Molecular Analysis of Fungal Populations in Patients with Onychomycosis Using Next Generation Sequencing (NGS) and Real-Time PCR

Adapted from a poster
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Bako Diagnostics

INTRODUCTION

Onychomycosis is the clinical term for a fungal infection of the nail. It constitutes an important public health problem due to its high incidence, increasing prevalence and an increased risk of complications such as cellulitis and skin ulcerations, both of which may lead to loss of digits or limb. The most common fungal pathogens in onychomycosis are keratin metabolizing dermatophytes including Trichophyton rubrum and Trichopyton mentagrophytes. Other fungal organisms, however, are also implicated in onychomycosis including saprophytic molds and yeast. There is some debate as whether non-dermatophyte fungi are causative or commensal in onychomycotic nails. Studies have reported distribution of fungal species in fungal nail infections,[1] but a systematic examination of the fungal nail population could be more useful in this regard. Conventional methods for diagnosis of onychomycosis are fungal nail culture and direct microscopic examination using Periodic Acid Shift reaction (PAS) and/or Gomori methenamine silver (GMS). These are widely used and considered the gold standard. However, molecular methods such as polymerase chain reaction (PCR) may have an advantage of time, sensitivity and specificity over conventional methods. In addition, next generation sequencing (NGS) technology is well suited for studying fungal organism distribution in onychomycotic nails.

STUDY OBJECTIVES:

1. Establish performance of a multiplex PCR fungal assay compared to the conventional gold standard method of histologic staining and microscopic examination with PAS/GMS for fungal pathogen identification.

2. Characterize fungal organism diversity and distribution in a clinical sample population with onychomycosis using NGS technology and sequence mapping to the UNITE database[3]. Compare identification results with a multiplex real time PCR fungal assay.

MULTIPLEX PCR ASSAY FOR FUNGAL ORGANISMS IN HUMAN NAIL

Bako Diagnostics processes a high volume of fungal nails per year using conventional and molecular techniques. The Fungal Nail PCR assay developed at Bako is a real time assay capable of producing results within a 24 hour period. The assay is broadly specific and can detect 15 different genera or species of fungal pathogens implicated in onychomycosis including dermatophytes, saprophytes and yeast (Figure 1). The PCR assay performance has been measured against gold standard conventional methods such as PAS/GMS staining with microscopy using identical samples and is highly correlative (Table 2a). The historical distribution of fungi classes in onychomycotic nails using this assay is reported (Table 2b).

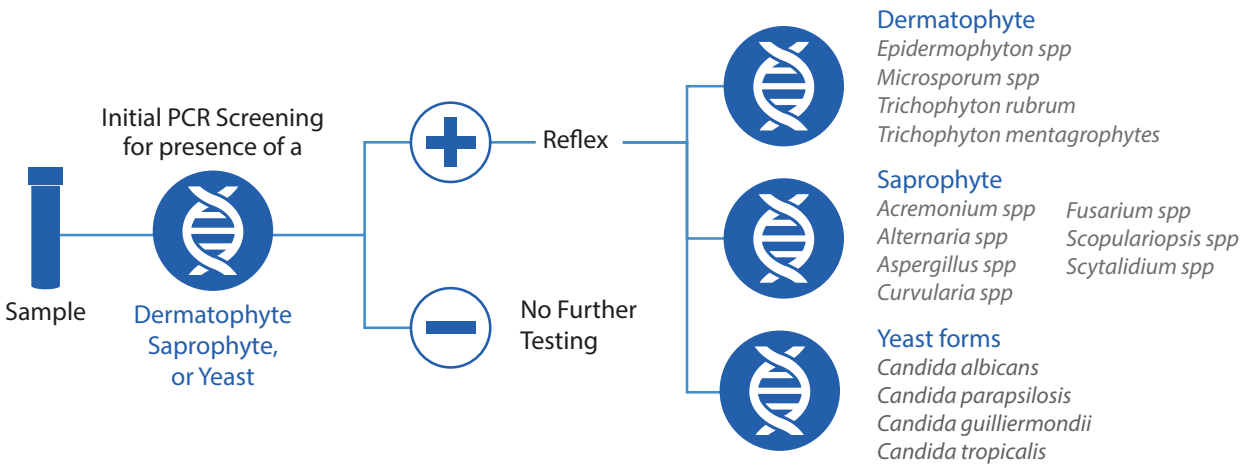


Figure 1. Schematic of Bako Fungal nail PCR assay. Clinical nail samples are screened for three different groups of fungi followed by reflex testing for positive screen results. Fifteen different fungal genera and species can be detected with this assay.

A

	PCR +	PCR -	Total
Histology +	21722	5703	**27425**
Histology -	1402	17569	**18971**
Total	**23124**	**23722**	**46396**

Accuracy	85%
Sensitivity	79%
Specificity	**93%**
PPV	**94%**
NPV	**75%**

B

Number of Organisms Detected in Positive Samples (%)	
Dermatophytes	16,239 (70%)
Saprophytes	8,815 (38%)
Yeast	3,248 (14%)

Table 2. A. Onychomycotic nails were examined by the Bako Fungal PCR assay and compared to conventional PAS/GMS staining with microscopy. Specificity and PPV for the PCR assay are 93% and 94% respectively. Sensitivity and NPV are lower, but sampling distribution of dissected nail is a contributor. B. Distribution of organisms detected in the 23,124 (50%) PCR-Positive Samples.

FUNGAL ITS AMPLICON DESIGN

For NGS amplicon sequencing we selected a region separate than that used in the multiplex PCR. This novel PCR amplicon targets the ITS2 and 28S region of the fungal ribosomal DNA gene (Figure 2a). This region has been reported to provide a greater taxonomic diversity and richness than other regions of the ribosomal gene locus[2]. In our testing, the amplicon was shown to produce a highly represented and uniform PCR product size across multiple species tested (Figure 2b). The ~600 bp size fits well with the MiSeq 300 x 2 reads.

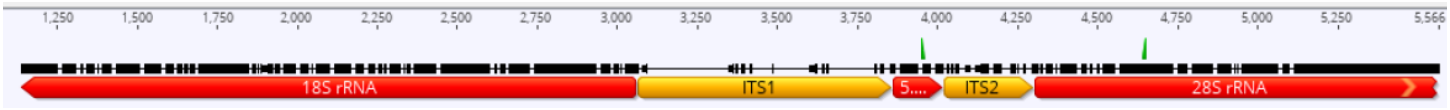
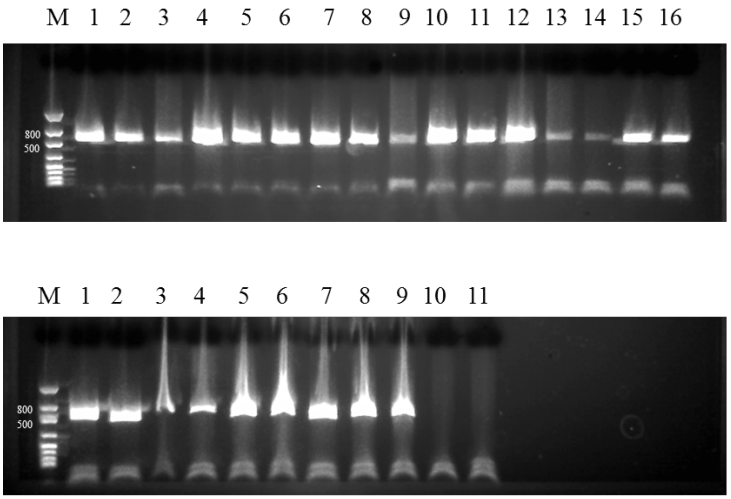


Figure 2a. Location of amplicon primers in the ribosomal region (green triangles). The amplicon sequence begins in the 5.8S rRNA, spans ITS2 and ends in the 28S rRNA gene.



Top Gel

M. Size Marker
1. Alternaria
2. Aspergillus
3. Chaetomium
4. Cladosporium
5. Curvularia
6. Epicoccum
7. Fusarium
8. Paecilomyces
9. Penicillium
10. Scopulariopsis
11. Scytalidium
12. Acremonium
13. Aspergillus terreus
14. Fusarium solani
15. Fusarium oxysporum
16. Fonsecaea

Bottom Gel

M. Size Marker
1. C. albicans
2. C. parapsilosis
3. C. guilliermondii
4. C. glabrata
5. C. krusei
6. C. tropicalis
7. M. pachydermatis
8. T. mentagrophytes
9. T. rubrum
10. Human gDNA
11. NTC

Figure 2b. Agarose gel analysis across different fungal organisms amplified with the new NGS primer pair. Amplification products are generally uniform in size and intensity.

ANALYSIS OF NGS FROM ONYCHOMYCOTIC NAILS

Residual DNA extracted from 384 clinical nail samples processed at Bako and positive for fungal elements by PAS/GMS staining were used to generate an amplicon library and sequenced on the Illumina MiSeq instrument to a depth of 50,000 reads each. Two hundred twelve samples met quality criteria and were further processed. Fastq files were analyzed using the ITS Metagenomics UNITE 20160131db from Illumina Professional Services to map sequences to the genomic ITS region. Rarefaction analysis was used to examine fungal diversity in the population of onychomycotic nails. (Figure 3). Comparison of sequencing results to the multiplex PCR assay are shown in Table 3.

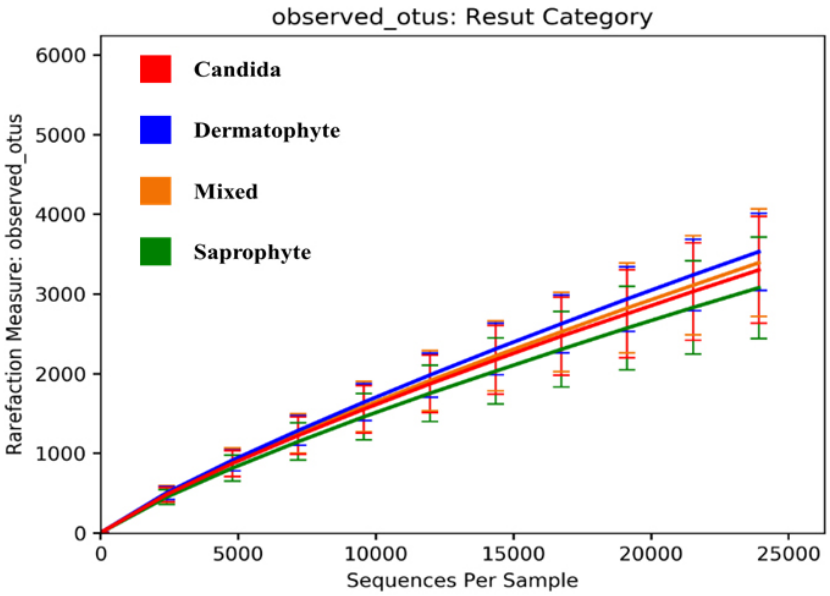


Figure 3. Rarefaction analysis using a sub-set (96) of the fungal nail specimens. Results are reported by specimen category according to PCR. OTUs were calculated at a sequencing depth of 24,000. Data indicate sufficient coverage of sample diversity at the specified sequencing depth (50K reads/sample). No statistical difference is seen between samples that are PCR positive for different fungal groups

FUNGAL IDENTIFICATION: PCR COMPARISON WITH NGS

PCR Positive	PPA by Sample	PPA by Organism
All	95%	91%
Dermatophyte	98%	98%
Yeast	88%	85%
Saprophyte	93%	92%
Mixed Organisms*	90%	80%

* Two or more fungal groups detected in same sample by PCR

Table 3: The percent positive agreement (PPA) with NGS was determined for 252 positive fungal PCR results from 212 onychomycotic nail samples. Results are reported per sample (PCR and NGS agree) and per organism (PCR and NGS agree for every organism detected in the sample).

FREQUENCY OF FUNGAL GENERA IN ONYCHOMYCOTIC NAILS

Sequencing results from 212 clinical onychomycotic nail specimens were sorted by the number of sequencing tags per OTU per sample. Taxa in the 95th percentile were then determined for each sample. These results were used to determine the frequency of each taxa present across all samples. The number of times each taxa was the majority organism (top hit) in the sample was compared to its frequency to generate a potency factor (Table 4).

Fungal Genus	Frequency (Rank)	Frequency as Top Hit	Potency
*Neoscytalidium	30 (15)	17	57%
*Trichophyton	113 (5)	59	52%
*Microascus	26 (18)	13	50%
*Acremonium	8 (32)	4	50%
* Aspergillus	51 (9)	22	43%
*Epidermophyton	42 (10)	18	43%
*Candida	156 (2)	31	20%
*Fusarium	144 (3)	17	12%
Penicillium	39 (11)	3	8%
Paraconiothyrium	81 (6)	5	6%
*Curvularia	79 (7)	3	4%
unidentified	52 (8)	1	2%
Chromocleista	189 (1)	1	1%
Cladosporium	133 (4)	0	0%
Alternaria	51 (9)	0	0%
Saccharomyces	38 (12)	0	0%
*Microsporum	6 (38)	0	0%
*Genera that are targets for the Bako Fungal Nail PCR Assay			

Table 4. Frequency of Fungal genera in 212 clinical nails from patients with an onychomycosis diagnosis. From the information collected, one can see that there are many genera that show up in the 95th percentile in a majority of the samples. For example, Candida and Fusarium ranked number 2 and 3 respectively. However, their potency is low (20% and 12% respectively) so their presence is less likely to be the cause of the clinical nail infection. On the other hand, Acremonium, ranked number 32 in frequency, has a higher potency (50%) and is thus more likely be a cause of clinical fungal nail infection when present. Trichophyton has a high frequency and potency as expected.

CONCLUSIONS

The Bako multiplex PCR assay for fungal identification in human nail samples agrees well with gold standard conventional methods of diagnosis for onychomycosis using PAS/GMS staining showing a high accuracy (85%), specificity (93%) and PPV (94%). Its advantages include fast turnaround time, high capacity, and specificity for a broad range of fungi and sensitivity equivalent to or greater than histology. Sampling issues are well known causes of error in these types of comparisons due to imbalanced distribution of fungal elements within different parts of the same nail used for each technique and can affect sensitivity (79%) and NPV (75%).

NGS of the fungal ITS region was undertaken for 384 clinical samples positive for fungal elements according to conventional PAS/GMS staining and PCR. Of those, 212 met quality criteria and were considered for further analysis. Rarefaction analysis indicates 50K reads per sample is adequate to cover the diversity seen with no difference between fungal groups. There is a 95% positive agreement between the PCR and NGS techniques at the sample level. In addition, NGS allows more in depth analysis of the fungal population in this large collection of nails. The Bako PCR assay can return positive results for more than one fungi, and NGS confirms 91% of all organisms detected by PCR.

Lastly, NGS analysis of onychomycotic nails shows promise in helping to understand the distribution of fungal organisms in clinical nail infections. This includes which fungi, when present, may be more likely to be the cause of infection rather than just a commensal attachment. Such information could lead to more focused treatment regimens.

METHODS

Histology: Clinical nail samples were prepared and examined for fungal elements according to Bako procedures for grossing, fixation, embedding and staining. Nails were examined with microscopy by a dermatopathologist.

Multiplex PCR: Clinical nail samples were extracted by bead beating followed by incubation in a Proteinase K lysis buffer for 30 minutes. DNA was 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. PCR reactions are assembled on a Hamilton Starlet workstation in 384 well format for the PCR screen using Bako screen primer sets and SYBR Green reaction mix (ThermoFisher, Waltham, MA) and analyzed on the QuantStudio 6 real time PCR machine using real time PCR and melt curve analysis. Positive screen samples were analyzed as above using Bako Reflex primer sets and SYBR green reaction mix on the ABI 7500. Raw data in the form of Ct and Tm values are auto analyzed by the Bako PCR Engine followed by manual review of results.

NGS: DNA from 384 clinical sample isolated through the PCR process workflow were used to generate the NGS amplicon for sequencing as follows: 12.5 ng DNA amplified with ITS primers, followed by index PCR, library normalization, pooling and sequencing on the MiSeq (Illumina) using 600 cycle V3 flow cell producing 100,000 paired end, 2x300 base reads (Omega Bioservices, Norcross, GA). Fastq files were analyzed by the ITS Metagenomics UNITE 20160131db Application from Illumina Professional Services (beta version). Data reduction and 95th percentile calculations for all samples were performed on the Stata Data Analysis and Statistical Software package (v15) from StataCorp (College Station, TX).

REFERENCES

*Original Poster: E. Gustafson, L. Bennett, I. Akinsanmi, B. Bakotic, Bako Pathology; Molecular Analysis of Fungal Populations in Patients with Onychomycosis Using Next Generation Sequencing (NGS) and Real-Time PCR, Association for Molecular Pathology, 2017.
Note: Bako is continuing efforts to make improvements in the assays described to better correlate with histopathology. Over time, Bako envisions further improvements to increase the accuracy and utility of the molecular assay described in the poster.

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