and Functional Test, Shandong Centers for Disease Control and Prevention, Jinan, Shandong, China
[5]Shandong Provincial Medical Center for Dermatovenereology, Jinan, Shandong, China
*Correspondence: F. Zhang. E-mail: zhangfuren@hotmail.com

## Abstract

**Background**  The current gold standard for diagnosing onychomycosis is direct microscopic examination and culturing. Fungal culture is a time-consuming procedure, while direct microscopy of potassium hydroxide (KOH) mounts suffers from low sensitivity. More rapid and sensitive methods for the diagnosis of onychomycosis are in high demand.

**Objective**  To establish an effective method for the diagnosis of onychomycosis by assessing the efficacies of fungal fluorescent staining and internal transcribed spacer (ITS) ribosomal DNA (rDNA) polymerase chain reaction (PCR)-based sequencing.

**Methods**  A total of 204 clinical specimens from patients with suspected onychomycosis were analysed. The gold standard for a true positive sample was positive by KOH, culturing or both methods. All specimens were also tested by fungal fluorescent staining and ITS rDNA PCR-based sequencing. We compared the detection, sensitivity and specificity for these two methods with conventional methods.

**Results**  In total, 126 (62%) and 102 (50%) were detected by fluorescent staining and PCR-based sequencing, respectively. According to the conventional diagnostic standard, the sensitivity of fluorescent staining and PCR-based sequencing was 97% and 78%, respectively, and specificities of 89% and 90%, respectively. Use of fluorescence enhanced the sensitivity of direct examination by 12% compared with KOH. PCR-based sequencing increased the sensitivity by 6% compared with culturing.

**Conclusions**  Fluorescence microscopy has a higher sensitivity for the detection of fungi in nail specimens compared with KOH and can be used as a rapid screening tool. PCR-based sequencing was faster and more sensitive compared with culture and when used in conjunction with fluorescence microscopy resulted in higher efficiency.

Received: 25 October 2017; Accepted: 22 January 2018

## Introduction

Onychomycosis is a nail disease caused by dermatophyte, yeast and mould infections of the nail plate. Its prevalence worldwide ranges from 10% to 30%.[1] Because onychomycosis requires long-term systemic antifungal treatment and different fungi may require different therapies, a confirmation of the aetiology and a precise mycological identification is necessary for treatment. Conventional diagnosis is based on direct microscopy [potassium hydroxide (KOH) mounts] of clinical specimens, followed by culture and morphological identification of the fungus. Direct

microscopy requires experienced investigators to identify the fungal elements and gives false-negative results in 5–15% of cases. Fungal culture is a time-consuming procedure and has a higher percentage of false negatives (30–50%).[2] Therefore, more rapid and sensitive methods for the identification of fungal species are needed.

In this context, molecular biological methods based on the polymerase chain reaction (PCR) have been developed to complement other methods of diagnosis. Most genetic studies of fungal pathogens have focused on the identification of cultured isolates. However, the direct application of PCR-based methods in clinical samples would allow early and specific diagnosis of fungal diseases and identification of the causative agents.[3,4] Meanwhile, prior studies have suggested that direct microscopy using fluorescence may be a sensitive technique, which could increase detection rates by 4–22% compared with KOH in the diagnosis of fungal infections.[5–7]

A limited number of studies have compared molecular diagnoses and conventional culture in the identification of clinical isolates for the diagnosis of onychomycosis, and most immunofluorescence studies have focused on deep fungal infections and the lack data of onychomycosis. Herein, we present the results of fungal fluorescent staining and internal transcribed spacer (ITS) ribosomal DNA (rDNA) PCR-based sequencing compared with conventional methods (microscopy of KOH mount and culture) for the diagnosis of onychomycosis.

## Materials and methods

### Patients and clinical specimens
A total of 204 nail specimens (107 toenail and 97 fingernail samples) from 187 patients with suspected onychomycosis were analysed. While the specimens were collected, informed consent was obtained based on the guidelines and agreements of the institutional ethical committee. All samples were collected by the same experienced mycologist. The specimen was obtained from the nail bed as close as possible to the advancing infected edge of the lesion. Patients who had used topical or systemic antifungal drugs within the previous 2 weeks were excluded from sampling. Specimens were evenly divided into four parts for examination by KOH microscopy, culturing, fungal fluorescent staining and PCR sequencing.

### Direct microscopy and culture
Direct microscopic examination was performed to assess the presence of fungal elements. The first nail material was tested by conventional methods with 10% KOH. The second portion of the nail samples was stained with fluorescent dyes and examined with fluorescence microscopy. A sample culture was performed in parallel on Sabouraud Dextrose Agar slants with and without cycloheximide, incubated at 25 °C for 4 weeks and checked periodically for growth. Isolates were identified at the species level by their macroscopic and microscopic appearances following lactophenol cotton blue staining.

### Molecular identification
Fungal DNA was extracted from nail samples using the Quick-DNA Fungal/Bacterial Miniprep Kit (D6005; Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The fungus-specific universal primers ITS1 (5′- TCCGTAGGT-GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA-TATGC-3′) were used to amplify the full ITS sequence,[8] and PCR was performed in a thermocycler (Veriti 96-Well Thermal Cycler; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. DNA sequencing was performed with 3500×L Dx. The sequencing results were evaluated using the nucleotide Basic Local Alignment Search Tool (BLAST) to determine the closest relatives on the NCBI website (http://www.ncbi.nlm.nih.gov). A way to circumvent this problem was to directly use the publicly available ITS sequence database at the Centraalbureau voor Schimmelcultures dermatophyte website (http://www.cbs. knaw.nl/dermatophytes/).

### Statistical analysis
KOH- and/or culture-positive samples were used as the gold standard to estimate the diagnostic sensitivity and specificity, as well as the negative and positive predictive values of each test. We identified three criteria for the diagnosis of non-dermatophyte mould onychomycosis: KOH positive, isolation in culture and dermatophyte exclusion.

## Results

### Clinical nail samples and participants
Of the 204 nail samples collected from 186 patients with suspected onychomycosis, 59% (120) were from females and 41% (84) from males. The ages ranged from 2 to 82 years, with a median age of 41 years. Distal and lateral subungual onychomycoses were the most prevalent type of clinical manifestation (73%), followed by superficial white onychomycosis (16%), total dystrophic onychomycosis (7%) and proximal subungual onychomycosis (4%) (Table 1).

### Detection of the four tests
Of the 204 nail samples, direct examination was positive in 103 samples (51%). Culturing was positive in 87 samples (43%). Direct examination and culturing were both positive in 69 samples (34%). Altogether, the combination of conventional methods revealed 121 (59%) positive specimens. Both direct microscopy and culturing were negative in 84 (41%) specimens. The detection percentage of fungal fluorescent staining and PCR-based sequencing was 62% and 50%, respectively. All conventional direct microscopy examination (KOH)-positive specimens were identified as positive using fungal fluorescent

| Clinical manifestation | Gender | | Total (*n* %) |
|---|---|---|---|
| | Male (*n*) | Female (*n*) | |
| DLSO | 53 | 95 | 148 (73%) |
| PSO | 3 | 6 | 9 (4%) |
| SWO | 18 | 14 | 32 (16%) |
| TDO | 10 | 5 | 15 (7%) |
| Total | 84 | 120 | 204 |

staining, and fungal fluorescent staining detected 23 specimens that were negative by KOH.

### Sensitivity, specificity and positive and negative predictive values

Conventional methods (KOH- and/or culture-positive) were used as the gold standard: 121 were positive and 84 were negative. We found the sensitivity of fungal fluorescent staining and ITS rDNA PCR-based sequencing to be 97% and 78%, respectively; specificities were 89% and 90%, respectively; positive predictive values were 93% and 92%, respectively; and negative predictive values were 95% and 74%, respectively. Use of fluorescence enhanced the sensitivity of direct examination by 12% compared with KOH. PCR-based sequencing increased the sensitivity by 6% compared with culturing. A comparison of all tested diagnostic methods for onychomycosis is shown in Table 2.

### Species identification of conventional cultures and PCR sequencing

Conventional cultures yielded pathogenic strains from 87 of the nail samples (41%), most of which were subsequently identified as dermatophytes (49 *T. rubrum*). The 38 remaining isolates were identified as non-dermatophytes (27 *Candida* species and 11 non-dermatophyte moulds). Of the 204 nail samples, 102 (50%) tested positive with PCR sequencing (66 *T. rubrum*, 28 *Candida* species and eight non-dermatophyte moulds) (Table 3). In total, 78 specimens were positive for both fungal culturing and PCR sequencing and yielded similar results. In 24 additional samples, which were culture negative, a positive

sequence was obtained: *T. rubrum* (18 samples), *Candida* species (five samples) and non-dermatophyte moulds (one sample).

### Discussion

Onychomycosis constitutes approximately half of all nail abnormalities that may have both psychosocial and economic effects.[9] Treatment success depends on accurate and rapid diagnosis of the pathogen. However, classic mycological diagnosis was an imperfect gold standard, whereas direct examination lacks specificity and culturing is frequently associated with weak sensitivity and false-negative results. The diagnostic accuracy of the KOH test and fungal culturing varies from 50% to 70%.[10] Improvement of traditional diagnostic methods and the development of new techniques will allow physicians to be more precise in the identification of pathogens causing nail infections.

In this study, we compared microscopic results using two different methods: KOH and fluorescence. Although KOH is rapid and economical, it requires sufficient experience to identify the fungal elements and gives false-negative results in 5–15% of cases.[11] Fluorescence offers the advantages of high sensitivity and specificity because it observes fungal elements and budding patterns more easily, especially for the detection of rare hyphae and spores, and the percentage of false-negative results is low compared with the culture method of diagnosis.[6,12] In this study, 103 (51%) positive results were found in nail samples using KOH, whereas 23 (11%) additional samples were detected with the use of fluorescence. Our results are consistent with previous studies, describing the importance of the use of fluorescence because it increases detection rates by 4–22% compared with KOH.[13–15] According to Abdelrahman *et al.*, use of fluorescence enhances the sensitivity and specificity of direct examination by 22% and 6%, respectively.[15] With the use of conventional methods as a gold standard, our results demonstrated a sensitivity of 97% and a specificity of 89% with fluorescence staining. The use of fluorescence enhanced the sensitivity of direct examination by 12% compared with KOH.

While direct examination is often sufficient to rapidly determine the presence of fungal elements in terms of specificity, culturing remains the reference for traditional diagnostic methods,

**Table 2** All results for four diagnostic methods in terms of overall detection rate, sensitivity, specificity and positive and negative predictive values

| Diagnostic method | Detection rate (%) | Sensitivity (%) | Specificity (%) | Positive predictive value (%) | Negative predictive value (%) |
|---|---|---|---|---|---|
| KOH | 51 | 85 | 100 | 100 | 82 |
| Fluorescent staining | 62 | 97 | 89 | 93 | 95 |
| Culture | 43 | 72 | 100 | 100 | 71 |
| PCR | 50 | 78 | 90 | 92 | 74 |

**Table 3** Comparative identification of dermatophytes and non-dermatophytes based on culture and PCR-based sequencing of nail specimens.

| Identified species | Culture (n) | PCR-based sequencing(n) | Culture and (or) PCR-based sequencing(n) |
|---|---|---|---|
| **Dermatophytes** | | | |
| *Trichophyton rubrum* | 49 | 66 | 66 |
| **Non-dermatophytes** | | | |
| Candida sp. | 27 | 28 | 33 |
| Aspergillus sp. | 7 | 4 | 8 |
| Alternaria sp. | 2 | 2 | 2 |
| Cladosporium sp. | 2 | 2 | 2 |
| Total | 87 | 102 | 111 |

even if it may be linked to false-negative results (30–50%).[2] Our results showed that among the 103 specimens positive by microscopy (KOH), 34 (33%) were negative by culturing. In this study, the sensitivity of culture was 72%, which was consistent with previous results ranging between 23% and 80%.[16] Negative cultures may be due to already dead fungi present in the distal part of the nail.

The identification of fungi in direct specimens using molecular biology is reliable and provides significantly improved results in comparison with culture.[17] Various PCR methods have been developed to detect one or several particular species with specific primers in clinical samples without further analysis. However, post-PCR techniques such as amplicon sequencing increases the sensitivity of pathogen detection in dermatological samples and is useful for the identification of infectious fungi at the species level from amplicons obtained with pan-fungal primers.[3] Most previous methods have focused on dermatophyte detection and species identification.[17–23] Few methods for the diagnosis of onychomycosis have considered yeasts and non-dermatophyte filamentous fungi such as *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Alternaria* spp. as possible infectious agents. Therefore, amplicon sequencing was shown to be efficient for the identification of infectious agents in onychomycosis.[3,24] In the present study, the detection rate of ITS rDNA PCR-based sequencing was 50%. PCR-based sequencing identified the infectious agent in 16 cases, where direct mycological examination (KOH) showed fungal elements, but negative results were obtained from fungal cultures, and infectious fungi were detected in 17 samples that were negative for microscopy (KOH) but positive for culture. PCR-based sequencing detected eight specimens that were negative for both microscopy (KOH) and culturing. Although a false-positive PCR result cannot be excluded, it is more likely that these represented false-negative results of the conventional diagnostic methods.[19] Divergences between PCR-based sequencing and culture results may be related to the aliquots, which may not

contain any further viable fungal elements for culture, whereas PCR may detect DNA contained in dead fungal elements.[11] Using conventional methods as the gold standard, PCR demonstrated sensitivities up to 78%, higher than culture (72%), whereas the specificity was 90%.

In onychomycosis, the infectious agents are primarily dermatophytes, especially *T. rubrum* and, to a lesser extent, *T. interdigitale*.[3] Non-dermatophytes account for 10–20% of onychomycosis in temperate climates. Of the non-dermatophytes, *Candida* species is the most common, and moulds, such as *Scytalidium*, *Aspergillus* and *Fusarium* species account for the majority of the remainder.[24,25] In the present study, a total of 111 isolates were identified at the species level by culture and PCR. *T. rubrum* was detected in 66 (60%) specimens; however, other pathogenic dermatophytes were not observed. Non-dermatophytes were detected in 45 (40%) specimens, including 33 (30%) yeasts and 12 (10%) moulds. This frequency rate was similar to a study in which non-dermatophytes were detected in as many as 38% of the tested cases, but much higher than in the previously reported surveys in which the prevalence rates of non-dermatophytic onychomycosis ranged from 1.45% to 17.6%.[24]

Recent data have supported the use of fluorescence microscopy and molecular biology for the diagnosis of onychomycosis. We concluded that fluorescence microscopy can be used as a rapid screening tool for the identification of fungi in nail specimens and when used in conjunction with DNA-based species identification results in higher efficiency, providing clinicians increased confidence in the diagnosis and treatment of patients. This study also indicated that non-dermatophytes, which were detected at high rates, are frequently involved in the pathogenesis of onychomycosis.

## References

1 Gupta AK, Simpson FC. Diagnosing onychomycosis. *Clin Dermatol* 2013; **31**: 540–543.

2 Gräser Y, Czaika V, Ohst T. Diagnostic PCR of dermatophytes – an overview. *JDDG. Journal der Deutschen Dermatologischen Gesellschaft* 2012; **10**: 721–725.

3 Verrier J, Monod M. Diagnosis of dermatophytosis using molecular biology. *Mycopathologia* 2016; **182**: 193–202.

4 Litz CE, Cavagnolo RZ. Polymerase chain reaction in the diagnosis of onychomycosis: a large, single-institute study. *Br J Dermatol* 2010; **163**: 511–514.

5 Bonifaz A, Rios-Yuil JM, Arenas R et al. Comparison of direct microscopy, culture and calcofluor white for the diagnosis of onychomycosis. *Rev Iberoam Micol* 2013; **30**: 109–111.

6 Idriss MH, Khalil A, Elston D. The diagnostic value of fungal fluorescence in onychomycosis. *J Cutan Pathol* 2013; **40**: 385–390.

7 Mikulska M, Furfaro E, Viscoli C. Non-cultural methods for the diagnosis of invasive fungal disease. *Expert Rev Anti-Infe* 2014; **13**: 103–117.

8 Didehdar M, Shokohi T, Khansarinejad B et al. Characterization of clinically important dermatophytes in North of Iran using PCR-RFLP on ITS region. *Journal de Mycologie Médicale/Journal of Medical Mycology* 2016; **26**: 345–350.

9 Nenoff P, Krüger C, Schaller J, Ginter-Hanselmayer G, Schulte-Beerbühl R, Tietz HJ. Mycology-an update Part 2: Dermatomycoses: Clinical picture and diagnostics. *JDDG. Journal der Deutschen Dermatologischen Gesellschaft* 2014; **12**: 749–777.

10 Amichai B, Davidovici B, Trau H, Lyakhovitsky A, Grunwald MH, Shemer A. A rationale for systemic treatment in onychomycosis with negative results on fungal examination. *Clin Exp Dermatol* 2011; **36**: 724–727.

11 Petinataud D, Berger S, Ferdynus C, Debourgogne A, Contet-Audonneau N, Machouart M. Optimising the diagnostic strategy for onychomycosis from sample collection to FUNGAL identification evaluation of a diagnostic kit for real-time PCR. *Mycoses* 2016; **59**: 304–311.

12 Weinberg JM, Koestenblatt EK, Tutrone WD, Tishler HR, Najarian L. Comparison of diagnostic methods in the evaluation of onychomycosis. *J Am Acad Dermatol* 2003; **49**: 193–197.

13 Gupta AK, Zaman M, Singh J. Diagnosis of trichophyton rubrum from onychomycotic nail samples using polymerase chain reaction and calcofluor white microscopy. *J Am Podiatr Med Assoc* 2008; **98**: 224–228.

14 Joseph J, Murthy S, Garg P, Sharma S. Use of different stains for microscopic evaluation of corneal scrapings for diagnosis of microsporidial keratitis. *J Clin Microbiol* 2006; **44**: 583–585.

15 Abdelrahman T, Letscher Bru V, Waller J, Noacco G, Candolfi E. Dermatomycosis: comparison of the performance of calcofluor and potassium hydroxide 30% for the direct examination of skin scrapings and nails. *J Med Mycol* 2006; **16**: 87–91.

16 Rothmund G, Sattler EC, Kaestle R *et al.* Confocal laser scanning microscopy as a new valuable tool in the diagnosis of onychomycosis - comparison of six diagnostic methods. *Mycoses* 2013; **56**: 47–55.

17 Dhib I, Fathallah A, Yaacoub A, Hadj Slama F, Said MB, Zemni R. Multiplex PCR assay for the detection of common dermatophyte nail infections. *Mycoses* 2014; **57**: 19–26.

18 Spiliopoulou A, Bartzavali C, Jelastopulu E, Anastassiou ED, Christofidou M. Evaluation of a commercial PCR test for the diagnosis of dermatophyte nail infections. *J Med Microbiol* 2014; **64**: 25–31.

19 Paugam A, L'Ollivier C, Viguié C *et al.* Comparison of real-time PCR with conventional methods to detect dermatophytes in samples from patients with suspected dermatophytosis. *J Microbiol Meth* 2013; **95**: 218–222.

20 Kondori N, Abrahamsson AL, Ataollahy N, Wennerås C. Comparison of a new commercial test, Dermatophyte-PCR kit, with conventional methods for rapid detection and identification of Trichophyton rubrum in nail specimens. *Med Mycol* 2010; **48**: 1005–1008.

21 Luk NM, Hui M, Cheng TS, Tang LS, Ho KM. Evaluation of PCR for the diagnosis of dermatophytes in nail specimens from patients with suspected onychomycosis. *Clin Exp Dermatol* 2012; **37**: 230–234.

22 Pankewitz F, Nenoff P, Uhrlaß S, Bezold G, Winter I, Gräser Y. Development of a novel polymerase chain reaction-enzyme-linked immunosorbent assay for the diagnosis of Trichophyton rubrum onychomycosis. *Br J Dermatol* 2013; **168**: 1236–1242.

23 Brasch J, Beck-Jendroschek V, Gläser R. Fast and sensitive detection of Trichophyton rubrum in superficial tinea and onychomycosis by use of a direct polymerase chain reaction assay. *Mycoses* 2011; **54**: e313–e317.

24 Ebihara M, Makimura K, Sato K, Abe S, Tsuboi R. Molecular detection of dermatophytes and nondermatophytes in onychomycosis by nested polymerase chain reaction based on 28S ribosomal RNA gene sequences. *Br J Dermatol* 2009; **161**: 1038–1044.

25 de Berker D. Fungal nail disease. *N Engl J Med* 2009; **360**: 2108–2116.