Molecular Biology Techniques for Identifying Dermatophytes and Their Possible Use in Diagnosing Onychomycosis in Human Toenail

A Review

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Traditional methods of diagnosing onychomycosis, such as microscopy, histologic staining, and cultures, may not provide the clinician with documentation before initiating antifungal drug therapy. DNA technology now supplies the tools for increased sensitivity, speed, and accuracy in the diagnostic arena by allowing for the amplification, qualification, and quantitation of DNA. These techniques, already being used to identify many infectious agents, may soon be commonly applied to onychomycosis. This report reviews some of the DNA-based techniques that are currently being used to identify dermatophytes and their possible diagnostic use. (J Am Podiatr Med Assoc 97(2): 134-144, 2007)

The prevalence of onychomycosis, a general term for fungal nail infections, has been reported to range from 2% to almost 9% in the general US population[1] and may be increasing in North America.[2] When the ensuing nail infection is exclusively dermatophytic, it is known as "tinea unguium." This nail infection is predominantly caused by the dermatophyte species *Trichophyton*, principally *Trichophyton rubrum* and *Trichophyton mentagrophytes var interdigitale*,[3] which are capable of parasitizing keratinized human tissues.[4] These dermatophytes secrete metalloproteases and serine proteases, with *T mentagrophytes* reported to have higher proteolytic activity than *T rubrum*.[5] The seven-member gene family encoding the serine protease activity in *T rubrum* includes two proteases with high specificity for keratin, the main protein in nails, and others that may be involved in immediate- and delayed-type hypersensitivity skin reactions.[6]

Trichophyton rubrum and *T mentagrophytes* account for 80% of all dermatophytic nail infections and approximately 50% of all nail infections,[7] with *T rubrum* being the most significant agent.[8] Though considered non–life threatening, the presence of onychomycosis could cause more serious dermatophytic infections and complications in certain populations and should be given careful clinical treatment. Onychomycosis is much more frequent in patients with human immunodeficiency virus disease and may be linked to the level of immunosuppression,[9] and, although the condition is rarely invasive, reports have confirmed the presence of deep dermatophytic infections in an immunocompromised host.[10]

A report[11] on elderly patients found diabetes mellitus to be the most prevalent disease associated with onychomycosis. Recent epidemiologic data[12] indicate that the total prevalence of diabetes in the United States is 20.8 million people, 10.3 million of whom are older than 60 years. In general, onychomycosis is thought to affect one-third of the diabetic population (a rate 2.77 times higher than in healthy individuals[13]), representing approximately 7 million people.

The established diagnostic techniques of microscopy, staining, and cultures for diagnosing onychomycosis often cannot identify the specific infectious agent or can cause a 3- to 4-week lapse between the initial patient visit and the final diagnosis. Negative culture results can frustrate the clinician when there is a strong suggestion of onychomycosis, which can delay treatment. The advent of molecular biology techniques and specifically the application of polymerase chain reaction (PCR)[14] and related nucleic acid techniques provide the laboratory with a powerful but relatively simple method for detection and diagnosis of human infections by microorganisms.[15]

The purposes of this article are to briefly review the traditional diagnostic techniques, to explain the basics of the most common DNA techniques current-

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ly used in dermatophyte studies, and to compare these techniques as diagnostic tools for identifying dermatophytes and diagnosing onychomycosis.

Traditional Diagnostic Procedures for Onychomycosis

The traditional methods of dermatophyte morphotyping are hyphal microscopy of potassium hydroxide (KOH) wet-mount preparations (which identifies hyphae under low microscopic lighting), Calcofluor white stain, histologic staining using periodic acid (ρ-aminosalicylic acid)–Schiff, and cultures. Although a KOH preparation, Calcofluor white stain, or periodic acid–Schiff stain may be used to confirm the presence of fungi,[16] a fungal culture is required to identify the specific genus and species of the infectious agent.[17] Periodic acid–Schiff, although not as widely performed, is becoming a more important clinical tool in diagnosing onychomycosis. This stain, performed by a dermatopathologist, has been used in the past to study glycoproteins, histochemistry, and ultrastructure,[18-20] and the detection of glycoproteins allows for the visualization of hyphae. Calcofluor white, a fluorescent brightener, stains cellulose and chitin, fluoresces when exposed to ultraviolet light, and has been reported to have high sensitivity in identifying the presence of dermatophytes.[4] When diluted with KOH, this stain will dissolve mammalian cells and other nonfungal debris, although the tougher cell walls of the fungi remain intact. It requires a fluorescent microscope with an excitation wavelength less than 400 nm and a barrier filter at 420 nm.[21]

The periodic acid–Schiff histochemical stain is thought to increase diagnostic yield,[22] with some authors concluding that it is the most sensitive method for the diagnosis of onychomycosis that they have reviewed.[23] Another study[24] reported 85% sensitivity with periodic acid–Schiff, compared with 32% with Mycosel agar (Sabouraud's + chloramphenicol and cycloheximide) (BBL, Coffeyville, Maryland) and 57% with KOH with centrifugation (see the discussion of culture medium later in this section). The greatest sensitivity in this study was reported for a combination of periodic acid–Schiff plus cultures, whereas other studies have shown that a combined KOH and periodic acid–Schiff stain is a simple and accurate tool for diagnosing onychomycosis,[25] although periodic acid–Schiff staining may miss nonviable fungal elements.[22] A similar comparative study[26] used Calcofluor white as the gold standard and found that a combination of nail plate biopsy and periodic acid–Schiff was slightly more positive than Calcofluor white (78 and 76 of 93 cases, respectively) in identifying onychomycosis in a group of patients.

The traditional methods of hyphae visualization through KOH analysis, even when coupled with periodic acid–Schiff, do not provide specific identification of the etiologic agent and thus may not provide a good scientific basis for selecting the most appropriate antimycotic agent.[4] A recent study[24] on standard methods used for the diagnosis of onychomycosis reported that 25% of patients suspected of having onychomycotic nails on the basis of clinical examination in fact had negative results on all of the traditional diagnostic tests used in this study. Although nononychomycotic agents may explain these results, more sensitive diagnostic methods may still be needed.

In conjunction with microscopy, culture is the most widespread method by which laboratories identify the causative microorganism. Many types of culture medium have been studied[27] for dermatophytic growth, the most common being dermatophyte test medium and Sabouraud's dextrose agar, with added antibiotics, such as cycloheximide, chloramphenicol, and gentamicin, to inhibit most saprophytic mold and bacterial growth. Cycloheximide-free media are used as a secondary medium. Specimens are plated on a primary medium that selects against most nondermatophytic molds and bacteria and on a secondary medium that will allow for such growth. If growth occurs on both types of media, the infective agent is assumed to be a dermatophyte.[1]

Although the use of antigen-antibody responses can be used to diagnose some fungal infections, this reaction depends on a functioning immune response, which may be substantially decreased in immunocompromised patients.[28] In addition, many of the antigen assays do not have sufficient sensitivity or specificity for routine clinical use,[29] although some techniques have combined PCR with antibody detection using enzyme immunoassay to increase the sensitivity (see the discussion of PCR later here).[30] Although the use of circulating antibodies to diagnose fungal infection can be useful in some patients, its application has not been well studied in nail infections and is not presently being used in the detection of onychomycosis.[31]

Although most treatments for tinea unguium do not differentiate between dermatophytic agents, this knowledge may be advantageous to the clinician. Because it has been reported that *T mentagrophytes* contains more proteolytic activity than *T rubrum*,[5, 32] confirming the etiologic infectious agent may be clinically relevant. In addition, the identification of nondermatophytic infections with dermatophytic origins is important for proper treatment of onychomycosis because dermatophyte infection of the nail may create

conditions favorable to bacterial and saprophytic molds, which on culture often overgrow dermatophytes, preventing their isolation and proper identification.

Yeasts and nondermatophytic fungi have also been identified as being associated with onychomycosis, with the most common yeast involved being *Candida albicans*. Although the frequency of reported nondermatophyte onychomycosis can be as high as 50%, most reports are in the 2% to 5% range,[31, 32] although these organisms may not always be the agents causing the primary infection. Bacteria and saprophytic mold growing from a patient with a clinical impression of onychomycosis may not be the original pathogens. Laboratory isolation of the dermatophyte can be impeded by the saprophyte in the same way that isolation of dermatophytes can be impeded by bacteria in the dermatophytosis complex syndrome.[17] A study[31] of techniques confirming the etiologic significance of nondermatophytic filamentous fungi in onychomycosis and increasing the accuracy of identification of these agents has recently been reported.

Brief Review of Molecular Biology Techniques for Diagnostics

DNA-based analyses are currently used routinely in a wide range of clinical settings, including molecular genetics, oncology, microbiology, hematology, and immunology. The most applied DNA technology, PCR, involves the amplification of a specific target DNA using a heat-stable enzyme, Taq polymerase, and a set of DNA primers (short DNA oligonucleotides), leading to an exponential amplification of this specific DNA. As a result, minute quantities of DNA become detectable, and further analysis and testing are made possible. Although conventional PCR is not quantitative, its qualitative ability to detect and identify DNA is the basis for molecular diagnostic testing. The presence of short repeats in the genome separated by various distances can be exploited by using an application of PCR called repetitive-sequence-based PCR. In this technique, primers are designed that are complementary to these interspersed repetitive sequences but are outwardly facing. As a result, different-sized DNA fragments are produced consisting of unique DNA sequences between these "REP" elements and used to establish fingerprinting patterns that are strain-specific.[33] Today, the most common method of DNA fingerprinting in applied forensics is combining PCR with the analysis of short tandem repeats (STRs) in highly polymorphic regions of DNA containing short repeated nucleotide sequences.[34]

A related PCR technique, reverse transcriptase-PCR, adds the enzyme reverse transcriptase. This technique has the ability to follow gene expression and thus the viability of the organism by converting the messenger RNA that expresses gene activity into a type of DNA termed "complementary DNA." The complementary DNA can then be amplified using PCR, and the gene activity can be studied and even quantified using real-time PCR (see the discussion below).[35]

Many of the techniques currently in use rely on PCR amplification of a target molecule followed by a variety of postamplification analyses, such as restriction enzyme digestion and agarose gel electrophoresis, for detection. These restriction enzymes "cut" the DNA at specific sites, resulting in differences in DNA fragment length that appear as "bands" when analyzed on agarose gel. This DNA banding pattern reflects the polymorphism or changes in nucleic acid sequence around these recognition sites or differences in insertion or deletion of sequences between these sites. Thus these restriction enzyme digests can lead to differentiation of DNA between species. This technique is commonly referred to as "restriction fragment length polymorphism" and was the original basis of DNA "fingerprinting" used in DNA analysis.

Polymerase chain reaction allows for direct identification of the nucleic acids of the infectious agent and thus bypasses the need for cultures that are traditional in morphological studies. In fact, PCR is displacing some of the gold standard cell cultures, supplementing antigen-antibody testing[36] and serologic assays, especially in virus detection,[37] and may very well become the gold standard for testing in the future. The application of these molecular biology techniques to detect bacteria (especially in the clinical setting),[38-41] bacterial biofilms,[42] viruses,[43-46] and fungi[47-51] directly from clinical specimens and fluids is now becoming more routine.

A commonly used quantitation method in PCR is real-time quantitative PCR.[52, 53] In this technique, accumulation of the PCR product is monitored in "real time," and the amount of initial target DNA is quantified using calibration curves from the log-linear phase of amplification. An application of real-time PCR known as "LightCycler PCR,"[54] named after the instrument used (Roche Diagnostics, Mannheim, Germany), has proved to be a rapid method for the detection and quantitation of DNA and thus the infectious agent. In this method, the level of measured fluorescence is proportional to the amount of DNA synthesized in the closed PCR reaction, leading to quantitation of this reaction and a reduction in the risk of contamination. A recent use of LightCycler PCR for

the identification of fungal DNA in dermatologic specimens provided conclusive new diagnostic information that was not given by microscopy or culture in 23.7% of cases.[55] As a result, more accurate quantitation of the fungal pathogen is possible, an application that could be applied to diagnosis as well as analysis of the efficacy of antifungal therapy. For quantitation and detection of viable dermatophytes,[56] real-time quantitative reverse transcriptase–PCR is the most sensitive technique available.

The Barcode of Life is a recent project that promotes the identification of species on the basis of DNA barcoding in which a short DNA sequence of a specific gene, eg, a 648–base pair region in the mitochondrial gene cytochrome oxidase, is used for species identification.[57, 58] This project is based on the fact that significant variation in sequences of a chosen gene such as cytochrome oxidase occurs at a greater frequency between species than within species. Although presently being used for taxonomy of species, this technique may be able to be applied in the future when mixtures of species are present in a sample and identification of each species is needed.

Application of Molecular Biology Techniques for Studying Dermatophytes and Diagnosing Onychomycosis

DNA Targets Using PCR Methods

The study of dermatophytes using PCR and other molecular biology methods has been well reported. These techniques have been applied in studying the phylogeny and taxonomy of these fungi[59-65] as well as in the diagnosis of dermatophytosis either from cultured isolates[59-63] or directly from clinical samples.[16, 56, 66-72] The design of many PCR studies of dermatophytes and other fungi have applied varying types of PCR methods using nonspecific primers such as random amplification of polymorphic DNA[72-74] and arbitrarily primed PCR,[48, 75] as well as targeting mitochondrial DNA.[76, 77] Random amplification of polymorphic DNA and arbitrarily primed PCR are used when little is known about the target sequence, but they can result in nonspecific matches and can yield poor specificity. Many more studies have targeted specific genes, eg, actin,[56, 78, 79] chitin,[62, 78, 79] and topoisomerase II.[80-83]

Most dermatophyte PCR studies[63, 64, 66, 67, 69, 70, 84-95] of fungi, however, have focused on the genes coding for ribosomal RNA, including transcribed and nontranscribed DNA regions. The ribosomal RNA genes can serve as good targets for molecular analysis because they are present in high copy numbers, increasing the sensitivity of their detection.[96] They are also present in all fungal species and have been shown to contain enough variations so as to differentiate the types of fungi.[70, 84, 94]

The organization of the ribosomal genes is in units that have two types of internal transcribed spacer (ITS) regions (Fig. 1). The first ITS is placed between the small 18S ribosomal gene and the 5.8S gene (ITS1) and the second between the 5.8S gene and the gene for the large 25S ribosomal subunit (ITS2). These ITS regions contain nucleotide variations that can be used for strain typing.[84] The tandemly repeated unit of the ribosomal RNA genes thus consists of 18S-ITS1-5.8S-ITS2-25S. In addition, between these tandem units are DNA regions called "nontranscribed spacer regions" (NTSs) that are not transcribed into ribosomal RNA (Fig. 1). This NTS region contains two sets of tandemly repetitive subelements (TRS-1 and TRS-2) that vary in copy number and may also be used for strain typing (Fig. 2).[85] Thus the presence of polymorphisms in the ITS and NTS regions of ribosomal DNA provides a powerful means for strain and possible subtype identification.

Results of PCR Studies of Dermatophytes

For the development of specific PCR primers, emphasis has been on the 18S ribosomal RNA gene, the ITS regions, and the NTS sequences. Using the 18S gene, which contains highly variable and highly conserved regions, coupled with restriction fragment length polymorphism analysis, PCR studies have been used for the development of species- and genus-specific markers.[68, 69, 84] Using a combination of ITS analysis and multiple restriction enzymes to produce different restriction fragment length polymorphism patterns, it has been shown that these DNA techniques can be used as a rapid identification method for most dermatophyte species[93] and were recently used to recognize 23 DNA subtypes of *T mentagrophytes*.[95]

The NTS region of the *T rubrum* ribosomal RNA gene also allows for the study of DNA subtypes,[85, 86, 97] and recent reports have determined that multiple subtypes of *T rubrum* may be involved in fungal nail infections.[92] One study[86] showed that more than one *T rubrum* subtype based on restriction fragment length polymorphism was often observed in successive samples from a single patient receiving oral antifungal therapy taken from the same onychomycotic nail during a 1- to 3-year period. Samples taken from different nails of the same patient had either the same or a mixture of the same and different subtypes of *T rubrum*. In a small study[98] of skin samples from patients with tinea

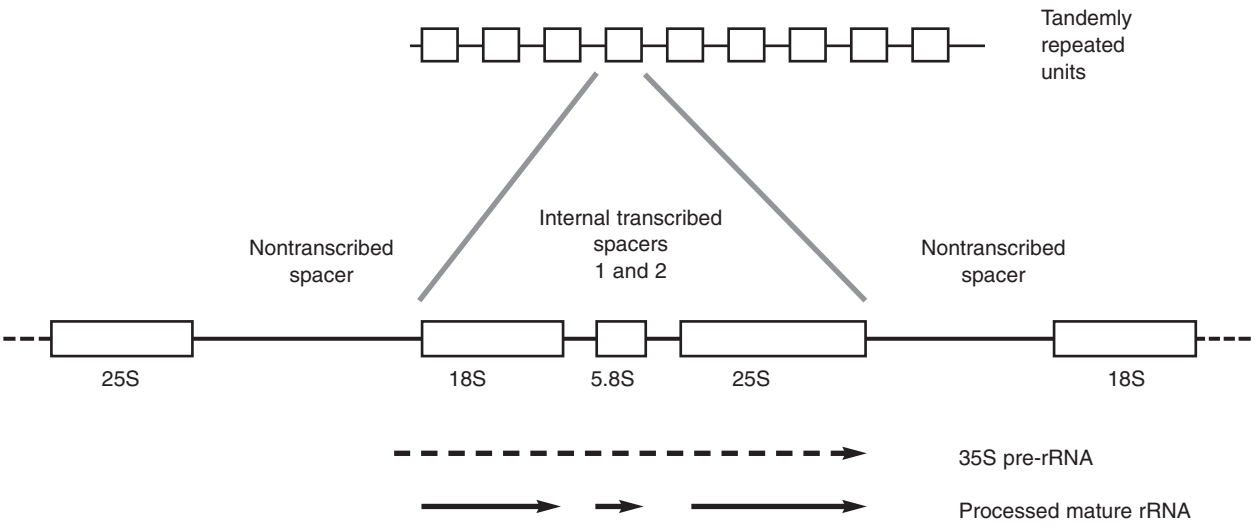


Figure 1. The ribosomal DNA repeats of fungi and higher organisms. The ribosomal genes are separated by two intergenic regions: the internal transcribed and nontranscribed spacers. Transcription of the 35S pre-RNA is initiated from an external transcribed spacer upstream of the 18S gene (region not shown). The gene for 5S ribosomal DNA, often present in the nontranscribed spacer region, is absent from the *Trichophyton rubrum* nontranscribed spacer. rRNA indicates ribosomal RNA. (Courtesy of Colin Jackson, PhD, and Richard Barton, PhD.)

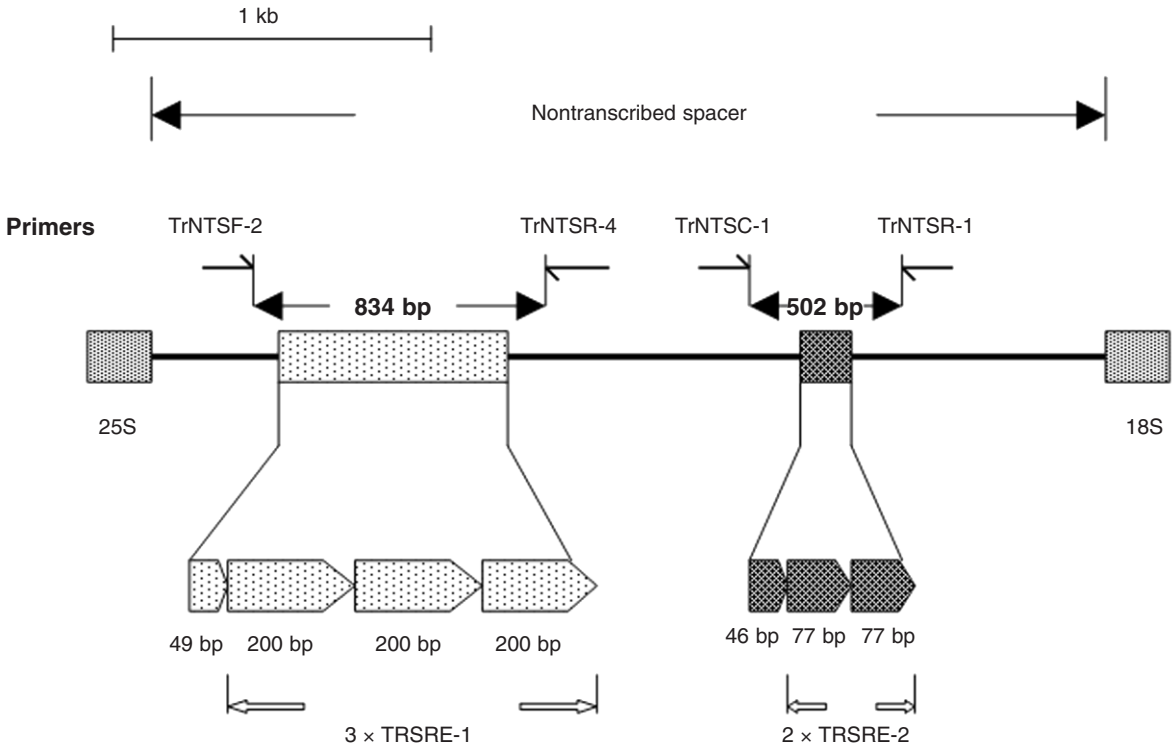


Figure 2. Schematic representation of the nontranscribed spacer region of *Trichophyton rubrum* showing the location of two sets of repetitive subelements, TRS-1 (TRSRE-1) and TRS-2 (TRSRE-2). The locations of the primer pairs used to amplify these repetitive regions for strain typing are indicated. The expanded section indicates the length in base pairs (bp) and the positions of the five complete and two partial repetitive elements. (Reprinted with permission from Jackson et al.[85])

pedis, however, only one subtype of *T rubrum* was isolated, suggesting a single etiology for this disease.

Although further study is needed, the use of these DNA techniques to study different subtypes of *T rubrum* should have a major impact on determining whether a relapse after treatment for onychomycosis is due to reinfection with a new dermatophytic strain or reinfection with the same strain and subtype. In addition, these results indicate that when studying antifungal susceptibility, the standard single colony isolated from a clinical sample may not truly represent all of the infecting dermatophytic strains.[92]

Aside from the ribosomal DNA genes, other specific genes are now being studied as potential targets in dermatophyte identification. Topoisomerase II is an enzyme needed to relieve torsional stress in DNA during processes that involve the constant winding and unwinding of DNA, as occurs in replication and transcription. DNA primers targeting the topoisomerase II genes have recently been used in PCR to rapidly identify 18 dermatophytes to the species level based on the PCR or PCR-restriction fragment length polymorphism product size, which varies from species to species.[82, 83, 97] These authors report that PCR could detect *T rubrum* and *T mentagrophytes* by a specific PCR-restriction fragment length polymorphism product size.

Another gene that has been targeted for studied dermatophytic identification is chitin synthase (*CHS1*),[99] an enzyme implicated in chitin repair. Chitin is a fibrous polysaccharide that constitutes the cell wall of many species of fungi, including dermatophytes. In this study, DNA from skin scrapings and hair samples from animals with clinically identified dermatophytosis were isolated and, using PCR, *CHS1* DNA specific for dermatophytes was amplified, demonstrating the potential use of PCR in the clinical diagnosis of dermatophytosis.

Recently, a polymorphic microsatellite marker, T1, which is based on repeats of the nucleotide GT, detected four polymorphic alleles of the *T rubrum–Trichophyton violaceum* clade using PCR analysis.[65] Using this T1 marker, Kardjeva et al[8] applied this technique to identify and subtype *T rubrum* directly from clinical samples, finding two of the alleles present in this patient population. Combining repetitive-sequence-based PCR techniques to obtain DNA fingerprinting of dermatophytes, Pounder et al[100] recently combined repetitive-sequence-based PCR with automated separation and identification of the DNA PCR fragments using fluorescence and microfluidic chips, allowing for identification of the dermatophytes in less than 24 hours. This technique, however, depends on first obtaining a pure culture for the analysis.

Use of Reverse Transcriptase–PCR to Assay Viability

Although PCR can detect the presence of dermatophytes, this technique merely detects the presence of DNA in the sample and does not necessarily indicate the viability of the infectious agent. A recent report[78] used reverse transcriptase–PCR to assess the viability of dermatophytes grown from cultures of skin scrapings taken from hyperkeratotic soles. Amplification of the messenger RNA, which represents the viability of the organism from these cultured clinical isolates, allowed for positive detection of dermatophytes in cases that were culture- and PCR-negative. This study was continued using the LightCycler with quantitative reverse transcriptase–PCR, allowing for the quantification of the dermatophyte actin messenger RNA isolated from clinical samples of skin scales and nail plates and thus quantitation of the active dermatophyte.[56] Further reports on following the expression of actin using reverse transcriptase–PCR in *T mentagrophytes* cultures treated with antifungal agents has shown that this method could be useful for evaluating fungicidal activity against dermatophytes.[79] Thus it may be possible in the future to use this method to assess fungal viability when testing the efficacy of antimycotic agents.[78]

DNA-Based Techniques Compared with Traditional Diagnostic Methods

In comparing methods for the clinical evaluation of onychomycosis, specificity, sensitivity, and time saved are of great importance. The sensitivity of cultures in identifying the causative agents has been historically poor. Multiple reasons for no-growth results on cultures have been enumerated in the literature, some of which include the use of old dermatophyte media, nails stored too long before sending for analysis, insufficient quantity of clinical specimens, and the use of inappropriate specimens that can also result in growth of contaminants.[101]

Reports of positive dermatophyte culture results range from 23%[24] to 77%,[102] with false-negative culture rates of at least 30%.[103] Because the conventional methods of culturing depend on measurement of the phenotypic characteristics of dermatophytes, they can be easily influenced by factors such as temperature and other medications, eg, chemotherapy, which may interfere with the metabolic process of the dermatophyte and affect interpretation of the *in vitro* culture results.[104] The PCR results of a recent study[8] involving direct analysis of clinically suspected onychomycotic nails detected etiologic fungus in close to

84% of cases in which 72% had failed to be identified using culture. Results of PCR were positive in all of the cases that were culture-positive. Polymerase chain reaction can be used to identify strains that do not present typical morphological characteristics or that will not grow in culture because a significant percentage of clinical samples from patients with dermatophytosis contain nonviable material.[4] In addition, the nonsterile conditions of the nail specimens can lead to the growth of bacterial and fungal contaminants in culture, which may obscure the true etiologic agent. Because PCR directly allows for analysis of the nucleic acid without the necessity of cultures, the problem of interfering organisms may be averted.

The presence of antibiotic-resistant bacteria presents a clinical problem when treating fungal infections. Antibiotic substances have been reported to be produced by fungi *in vivo* and may result in the selection of resistant bacteria in lesions, with *T rubrum* seeming particularly likely to be associated with such antibiotic-resistant bacteria. These bacteria tend to prevent pigmentation formation in cultures and, thus, when depending on colony morphology and pigmentation, misidentification in the clinical laboratory is a possible consequence.[4] The problem of identifying nondermatophytes as the causative agent in onychomycosis has been controversial. Even when a nondermatophyte grows out from a specimen that is hyphae-positive on the basis of microscopy, the culture must be secondarily grown to confirm that the nondermatophyte is the etiologic agent. Because a significant portion of clinical specimens (up to 20% in tinea unguium) contain nonviable dermatophyte material, the use of culture as a tool for identification can lead to a confused diagnosis.[4] This problem can be overcome by the use of molecular biology techniques, as identification of the dermatophyte from nonviable tissue using PCR is possible.

The sensitivity of using periodic acid–Schiff is reported to be 4 ng of carbohydrates,[20] whereas that for detecting target DNA using PCR has varied from 10 pg, or approximately 25 fungal genomes,[69] to as little as 1 to 10 fg or 1 to 10 organisms,[50] with similar sensitivity reported using real-time quantitative PCR for dermatophytes.[56] Using the lower limits of PCR detection, there is an increase of 1,000-fold in sensitivity for the PCR method over the periodic acid–Schiff histologic stain.

The PCR technique is not, however, without potential drawbacks. First, owing to the high sensitivity of the reaction, any contamination of the DNA sample and the PCR solutions could lead to additional amplification of other DNA besides the target sample. This procedure must be performed by trained technicians using sterile reagents to prevent false results. Second, although use of the PCR technique is now a basic protocol in all molecular biology laboratories, the cost of the solutions, enzymes, and thermocycler may still pose an obstacle compared with the microscopy, histopathology, and culture techniques commonly used in mycology laboratories. Third, because PCR is based on the enzymatic amplification of target DNA, it is susceptible to any inhibitors that may be present, which would interfere with the PCR reaction.[8, 105] Despite these technical difficulties, PCR and related molecular biology techniques will become important tools in diagnosing fungal infections.

Summary

Proper treatment of onychomycosis is possible only with proper diagnosis. An epidemiologic survey[102] of 2,001 patients showed that only 40% of the patients with clinically abnormal nails had onychomycosis based on mycologic evidence. Assuming an 80% cure rate with an oral antifungal agent, it has been estimated that if a physician were to start therapy for every patient with abnormal-appearing nails, then approximately one-third of the patients with abnormal nails would be cured.[106] Because increasingly larger percentages of patients may be presenting with nail dystrophy of nononychomycotic origins,[24] it is important to identify the agent causing the infection before oral antifungal drugs are prescribed. Despite the newer oral antifungal agents that have improved prospects for successful treatment of onychomycosis, studies still show mycologic cure rates ranging from approximately 40% to 80%.[107, 108] Not having the identity of the infectious agent may contribute to these therapeutic failures. Restriction enzyme analysis coupled with PCR primers and DNA probes targeted to the ribosomal RNA DNA region or to other genes, such as actin, chitin, and topoisomerase II, have identified and differentiated the two main etiologic agents of onychomycosis—*T rubrum* and *T mentagrophytes*—and have identified multiple subtypes of these dermatophytes.

An efficient and quick method for the diagnosis of onychomycosis is necessary before initiating antifungal drug therapy. Although KOH analysis using microscopy can conceivably be performed in the office given the proper instrumentation and histologic training, traditionally cultures and KOH are sent to a specialty laboratory for analysis. This results in a minimum 2- to 4-week time lag, which delays appropriate treatment. The use of molecular biology techniques has made identification of the etiologic agent possible in a much shorter time frame. Whereas the time for detection of the etiologic agent using a molecular bi-

ology technique can vary depending on the method and resources, it is usual that identification can be performed within 1 to 2 days, whereas the LightCycler PCR typically allows for DNA amplification to be completed in less than 20 min.[54] Complete identification and quantitation of the viable dermatophytes in skin tissue was recently reported to be less than 4 hours,[56] compared with the 2 to 4 weeks needed for identification using cultures.

Although the advent of new antifungal therapy has improved efficacy and tolerance to many of these drugs, adverse effects of these medications can still occur, and liver function tests are often warranted.[3] Although rare, some of the adverse effects of terbinafine, one of the most common of the antifungal drugs, are diarrhea, pruritus, dyspepsia, abdominal pain, headache, visual disturbance, and neutropenia, as well as a recent observation of facial pigmentation.[109] Liver failure has been described as a possible complication with some antifungal drugs,[110] and their interactions with antidepressant medications, such as paxetine, have also been reported.[108] Given the importance of proper diagnosis and the potency of many of the antifungal drugs, the US Food and Drug Administration[110] now recommends that health-care professionals obtain nail specimens for laboratory testing before prescribing the medications for fungal nail infections, to confirm the diagnosis.

The aging population in the United States is increasing, with a total of approximately 35 million people older than 65 years[111] and projections in 2020 of approximately 55 million people in this age group. Some investigators[112] estimate that up to 30% of the population by age 60 years will have onychomycosis. The need for more sensitive and quicker methods for diagnosing onychomycosis will become even more pressing in the future.

Advances in DNA technologies now allow for the amplification, qualification, and quantitation of DNA isolated from clinical samples. Despite the possible problems with contamination, these protocols will become more widely accepted and used in the diagnostic arena as calibration and training increase. With the explosion of scientific knowledge, the transition from research to clinical application is no longer theoretical. Polymerase chain reaction and DNA technologies are already being used to study dermatophytes and onychomycosis. Podiatric physicians need to be prepared to understand and apply these new techniques as they become available for the diagnosis of diseases, especially as they relate to podiatric medicine.

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