Fungal Diversity and Onychomycosis

An Analysis of 8,816 Toenail Samples Using Quantitative PCR and Next-Generation Sequencing

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Background: Onychomycosis is a fungal infection of the nail that is often recalcitrant to treatment and prone to relapse. Traditional potassium hydroxide and culture diagnosis is costly and time-consuming. Therefore, molecular methods were investigated to demonstrate effectiveness in diagnosis and to quantify the microbial flora present that may be contributing to disease.

Methods: A total of 8,816 clinically suspicious toenail samples were collected by podiatric physicians across the United States from patients aged 0 to 103 years and compared with a control population (N = 20). Next-generation sequencing and quantitative polymerase chain reaction were used to identify and quantify dermatophytes, nondermatophyte molds, and bacteria.

Results: Approximately 50% of suspicious toenails contained both fungi and bacteria, with the dermatophyte *Trichophyton rubrum* contributing the highest relative abundance and presence in 40% of these samples. Of the remaining 50% of samples, 34% had bacterial species present and 16% had neither. Fungi only were present in less than 1% of samples. Nondermatophyte molds contributed to 11.0% of occurrences in fungus-positive samples. All of the control samples were negative for fungi, with commensal bacterial species composing most of the flora population.

Conclusions: Molecular methods were successful in efficiently quantifying microbial and mycologic presence in the nail. Contributions from dermatophytes were lower than expected, whereas the opposite was true for nondermatophyte molds. The clinical significance of these results is currently unknown. (J Am Podiatr Med Assoc 109(1): 57-63, 2019)

Use of genomic techniques has allowed the identification of fastidious and noncultivable microorganisms in nail samples. Consequently, a better understanding of the role of biodiversity in onychomycosis and chronic nail infections can be elucidated. Metagenomics techniques can now determine the entire genetic composition of a microbial community. Even organisms in low abundance or dormant can be identified through 16S (bacterial) and 18S (fungal) ribosomal DNA sequences. Knowledge of the particular microbe combinations that result in disease can then be applied to the creation of targeted treatments and improving cure rates. Therefore, molecular genetics–based diagnostics were used to accurately and rapidly identify microbes present in patients with onychomycosis to improve treatment recommendations.

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Methods

Patient Population and Sample Collection

A total of 8,816 toenail samples collected by podiatric physicians from clinically suspicious toenails across the United States were submitted

to Pathogenius Laboratories (Lubbock, Texas) for laboratory analysis between January 1, 2013, and January 31, 2016. The patient population was composed of 3,487 males and 5,329 females aged 0 to 103 years (61 reported no age selected). A second population (N = 20) of healthy nails was examined as controls. All of the control nails were investigated for the presence of bacteria and fungi, and six were further examined for species. Fingernail samples were excluded from this population assuming that nail specimens were accurately labeled by physicians at the time of analysis and because the podiatric physician's scope of practice is often, although not always, limited to foot and ankle disease. In addition, the organisms that are found in fingernails are not the same or in the same distribution as those found in toenails.

DNA Extraction and Amplification

The DNA isolation was performed using the High Pure polymerase chain reaction (PCR) template preparation kit (Roche Diagnostics, Indianapolis, Indiana) following a modified manufacturer's protocol. The extraction procedure was modified to include a beading step for tissue and cell disruption using 5-mm steel beads, 0.5-mm zirconium oxide beads, and the TissueLyser II instrument (Qiagen, Germantown, Maryland). The lysate was then run through the glass fiber fleece column following the manufacturer's protocol provided with the Roche High Pure PCR template preparation kit.

DNA was amplified through quantitative PCR including a 5-min predenaturation at 95°C, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 40 seconds, extension at 72°C for 60 seconds, and a final extension at 72°C for 10 minutes. DNA was purified using Agencourt AMPure beads (Beckman Coulter, Indianapolis, Indiana) and the Qiagen MinElute PCR purification kit according to the manufacturer's instructions. DNA was then pooled based on amplification strength and attached to Ion Sphere particles (Thermo Fisher Scientific) through emulsion PCR. Ion Sphere particles were enriched and sequenced using next-generation sequencing technology (Personal Genome Machine, Life Technologies).

Bioinformatic and Biostatistical Analyses

Denoising was performed to remove short sequences, singleton sequences, and noisy reads, followed by chimera detection. Correction of sequencing errors and chimera removal was performed by first trimming sequences back using a running average of Q25. Trimmed sequences were then run through USEARCH[1] to cluster the sequences at 4% divergence, perform operational taxonomic unit (OTU) selection and chimera depletion using the UPARSE[2] OTU selection algorithm, and map each sequence to the corresponding nonchimera OTU. Mapped sequences were then grouped by OTU, and quality score–based sequence correction was performed.

Corrected sequences were run through a taxonomic analysis pipeline (RTL Genomics, Lubbock, Texas) to determine the taxonomic classifications. First, quality checking on each corrected sequence was performed to confirm primer removal and a minimum sequence length of 250 base pairs. Selection of OTUs was then performed using the UPARSE OTU selection pipeline.[1,2] Selected OTUs were aligned using MUSCLE,[3,4] and a phylogenetic tree was generated using FastTree.[5,6] The selected OTU sequences were globally aligned using USEARCH[1] against a database of 16S sequences for bacterial assays and internal transcribed spacer sequences for fungal assays. Sequences were gathered from GenBank and are classified to at least genus level with at least partial sequences of the target genes. Confidence values were assigned to each OTU classification, and the lowest common ancestor was determined based on these confidence values. The top hit and lowest common ancestor was reported for each OTU. For the analysis in this study, we included only the data from microbial species that comprised at least two orders of magnitude (two $\log_{10}$ values) of the total bacterial population. Therefore, microbial species that represented less than 1% of the entire sample are not reported in this study.

Results

Of the 8,816 samples, approximately 50% (n = 4,328) were positive for fungal organisms (as well as bacteria), with 19,351 unique fungal occurrences. Therefore, the data are consistent with clinical and scientific observations in onychomycosis literature.[7] Of the remaining 50% of samples, 34% (n = 2,995) had bacterial species present and 16% (n = 1,443) had neither. Fungi only were present in less than 1% of samples (n = 50). Contradictory to the currently accepted estimate of 90%,[8] the dermatophyte *Trichophyton rubrum* was present in 40% of fungus-positive samples and contributed the highest relative abundance (Figs. 1 and 2). *Pithomyces chartarum* was the second most commonly found organism (occurring in 20% of fungus-positive

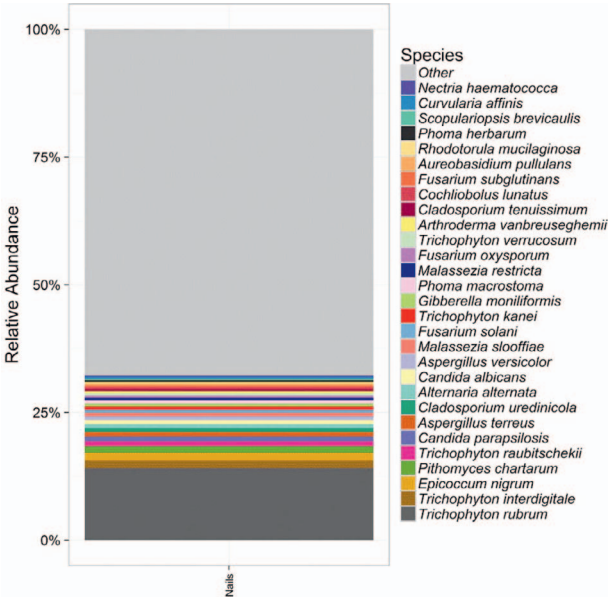

Figure 1. Average relative abundance of the top 30 fungal species determined by internal transcribed spacer sequences in 8,816 nail samples.

samples) and has been previously implicated in onychomycosis.[9] Related species *Pithomyces sacchari* and *Pithomyces maydicus* have similarly been recovered from skin, nails, and respiratory tracts.[10]

Following these, saprophytic yeasts such as *Epicoccum nigrum* (occurring in 18% of fungus-positive samples) and *Alternaria alternata* (occurring in 11% of fungus-positive samples) were overly abundant. The latter are considered emerging pathogens in onychomycosis, especially in warm and humid environments,[11] and the former are associated with respiratory fungal allergies, including allergic asthma, rhinitis, hypersensitivity pneumonitis, and allergic fungal sinusitis.[12]

Nondermatophyte molds (NDMs) represented 11.0% of occurrences (2,125 of 19,351) in fungus-positive samples. The most commonly observed NDMs included various species of *Candida*, *Aspergillus*, *Fusarium*, and *Acremonium* (Fig. 3). Many of these species are ubiquitously present in the environment or are common commensal organisms, although there is emerging evidence that some may have pathogenic potential or have been previously linked to onychomycosis (Table 1). Therefore, the frequent presence in chronically infected nails (and lack of presence in the control population [Table 2]) suggests utility in further investigating targeted treatment options.

Most bacteria identified were *Staphylococcus*

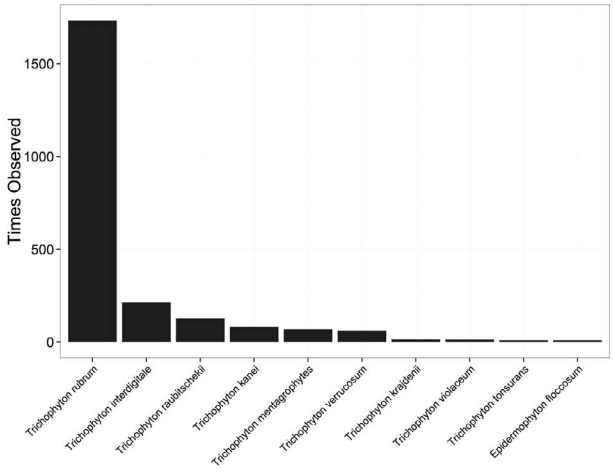

Figure 2. Occurrence of the most common dermatophytes in fungal (18S) species identification.

species (Fig. 4), including common commensals such as *Staphylococcus epidermis* (occurring in 14% of bacterium-positive samples), *Staphylococcus pettenkoferi* (occurring in 11% of bacterium-positive samples), and *Staphylococcus lugdenensis* (occurring in 5% of bacterium-positive samples), which were also all present in the control population (Table 2). Not all species isolated were so benign, however. Also highly prevalent was *Corynebacterium tuberculostearicum*, which was present in 8% of bacterium-positive nail specimens. The clinical relevance of this species has been difficult to assess because it is commonly present on the skin and mucosal surfaces (and was also present in the control population [Table 2]) but has been considered relevant in less than half of the cases where it has been identified in patients with chronic disease.[13]

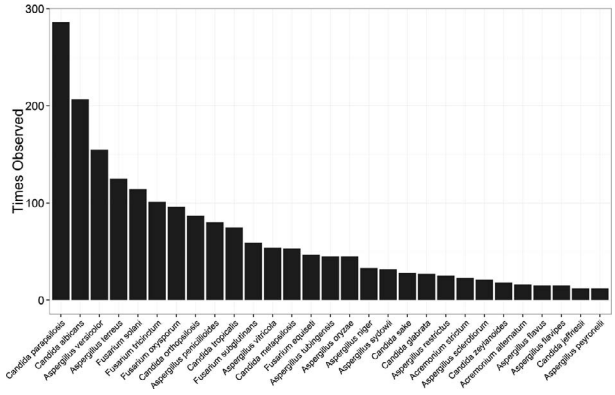

Figure 3. Occurrence of the most common nondermatophyte molds in fungal (18S) species identification.

Table 1. Most Commonly Identified Nondermatophyte Molds Observed in Onychomycosis-Suspected Nails

Nondermatophyte Mold	Evidence of Involvement in Onychomycosis	Evidence of Virulence/ Pathogenicity	Little/No Evidence of Pathogenicity
Candida parapsilosis	Yes[22,23]		
Candida albicans	Yes[22,23]		
Candida orthopsilosis	Yes[22]		
Candida tropicalis	Yes[23,24]		
Candida metapsilosis	Yes[22]		
Candida sake	No[a]	Yes[25-27]	
Candida glabrata	Yes[23]		
Candida zeylanoides	Yes[28,29]		
Candida jeffriesii	No[a]	No	Xylose-fermenting, associated with rotting wood and the intestines of wood-boring beetles[30]
Aspergillus versicolor	Yes[31-34]		
Aspergillus terreus	Yes[35-37]		
Aspergillus penicillioides/vitricola	No[a]	No	Extreme halophile,[38] pathogenesis is extremely rare even in the immunocompromised
Aspergillus tubingenesis	Yes[36]		
Aspergillus oryzae	No[a]	No	Domesticated, used in industrial fermentation processes[39]
Aspergillus niger	Yes[33,36]	No	
Aspergillus sydowii	Yes[36]		
Aspergillus restrictus	No[a]	No	Xerophilic, allergen, asthma, found commonly in house dust,[40] pathogenic in immunocompromised, one anecdotally reported case of onychomycosis
Aspergillus sclerotiorum	No[a]	No	Soil fungus, rare reports of onychomycosis in genetically predisposed (diabetic) individuals
Aspergillus flavus	Yes[33,35,36]		
Aspergillus flavipes	No[a]	No	Few reports of pathogenicity in immunocompromised individuals
Aspergillus peyronelii	No[a]	No	No evidence

[a]In the absence of evidence of previous connections to onychomycosis, presence or absence of reports of pathogenicity are identified.

Discussion

Contrary to the current literature, a molecular analysis of 8,816 toenail samples suspected of onychomycosis revealed the presence of *T rubrum* in approximately 40% (as opposed to the expected 90%[8]) of fungus-positive samples, suggesting that the cause of disease may be more diverse than presently estimated. In comparison, a control population of healthy nails (N = 20) contained no fungal or yeast species, with commensal bacterial species such as *Staphylococcus epidermidis* composing most of the microbial flora. Just as importantly, molecular techniques were demonstrated as an accurate and efficient method for diagnosis. Moreover, there have been similar observations of an increased presence of NDMs in other geographic locations reported as well.[16,17] Ebihara et al[18] also report higher rates of NDMs and suggest that their observations were possible due to the sensitivity of the molecular methods used for diagnosis and that NDMs may currently be underestimated due to the difficulty of identifying them by conventional methods. Overall these results provide insight into the chronic nature of the disease as well as implications for treatment.

Table 2. Average Relative Percentage of Top Microbial Species Determined by 16S Comparison in Six Nail Samples

Bacterial Species	Observed (%)
Staphylococcus epidermidis	35.83
Corynebacterium tuberculostearicum	15.50
Staphylococcus warneri	13.00
Corynebacterium jeikeium	7.83
Staphylococcus lugdunensis	4.17
Enterococcus faecalis	2.83
Staphylococcus pettenkoferi	2.00
Anaerococcus tetradius	1.83
Kocuria rosea	1.67
Brevibacterium mcbrellneri	1.67
Corynebacterium confusum	1.00
Zimmermannella bifida	1.00

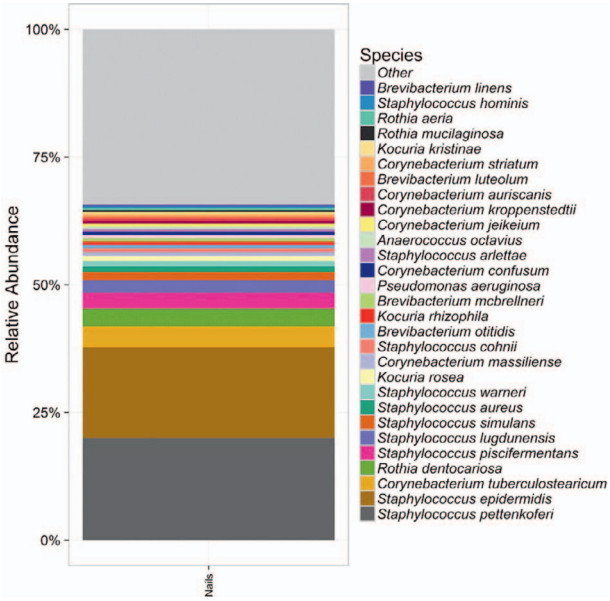

Figure 4. Average relative abundance of the top 30 bacterial species determined by 16S comparison in 8,816 nail samples.

As expected, *T rubrum* and dermatophytes were observed most often; however, saprophytic yeasts, NDMs, and bacteria were also observed on a consistent basis. Because the latter organisms are known to form commensal relationships, a connection to onychomycosis may not be straightforward; however, there is evidence in the literature to support this theory of a possible group contribution to disease (Table 1).

For example, *Rothia dentocariosa* was found in more than 3% of bacterium-positive samples and has pathogenic potential as the cause of dental caries and periodontal disease. As an opportunistic infectious organism, pathogenicity has been implicated in endocarditis, pneumonia, peritonitis, and lung infections. Therefore, it is possible that periodontal disease and dental procedures may be the first step in the inoculation of other distal peripheral tissues such as toenails.

Likewise, gastrointestinal host colonization with gram-positive commensal bacteria such as *Enterococcus faecalis*, which was present in almost 1% of bacterium-positive samples, is another area of concern with nail disease. *Enterococcus faecalis* can bind to dentin, alter host responses, and use serum as a nutritional source, therefore competing with other cells.[14] Thus, frequent pedal contamination with intestinal flora as a result of pooling water from baths and showers in community-acquired settings such as nursing homes may be a growing concern for nail infection. Similarly, Shemer et al[15] reported that 46% of swimming pool employees had both tinea pedis and onychomycosis in a recent study of 169 employees of public pools in Israel, suggesting that moisture-rich environments such as public swimming pools may also contribute to the development of nail disease.

Taken together, evidence from this analysis has led to the hypothesis that development of onychomycosis may depend on a plethora of microbes contributing synergistically to disease. Moreover, there is a possibility of biofilm formation, which displays similar characteristics (recurrence and recalcitrance to normally effective treatment) as observed in patients with onychomycosis.[19] This hypothesis supports the chronic nature of onychomycosis because antifungal therapies are targeted mainly at *T rubrum* and NDMs are often resistant to both oral and topical antifungal agents.[20] Thus, investigations into combining NDM treatment with traditional therapy seem worthwhile.

Implications for Diagnosis and Treatment

Based on the high cost and low accuracy of current potassium hydroxide and culture techniques used in standard diagnosis, use of empirical oral terbinafine without the benefit of confirmatory diagnosis has been suggested, reporting minimal effect on patient safety with a potential savings of millions of healthcare dollars annually.[21] There are at least two drawbacks to this approach, the first being that because both oral and topical antifungal therapies are specifically focused at *T rubrum*, we may be actively selecting for alternative organisms by targeting the wrong genus and species while encouraging the development of resistance. Second, other similar nail disorders cannot be excluded without nail sampling, such as trauma, psoriasis, lichen planus, or other nail disease. Alternatively, molecular methods are extremely specific, sensitive, and quantitative, which allows not only diagnosis but is invaluable in assessing response to treatment. In comparison, they are both faster (3-day turnaround time) and lower in cost than current methods. Laboratory tests with the required expertise are now becoming routinely available at steadily decreasing costs.

Conclusions

Through the evidence provided herein, dermatophytes and specifically *T rubrum* are unsurprisingly a main contributor to onychomycosis; however,

additional organisms, such as yeasts, NDMs, and possibly bacteria, may also be playing a larger role in disease development than previously believed. The clinical significance remains unknown, and further confirmatory tests will provide a better understanding. To improve treatment outcome, use of new molecular diagnostic techniques are recommended to confirm disease and to target therapy to the presence of specific microbes.

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Conflict of Interest: None reported.

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