DNA-based detection for onychomycosis correlates better to histopathology than does fungal culture

Erik Gustafson PhD, Wayne Bakotic DO, Lori Bennett, Laurie Page, Laurence McCarthy PhD

Affiliations: Bako Diagnostics, Alpharetta, Georgia, USA

Corresponding Author: Erik Gustafson, Ph.D., Bako Diagnostics, 6240 Shiloh Rd, Alpharetta, GA 30005, 678-679-4170, egustafson@bakodx.com

Abstract

Onychomycosis is a prevalent disease of the nail. Traditional methods for diagnosis include direct microscopy with potassium hydroxide (KOH microscopy) and fungal culture. Other techniques using histochemical staining have higher sensitivity, but cannot identify genus or species of the infecting agent. PCR assays are sensitive, specific, and capable of genus and species level identification. We describe a real-time PCR assay for 15 different fungi that are associated with onychomycosis. Of 425 clinical samples suspected of onychomycosis analyzed by fungal culture and PCR, 219 samples were positive for both (52% agreement). Of the 206 discordant samples, 95% were resolved in favor of PCR by DNA sequencing. On a larger data set of 2,452 samples, positivity rates for histopathology, PCR, and culture were 85%, 73%, and 54% respectively. Further, 48% of PCR positive and 51% of histopathology positive samples were negative by culture. PCR outperformed culture compared to histopathology for sensitivity (80% versus 49%), specificity (92% versus 79%), positive predictive value (94% versus 77%), and negative predictive value (76% versus 52%). These results indicate the culture method lacks the sensitivity to be a reliable assay for onychomycosis, that PCR and histopathology are highly concordant, and that PCR provides the highest degree of diagnostic accuracy available.

Keywords: onychomycosis, PCR, fungal culture, histopathology

Introduction

Onychomycosis is the fungal infection of the nail [1, 2]. It is the most frequently occurring nail disease with an incidence of 2-13% in the general population but increasing to 48% by age seventy [3]. The most common pathogens implicated in onychomycosis are a class of keratin metabolizing organisms designated as the dermatophytes [4, 5] of which the most prevalent are *Trichophyton rubrum* and *Trichophyton mentagrophytes*. These two alone account for the majority of all documented onychomycosis cases [2, 6, 7]. Other fungal pathogens are also reported as a cause of onychomycosis and include various saprophytic molds as well as yeasts [8-11].

Traditionally onychomycosis has been diagnosed by either direct microscopy (KOH preparation) or by fungal culture. Although historically popular, each of these methods has been found to not accurately detect a significant number of cases [3, 12-16]. Newer techniques such as histologic staining and PCR offer better performance. For histopathology, formalin-fixed paraffin-embedded sections are subjected to periodic acid-Schiff (PAS) reaction, [17, 18] and/or staining with Gomori methenamine silver, [18, 19] and examined microscopically for the presence of fungal elements. Although offering excellent sensitivity, histopathology requires a longer turnaround time than direct KOH microscopy or PCR and is unable to identify the genus or species of the infecting agent.

PCR testing has the capability of offering faster turnaround times, as well as improved sensitivity and specificity over other methods [7, 12, 13]. Further, PCR is able to also identify the genus and species of the infecting organism(s) [14, 20]. Sensitive detection coupled with fungal identification is beneficial when making decisions associated with anti-fungal

therapy [21-23]. For these reasons, molecular tests are increasingly used for the improved clinical diagnosis of onychomycosis [15].

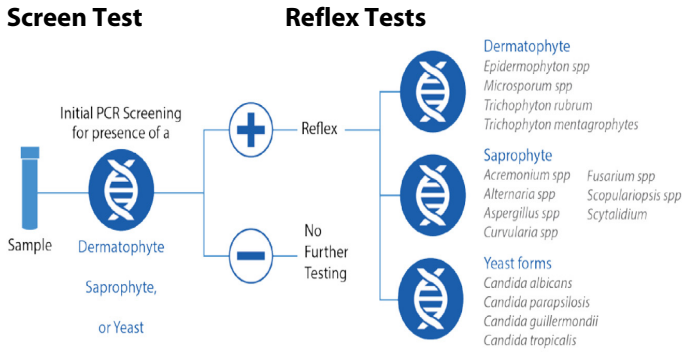

Figure 1. *Description of two-step fungal PCR Assay.* ***Screen Panel****. Nail samples suspected of onychomycosis are discretely screened for the presence or absence of three classes of fungi.* ***Reflex Panels****. Nail samples that have a positive Screen Test for one or more classes of fungi will have the sample run with the corresponding Reflex Test(s).*

Our laboratory now performs histological staining, PCR, and culture for onychomycosis diagnosis, and has accumulated data for a large number of nail samples collected from geographically dispersed patients suspected of having onychomycosis. These data were used to compare culture, histopathology and PCR methods.

Methods

Sample collection

Nail samples from suspected cases of onychomycosis were collected by podiatrists in the normal course of their patient care and submitted to Bako Diagnostics for clinical laboratory testing. Samples were collected as dry keratin tissue and include distal nail clippings and scrapings and shipped dry. Minimum requirements for PCR testing were 0.3cm^2 of specimen. Samples were aseptically minced and thereafter portions submitted to histopathology for embedding and staining, to the molecular department for DNA isolation and PCR testing, and to microbiology for fungal culture. All samples used for the studies described here were tested with histopathology, culture, and PCR.

Microbial Culture

Upon receipt at the laboratory, the nail samples were inoculated onto potato dextrose and mycobiotic agars [24]. All work was performed in a biosafety cabinet. Inoculated plates were wrapped in parafilm and then incubated at 31.8° C. The cultures were visually inspected for fungal growth at days 3, 7, 14, 21, and 28. Fungi were identified by mass spectroscopy (bioMerieux MALDITOF). Fungi not identified by mass spectrometry were identified microscopically using lacto-phenol aniline blue stain (Remel, San Diego, CA).

PCR

Clinical nail samples were first subjected to bead beating followed by incubation at 56C in a Proteinase K lysis buffer for 30 minutes (Omega Biotek, Norcross, GA). DNA was thereafter purified from crude extracts using a magnetic bead purification procedure with the Mag-Bind Plant Double Stranded DNA kit (Omega Biotek, Norcross, GA) automated on the Hamilton MicroLab STAR workstation. The extracted sample material was then used for the PCR screening test and/or one or more PCR reflex tests.

The PCR assays described here can detect up to 15 different fungi associated with onychomycosis. The test consists of distinct steps, the first being a screening test to determine the presence or absence of one or more of three types of fungi — dermatophytes, saprophytes, and yeast. No specific genus or species identification is determined at this stage. This screening test is designed to screen out negative samples and/or identify those samples that require further analysis to identify the causative agent(s). In the event a positive result is obtained for one or more fungal types, specific reflex test(s) are performed to determine the genus and/or species of fungi present in the nail (**Figure 1**).

PCR screening tests were assembled on a Hamilton Starlet workstation in 384-well format with specific primer sets and SYBR Green reaction mix (ThermoFisher, Waltham, MA) and then analyzed on the QuantStudio 6 real time PCR machine (ThermoFisher, Waltham, MA) using real time PCR and melt curve analysis. Positive screen test samples were then analyzed as above using primer sets specific for the indicated reflex test and SYBR green reaction mix on the ABI 7500 (ThermoFisher, Waltham, MA). Gene targets include ribosomal RNA

gene and mitochondrial gene regions of the fungal genomes designed to detect fungal classes (screen test) and other specific molecular targets to identify the genus/species of the disease-causing agent (reflex tests). Raw data in the form of Ct and Tm values were auto analyzed by an internally developed PCR Analysis Engine designed to assign results compared to validated Ct cutoff and Tm ranges for each target, and followed by manual review of results before release.

DNA Sequencing

Forward and reverse primers were designed in fungal consensus regions in the 18S rRNA gene to generate amplicons ~485 bp in length. PCR amplification was performed using GeneAmp® Fast PCR Master Mix (2×), (ThermoFisher, Waltham, MA). Amplification was performed on the 7500 Fast Real-Time PCR System (ThermoFisher, Waltham, MA) and confirmed by gel electrophoresis analysis with the FlashGel™ system, 1.2% agarose (Lonza Rockland, Inc, Rockland, ME). Amplicons were purified using the QIAquick 96 PCR Purification Kit (Qiagen, Hilden, DE) and eluted into Molecular Grade Water. Sequencing of purified amplicons was performed by the Georgia Genomics Facility (University of Georgia, Athens, GA). Raw data files were retrieved for analysis. Forward and reverse sequences were aligned and assembled by Geneious 7.1.6 software (Biomatters Ltd, Auckland, NZ) using the de novo assembly function. Resulting sequences were analyzed using BLAST for genera identification.

Histopathology Staining

PAS staining was conducted on tissue sections using an automated process on the Sakura Linear stainer (Sakura Finetech, Torrance, CA). Briefly slides were immersed in xylene to remove paraffin and then rehydrated by successive rinses in 100% alcohol followed by 95% alcohol and then a water rinse. They were then oxidized in 1% periodic acid for 13 minutes, rinsed with tap water and stained with Schiff reagent for 20 minutes. Following another water rinse, the slides were counter-stained with hematoxylin for 50 seconds, rinsed in water, and treated in Define solution as a clarifying agent for 20 seconds. After another water rinse, the procedure was completed by several washes in 95% and 100% alcohol and then xylene to dehydrate and mount.

Table 1. *Data Analysis Format and Definitions.*

		Comparator Method	
		Positive	Negative
Reference Standard	Positive	True Positive (TN)	False Negative (FN)
	Negative	False Positive (FP)	True Negative (TN)

Accuracy- the overall agreement between two methods = (TP+TN)/(TP+FP+FN+TN).
Sensitivity- proportion of patients with the disease who test positive = TP/(TP+FN).
Specificity- proportion of patients without the disease who test negative = TN/(TN+FP).
Positive Predictive Value (PPV)- proportion of patient with positive results who have the disease = TP/(TP+FP).
Negative Predictive Value (NPV)- proportion of patients with negative results who do not have the disease = TN/(TN+FN).
Reference Standard- the method that is being used as the source of the "correct" result.
Comparator method- the method that is being compared to the reference standard and has its performance described by accuracy, sensitivity, specificity, PPV and NPV.

Gomori methenamine silver staining is conducted on tissue sections using an automated process on the Prisma Stainer (Sakura Finetech, Torrance, CA). Briefly, slides are immersed in xylene to remove paraffin and then rehydrated by successive rinses in 100% alcohol followed by 95% alcohol and a water rinse. They are then oxidized in 7% chromic acid solution for 10 minutes, followed by a 4-minute water rinse. Slides are next rinsed in 1% sodium bisulfate and rinsed in water before being treated in a 0.1% gold chloride solution for three minutes to enhance staining. Water rinses and a 5% sodium thiosulfate rinse to remove excess silver nitrate proceeds counter staining in Light Green solution for three minutes. Water rinses and washes in 100% alcohol and xylene for dehydration before mounting completes the staining procedure.

Data comparison and calculations:

The culture, histopathology and PCR methods were compared to each other with accuracy, sensitivity, specificity, and predictive values of both positive and negative results calculated. Analyses were formatted and calculated as defined in **Table 1**.

Table 2. *Result distribution of culture and PCR methods for 425 nail samples.*

		Fungal Culture					
	Result	**Yeast**	**Dermatophyte**	**Saprophyte**	**Negative**	**Total**	**%**
PCR	Yeast	26 (4)	1	3	6 (4)	36	8%
	Dermatophyte	7 (7)	59 (15)	43 (41)	89 (86)	198	47%
	Saprophyte	0	1	28 (1)	21 (13)	50	12%
	Negative	5 (4)	7 (6)	23 (11)	106 (16)	141	33%
	Total	38	68	97	222	425	
	%	9%	16%	23%	52%		

Parentheses indicate samples that were subjected to DNA sequencing.

Results

An initial validation set of the PCR test's performance used 425 clinical samples submitted for fungal culture. **Table 2** shows the distribution of the 425 results with respect to dermatophyte, saprophyte, yeast and negative results obtained with the two methods. Culture positive samples were observed for 203 samples (48%) with 68 (16%) of the total reported as dermatophyte, 97 (23%) reported as saprophytes, and 38 (9%) as yeast. For the same 425 samples, 284 (67%) were positive by PCR with 198 (47%) of the total reported as dermatophyte, 50 (12%) as saprophyte, and 36 (8%) as yeast. A total of 206 discordant results between the culture and PCR methods were observed for an overall concordance of 52% (219/425).

Sufficient sample quantities allowed 172 of the total 206 discordant samples to be tested further by sequencing of a 485 base pair region of the fungal 18S ribosomal RNA gene. Results show that 167 samples (97.1%) resolved in favor of the original PCR assay result whereas only four samples (2.3%) resolved in favor of the original culture result. One sample (0.6%) resolved in favor of neither method (**Table 3**). Notably, of the original 139 samples that were dermatophyte negative by culture but dermatophyte PCR positive, 134 (96%) were positive for a dermatophyte by sequencing. This included 41 dermatophyte PCR positive samples that grew only saprophytes on culture. Eleven other saprophyte-positive cultures that were saprophyte PCR negative were also sequenced. Ten of these were negative by sequencing suggesting the saprophyte reported was a contaminant. It is also possible that the cultured saprophytes present were valid, but not recognized by the different primer sets used for either the PCR assay or DNA sequencing. However, during validation of the assay, multiple strains of off target organisms were tested to verify specificity. This and the broad nature of the saprophyte PCR screen makes this scenario of unrecognized saprophytes unlikely. Lastly, of the 15 culture positive yeast samples that were sequenced, 7 sequenced as a dermatophyte and four were negative by sequencing, which confirmed the prior PCR results. As a control for the sequencing method, 36 of 219 concordant samples were sequenced and gave the same result as originally reported by the two methods. The results of PCR compared to culture following discordant resolution by sequencing (**Table 4**) show a concordance of 91% (385/425).

Table 3. *Sequencing results of discordant samples between fungal culture and PCR methods.*

Sequence Result	Culture agree	PCR Agree	Neither agree
Yeast		3	
Dermatophyte	3	134	
Saprophyte	1	13	1
Negative		17	
Total	4	167	1
%	2.3	97.1	0.6

A large sequentially selected clinical sample set of 2,452 samples was selected for assessment as each was tested by culture, histopathology, and PCR. Of the 2,452 samples, 1,707 (70%) tested positive by at least one of the methods and 745 (30%) tested

Table 4. *Result distribution of culture and PCR methods on 425 nail samples with sequencing to resolve discordant samples.*

		Culture + Sequencing					
		Yeast	Dermatophyte	Saprophyte	Negative	Total	
PCR + Seq	Yeast	29	1	3	4	37	9%
	Dermatophyte	0	192	3	3	198	47%
	Saprophyte	0	1	42	7	50	12%
	Negative	1	4	13	122	140	33%
	Total	30	198	61	136	425	
		7%	47%	14%	32%		

negative by all three methods (**Table 5A)**. Of the 1,707 positive samples as the denominator, histopathology detected fungi in 85%, PCR in 73% and culture in 54%, respectively (**Table 5B)**, similar to the positivity rate observed in the 425-sample data set that compared PCR to culture (**Table 2**).

Table 6 indicates that PCR matches with histopathology much more closely than culture in all aspects including accuracy (85% versus 61%), sensitivity (80% versus 49%), specificity (92% versus 79%), positive predictive value (94% versus 77%), and negative predictive value (76% versus 52%).

Table 5 *A*. *Distribution of test results from three diagnostic methods on 2452 nail samples suspected of onychomycosis.*

No. Samples	Culture	PCR	Histology	% Samples tested
745	-	-	-	30%
609	+	+	+	25%
550	-	+	+	22%
189	-	-	+	8%
179	+	-	-	7%
101	+	-	+	4%
50	-	+	-	2%
29	+	+	-	1%
1707	Any Method Positive			70%
745	Negative			30%
2452	Total			100%

B. *Sensitivity for each method.*

No.	Method	%
1449	Histology Positive	85%
1238	PCR Positive	73%
918	Culture Positive	54%

Discussion

The purpose of this study was to compare our fungal PCR assay to fungal culture and histopathology staining for the diagnosis of onychomycosis. Onychomycosis constitutes an important public health problem owing to its high incidence, increasing prevalence, and associated complications [1, 5, 25, 26] Persons with onychomycosis have been shown to be at increased risk to develop cellulitis and skin ulcerations, both of which may lead to loss of digits or limb [1, 26, 27]. In addition to advanced age and immunological deficiencies, additional predisposing factors are chronic microtrauma to the nail apparatus, onycholysis, onychoschizia, and genetic predisposing factors [5].

In current practice, direct KOH microscopy and fungal culture are most widely used to diagnose onychomycosis. Direct KOH microscopy of nails is generally performed in 10-20% KOH solution followed by examination under a microscope for fungal elements. Direct KOH microscopy is a rapid and economical method, but can lack sensitivity, with false negative results of 5% to 15% versus fungal

Table 6. *PCR and culture performance compared to histopathology as the reference method on 2452 samples assayed with all three methods.*

	Culture +	Culture -	PCR+	PCR-
Histology +	710	739	1159	290
Histology -	208	795	79	924
Accuracy	61%		85%	
Sensitivity	49%		80%	
Specificity	79%		92%	
PPV	77%		94%	
NPV	52%		76%	

culture [3, 15, 16]. This method is non-specific in that it cannot differentiate between dermatophytes and other fungal species [3, 28, 29]. Recent articles have reported that in the diagnosis of onychomycosis, direct KOH microscopy sensitivity is 64% compared to fungal culture or a combination of direct KOH, fungal culture, and PAS staining [28, 30]. A dye such as calcofluor white may be used to help with the visualization, which may increase sensitivity [31].

Fungal culture has historically been the de facto reference standard for onychomycosis. Two big drawbacks are the long turnaround time required for test results, which can be several weeks, and its broadly reported poor sensitivity. Recent articles have claimed that in the diagnosis of onychomycosis, fungal culture sensitivity ranged from 30%-57% [2, 15, 16, 28-30]. Hypotheses to explain these observations include dead organisms that will not grow in culture [3, 16, 25], inaccessibility of the organism trapped in nail keratin to the growth media [3, 14], prior treatment with anti-fungal agents that inhibit culture growth [12, 14-16], sampling error [14, 20], and the slow growth of dermatophytes in culture [32].

For these reasons, the use of fungal culture for onychomycosis diagnosis has been criticized. Spiliopoulou et al. [14] reported that out of 418 samples from patients suspected of onychomycosis over a 3-year period, 10.5% were positive for dermatophytes using culture, whereas three times as many, 30.1%, were positive for dermatophytes by a commercial dermatophyte PCR assay. Mehlig et al. [12] reported that out of 253 patients tested for onychomycosis 34.4% were positive by KOH direct microscopy, 31.6% by culture, and 50.6% by a commercial multiplex PCR test. In another report Dhib et al. [13] reported that 91.3% of culture negative specimens were PCR positive in their study. There are many other reports documenting the poor culture sensitivity [2, 3, 15, 16, 20, 29, 33-38].

Our current results comparing 425 results of PCR to fungal culture indicated 48% were culture positive and 67% were PCR positive, both in line with the literature. Although the poor sensitivity of fungal culture is evident in our data, its inability to detect a significant number of dermatophytes is concerning. Dermatophytes are difficult to culture as they are slow growing. Their growth can be inhibited by saprophytic molds that can overgrow the culture and obscure slower growing dermatophytes [13-16, 20, 29]. Our results demonstrate that 41 of 97 (42%) of cultures reported to be positive for saprophytes and negative for dermatophytes were identified as positive for dermatophytes as detected by PCR and confirmed by DNA sequencing. This is similar to that reported by Wisselink et al. [39] who found that 24% of cultures with mold detected dermatophyte DNA by PCR. When DNA sequencing was used to resolve the disparities in our data between culture and PCR, PCR results were typically supported by DNA sequencing. A similar conclusion was drawn by Sato et al. [38] when comparing a PCR microarray combination to culture results for onychomycosis. When discrepancies were sequenced, it was the PCR method that was found to be correct.

Histologic staining of formalin-fixed paraffin-embedded nail biopsy sections stained with PAS [17, 18] and/or Gomori methenamine silver has proven to be quite useful in diagnosing onychomycosis, although there are differing opinions on the superiority of PAS versus Gomori methenamine silver stains for identifying fungal elements in nail samples [17, 19]. Our experience is that the two stains complement each other and help in the visualization of fungal structures. In addition, using histopathology staining, other information can be garnered such as direct visualization of fungal elements such as spores and hyphae, the degree of nail plate involvement, as well as the observation and identification of other primary and compounding causes of onychodystrophy. Of the two, PAS is the more widely used staining technique. Histopathology staining with PAS microscopy is significantly more sensitive than direct KOH microscopy or culture, with sensitivities ranging from 84-100% to diagnose onychomycosis [28, 30, 36, 40, 41]. Drawbacks of PAS microscopy include higher cost, a longer turnaround time than direct KOH or PCR, and an inability to distinguish among fungal species. One recent study utilized a meta-analysis methodology of 13 studies over a 16-year

period comparing over 2000 patient samples with direct KOH microscopy, culture, and histopathology. The study concluded that histopathology staining with PAS outperformed the other two techniques, but that combining tests with complementary attributes would establish the most accurate diagnosis [40].

PCR testing for onychomycosis adds the benefit of a fast turnaround time, good sensitivity and specificity, and the ability to identify organisms at the genus and species level [14, 20]. The fungal nail assay described here takes the approach of first screening nails suspected of onychomycosis for three types of fungi — dermatophytes, saprophytes, and yeast. As a substantial portion of nail samples submitted for analysis are negative for fungal elements, this screening approach can save time and cost for both the laboratory and the patient. Only in the event a screening test is positive, is reflex testing performed to identify the genus or species of the infecting organism. Together, the three reflex assays are capable of detecting 15 of the fungi most frequently associated with onychomycosis. Speciation or further identification of the pathogenic fungi is beneficial when making decisions associated with anti-fungal therapy as not all antifungal agents are effective against every fungus, and different treatment regimens may be indicated for different class or organisms [21, 23, 42-44]. Additionally, treatment for onychomycosis can be expensive and can require the use of oral antifungals that may produce side effects. For these reasons, the benefits of molecular tests for the clinical diagnosis of onychomycosis are becoming recognized [16].

Our data indicates that either PCR or histopathology staining can offer better performance than fungal culture as both are more sensitive. It is evident from our data and others that no single method can be used as the ideal test for onychomycosis. It is now common to use combined methods as a diagnostic standard to take advantage of complementary performance characteristics and overcome limitations of any one method. A recent report indicates real-time PCR, combined with histopathology had the highest efficacy of any method or combination of methods tested for diagnosing onychomycosis [45]. Our study again indicates histopathology and the PCR test described have good concordance.

Conclusion

In conclusion, our data suggest that our fungal PCR assay has demonstrated superior sensitivity and specificity to fungal culture, with faster turnaround time in addition to the capability to determine fungal genus and species. Speciation of the pathogenic fungi is beneficial when making decisions associated with anti-fungal therapy as different organisms respond differently to various antifungal medications. In addition, the PCR assay is highly concordant with the results of the sensitive histopathology techniques that also identify acute and chronic trauma, neoplastic processes, and other non-infectious causes of onychodystrophy. Combined, PCR and histopathology provide the most comprehensive evaluation of nail unit dystrophy enabling the selection of appropriate treatment for the patient's disease. By using these technologies in tandem, clinicians receive the highest sensitivity and specificity available for onychomycosis.

Potential conflicts of interest

The authors declare no conflicts of interests.

References

1. Bristow IR, Spruce MC. Fungal foot infection, cellulitis and diabetes: a review. *Diabet Med.* 2009;26:548-551. [PMID 19646196].
2. Chandran NS, Pan JY, Pramono ZA, Tan HH, Seow CS. Complementary role of a polymerase chain reaction test in the diagnosis of onychomycosis. *Australas J Dermatol.* 2013;54:105-108. [PMID 23425129].
3. Emam SM. Real- time PCR: A rapid and sensitive method for diagnosis of dermatophyte induced onychomycosis, a comparative study. *Alexandria J. of Med.* 2016;52(Issue 1):83-90. [DOI: 10.1016/j.ajme.2015.05.001].
4. Borman AM, Campbell CK, Fraser M, Johnson EM. Analysis of the dermatophyte species isolated in the British Isles between 1980 and 2005 and review of worldwide dermatophyte trends over the last three decades. *Med Mycol.* 2007;45:131-141. [PMID 17365649].

5. Elewski BE. Onychomycosis: pathogenesis, diagnosis, and management. *Clin Microbiol Rev*. 1998;11:415-429. [PMID 9665975].
6. Piraccini BM, Alessandrini A. Onychomycosis: A Review. *J Fungi (Basel)*. 2015;1:30-43. [PMID 29376897].
7. Gupta AK, Versteeg SG, Shear NH. Onychomycosis in the 21st Century: An Update on Diagnosis, Epidemiology, and Treatment. *J Cutan Med Surg*. 2017;21:525-539. [PMID 28639462].
8. Gupta AK, Drummond-Main C, Cooper EA, et al. Systematic review of nondermatophyte mold onychomycosis: diagnosis, clinical types, epidemiology, and treatment. *J Am Acad Dermatol*. 2012;66:494-502. [PMID 21820203].
9. Hwang SM, Suh MK, Ha GY. Onychomycosis due to nondermatophytic molds. *Ann Dermatol*. 2012;24:175-180. [PMID 22577268].
10. Morales-Cardona CA, Valbuena-Mesa MC, Alvarado Z, Solorzano-Amador A. Non-dermatophyte mould onychomycosis: a clinical and epidemiological study at a dermatology referral centre in Bogota, Colombia. *Mycoses*. 2014;57:284-293. [PMID 24279435].
11. Farwa U, Abbasi SA, Mirza IA, et al. Non-dermatophyte moulds as pathogens of onychomycosis. *J Coll Physicians Surg Pak*. 2011;21:597-600. [PMID 22015119].
12. Mehlig L, Garve C, Ritschel A, et al. Clinical evaluation of a novel commercial multiplex-based PCR diagnostic test for differential diagnosis of dermatomycoses. *Mycoses*. 2014;57:27-34. [PMID 23734586].
13. Dhib I, Fathallah A, Yaacoub A, et al. Multiplex PCR assay for the detection of common dermatophyte nail infections. *Mycoses*. 2014;57:19-26. [PMID 23734576].
14. 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*. 2015;64(Pt 1):25-31. [PMID 25418736].
15. Petinataud D, Berger S, Ferdynus C, et al. 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. [PMID 26806228].
16. Kizny Gordon A, McIver C, Kim M, Murrell DF, Taylor P. Clinical application of a molecular assay for the detection of dermatophytosis and a novel non-invasive sampling technique. *Pathology*. 2016;48:720-726. [PMID 27780596].
17. Barak O, Asarch A, Horn T. PAS is optimal for diagnosing onychomycosis. *J Cutan Pathol*. 2010;37(10):1038-1040. [PMID: 20412344].
18. Reza Kermanshahi T, Rhatigan R. Comparison between PAS and GMS stains for the diagnosis of onychomycosis. *J Cutan Pathol*. 2010;37:1041-1044. [PMID 19922486].
19. D'Hue Z, Perkins SM, Billings SD. GMS is superior to PAS for diagnosis of onychomycosis. *J Cutan Pathol*. 2008;35:745-747. [PMID 18331569].
20. 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 Methods*. 2013;95:218-222. [PMID 24001882].
21. Jo Siu WJ, Tatsumi Y, Senda H, et al. Comparison of in vitro antifungal activities of efinaconazole and currently available antifungal agents against a variety of pathogenic fungi associated with onychomycosis. *Antimicrob Agents Chemother*. 2013;57:1610-1616. [PMID 23318803].
22. Gupta AK, Paquet M, Simpson FC. Therapies for the treatment of onychomycosis. *Clin Dermatol*. 2013;31:544-554. [PMID 24079583].
23. Gupta AK, Gupta G, Jain HC, et al. The prevalence of unsuspected onychomycosis and its causative organisms in a multicentre Canadian sample of 30 000 patients visiting physicians' offices. *J Eur Acad Dermatol Venereol*. 2016;30:1567-1572. [PMID 27168494].
24. Balows, Albert. Clinical microbiology procedures handbook. Henry D. Isenberg, editor. 2nd ed. Washington DC: ASM Press, 2004.
25. Ghannoum MA, Hajjeh RA, Scher R, et al. A large-scale North American study of fungal isolates from nails: the frequency of onychomycosis, fungal distribution, and antifungal susceptibility patterns. *J Am Acad Dermatol*. 2000;43:641-648. [PMID 26806228].
26. Mayser P, Freund V, Budihardja D. Toenail onychomycosis in diabetic patients: issues and management. *Am J Clin Dermatol*. 2009;10:211-220. [PMID 19489654].
27. Papini M, Cicoletti M, Fabrizi V, Landucci P. Skin and nail mycoses in patients with diabetic foot. *G Ital Dermatol Venereol*. 2013;148:603-608. [PMID 24442040].
28. Blake N, Zhu J, Hernandez G, Juliano PJ. A Retrospective Review of Diagnostic Testing for Onychomycosis of the Foot. *J Am Podiatr Med Assoc*. 2015;105:503-508. [PMID 26667504].
29. 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. [PMID 20370367].
30. Shenoy MM, Teerthanath S, Karnaker VK, et al. Comparison of potassium hydroxide mount and mycological culture with histopathologic examination using periodic acid-Schiff staining of the nail clippings in the diagnosis of onychomycosis. *Indian J Dermatol Venereol Leprol*. 2008;74:226-229. [PMID 18583788].
31. Haldane DJ, Robart E. A comparison of calcofluor white, potassium hydroxide, and culture for the laboratory diagnosis of superficial fungal infection. *Diagn Microbiol Infect Dis*. 1990;13:337-339. [PMID 1706243].
32. Li XF, Tian W, Wang H, et al. Direct detection and differentiation of causative fungi of onychomycosis by multiplex polymerase chain reaction-based assay. *Eur J Dermatol*. 2011;21:37-42. [PMID 21245019].
33. Jensen RH, Arendrup MC. Molecular diagnosis of dermatophyte infections. *Curr Opin Infect Dis*. 2012;25:126-134. [PMID 22248981].
34. Bergmans AM, van der Ent M, Klaassen A, et al. Evaluation of a single-tube real-time PCR for detection and identification of 11 dermatophyte species in clinical material. *Clin Microbiol Infect*. 2010;16:704-710. [PMID 19624518].
35. Brillowska-Dabrowska A, Nielsen SS, Nielsen HV, Arendrup MC. Optimized 5-hour multiplex PCR test for the detection of tinea unguium: performance in a routine PCR laboratory. *Med Mycol*. 2010;48:828-831. [PMID 20105101].
36. Litz CE, Cavagnolo RZ. Polymerase chain reaction in the diagnosis of onychomycosis: a large, single-institute study. *Br J Dermatol*. 2010;163:511-514. [PMID 20491764].
37. 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. [PMID 22300052].
38. Sato T, Takayanagi A, Nagao K, et al. Simple PCR-based DNA microarray system to identify human pathogenic fungi in skin. *J Clin Microbiol*. 2010;48:2357-2364. [PMID 20421438].
39. Wisselink GJ, van Zanten E, Kooistra-Smid AM. Trapped in keratin; a comparison of dermatophyte detection in nail, skin and hair samples directly from clinical samples using culture and real-time PCR. *J Microbiol Methods*. 2011;85:62-66. [PMID 21277340].

40. Velasquez-Agudelo V, Cardona-Arias JA. Meta-analysis of the utility of culture, biopsy, and direct KOH examination for the diagnosis of onychomycosis. *BMC Infect Dis*. 2017;17:166. [PMID 28222676].
41. Haghani I, Shokohi T, Hajheidari Z, Khalilian A, Aghili SR. Comparison of diagnostic methods in the evaluation of onychomycosis. *Mycopathologia*. 2013;175(3-4):315-321. [PMID 23371413].
42. Queller JN, Bhatia N. The Dermatologist's Approach to Onychomycosis. *J Fungi (Basel)*. 2015;1:173-184. [PMID 29376907].
43. Westerberg DP, Voyack MJ. Onychomycosis: Current trends in diagnosis and treatment. *Am Fam Physician*. 2013;88:762-770. [PMID 24364524].
44. Baudraz-Rosselet F, Ruffieux C, Lurati M, Bontems O, Monod M. Onychomycosis insensitive to systemic terbinafine and azole treatments reveals non-dermatophyte moulds as infectious agents. *Dermatology*. 2010;220:164-168. [PMID 20110639].
45. Hayette MP, Seidel L, Adjetey C, et al. Clinical evaluation of the DermaGenius® Nail real-time PCR assay for the detection of dermatophytes and Candida albicans in nails. *Med Mycol*. 2018. [PMID 29762721].