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Amplicon-Based Next-Generation Sequencing for Detection of Fungi in Formalin-Fixed, Paraffin-Embedded Tissues

Correlation with Histopathology and Clinical Applications

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Invasive fungal infections are increasing in prevalence because of an expanding population of immunocompromised individuals. To reduce morbidity and mortality, it is critical to accurately identify fungal pathogens to guide treatment. Current methods rely on histopathology, fungal culture, and serology, which are often insufficient for diagnosis. Herein, we describe the use of a laboratory-developed internal transcribed spacer—targeted amplicon-based next-generation sequencing (NGS) assay for the identification of fungal etiology in fungal stain—positive formalin-fixed, paraffin-embedded tissues by using Illumina MiSeq. A total of 44 specimens from 35 patients were included in this study, with varying degrees of fungal burden from multiple anatomic sites. NGS identified 20 unique species across the 54 total organisms detected, including 40 molds, 10 yeasts, and 4 dimorphic fungi. The histopathologic morphology and the organisms suspected by surgical pathologist were compared with the organisms identified by NGS, with 100% (44/44) and 93.2% (41/44) concordance, respectively. In contrast, fungal culture only provided an identification in 27.3% (12/44) of specimens. We demonstrated that NGS is a powerful method for accurate and unbiased fungal identification in formalin-fixed, paraffin-embedded tissues. A retrospective evaluation of the clinical utility of the NGS results also suggests this technology can potentially improve both the speed and the accuracy of diagnosis for invasive fungal infections. *(J Mol Diagn 2020, 22: 1287—1293; https://doi.org/10.1016/j.jmoldx.2020.06.017)*

Invasive fungal infections are on the rise worldwide because of the rapid expansion of immunocompromised patients.[1,2] As prompt identification of fungal etiology is key for administering effective antifungals to reduce morbidity and mortality, more sensitive and accurate diagnostic methods are warranted. However, current methods rely mainly on histopathologic examination, fungal culture, and serology, which are often insufficient in diagnosing invasive fungal infections, even when used in combination.[1] Although fungal serology is available for a limited subset of organisms, primarily dimorphic fungi and *Aspergillus* spp, the specificity is problematic, with cross-reactivity and previous exposure confounding the result interpretation.[1,3] Both fungal culture and histopathologic examination, considered the current gold standards, lack sensitivity.[4] In addition, specimen collection is a universal challenge for clinical microbiology laboratories because histopathology requires formalin-fixed tissues, which leads to reduced availability of fresh tissues for fungal culture.

Histopathologic examination provides a description of fungal morphology observed in tissue using special stains (Grocott or Gomori methenamine silver, hematoxylin and

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eosin, and periodic acid-Schiff stains), but cannot provide accurate species-level, or often even the genus-level, identification.[3,5] Although certain morphologies may be more specific (eg, *Coccidioides* spp are classified as spherules and endospores), many organisms share common morphologies and there is variation in observed morphologies that deviate from the classic characterization, leading to misidentification of fungi.[3] For instance, a wide array of fungi sharing nearly identical histopathologic morphology can be responsible for mycetoma, including *Aspergillus* spp, *Scedosporium apiospermum*, *Acremonium* spp, and *Curvularia* spp, making diagnosis based on histopathology difficult.[6] Multiple studies have demonstrated discordant results (20% to 80% disagreement) between histopathology and fungal culture.[3,7–9] The highest rates of discrepancies occur in the cases of invasive septate molds,[3] which comprise the largest morphologic group of pathogenic fungi. Another challenge is that fungal culture may take several days or weeks and have low yields, limiting the utility of relying on both culture and histopathology to make final diagnosis.[8] These challenges highlight the need for better tests that can rapidly detect fungus directly from fixed tissues with higher sensitivity and accuracy.

Currently, limited molecular assays are commercially available for formalin-fixed, paraffin-embedded (FFPE) tissue. With increasing diversity of both pathogenic and opportunistic fungi, development of novel molecular assays that can broadly detect and identify fungi to the species level directly in FFPE tissue is crucial. Herein, we describe the use of a laboratory-developed ribosomal internal transcribed spacer (*ITS*)–targeted amplicon-based next-generation sequencing (NGS) assay for the identification of fungal etiology in fungal stain–positive FFPE tissues.

Materials and Methods

Ethics

This study has been determined to be exempt by the Institutional Review Board at the University of California, Los Angeles.

Sample Selection

FFPE blocks were selected by a surgical pathologist (K.L.L.) using the following criteria: presence of fungal organisms by hematoxylin and eosin, periodic acid-Schiff, and/or Grocott or Gomori methenamine silver stain; and age of block was <3 years. Sequencing results were not utilized in any way to impact patient care. Cases with scant tissue (<0.3 cm^2) were excluded. A total of 44 specimens comprising 35 unique cases were included in this study. Cases included autopsy ($n = 12$ cases) and nonautopsy ($n = 23$ cases) tissues from patients with diverse clinical histories and were taken from a wide spectrum of anatomic sources. Quantification of the overall fungal burden was performed on a scale where 1 indicates ≤50; 2, 50 to 100; and 3, ≥100 individual fungal elements (ie, hyphae or yeast) observed per slide.

ITS Amplicon-Based NGS Test

Four slices of FFPE tissue (8 μm thick) were cut and immediately placed into 1.5-mL tubes, sealed with parafilm to minimize oxygen exposure, and stored at 4°C. Extraction was performed within 48 hours after the slices were cut, using the Qiagen QIAmp DNA FFPE Tissue Kit (Germantown, MD) following manufacturer's instructions. Sequencing of the *ITS* has previously been shown to provide accurate identification in clinically prevalent fungi.[10–12] Thus, PCR amplification was performed using previously published PCR settings and primers ITS3F and ITS4R targeting the *ITS2* region[11] using the Takara High Fidelity PCR EcoDry Premix (Kusatsu, Japan). PCR amplicons were cleaned using the Zymo Select-a-Size DNA Clean & Concentrator Kit (Irvine, CA). Fragmentation size and DNA concentration were assessed using the Agilent High Sensitivity DNA kit via Bioanalyzer (Waldbronn, Germany) and Invitrogen Qubit 1X dsDNA HS Assay (Carlsbad, CA), respectively. The libraries were prepared for sequencing using the Qiagen QIAseq 1-Step Amplicon Library Preparation Kit. Amplicon-based NGS was performed using the Illumina MiSeq (San Diego, CA) with the 2 × 250 pair-read protocol. A negative water control starting at the PCR amplification step was included in each run to assess for contamination.

Bioinformatics

Sequencing analysis was performed using two commercial software platforms: Pathogenomix RIPSEQ version 1.0 (Santa Cruz, CA) using the GenBank Fungal 5 database and Three Coin Analytics AmpliSeer version 1.0 (Pleasanton, CA) using the UNITE database (*https://unite.ut.ee*, last accessed February 2, 2019). Quality control criteria were established to filter contaminants and potential spillover from highly positive samples. For Pathogenomix RIPSEQ, the following criteria were established for calling a true positive result: sequences for an organism must comprise >5% of total reads for each sample (% abundance as calculated by number of reads matching organism divided by total number of reads for that sample matching any fungal organism); minimum of 100 reads for that organism. For Three Coin Analytics AmpliSeer, the following criteria were set for all nondimorphic fungi: i) minimum of 1000 reads for each organism; ii) the number of reads for an organism in the sample must be at least 20× the number of reads of the same organism in the negative control of the same run; iii) the number of reads of a particular organism in a sample must comprise at least 1.5% of total reads for that organism across all samples; and iv) if multiple species in the same genus were detected, the species with <10% of the total reads for that genus is considered a spillover from the dominant species and ignored. No minimum read cutoff was set for all dimorphic fungi to maximize the sensitivity because of their

Table 1 Characteristics of Cases

Characteristic	*n* (%)
Specimens	44
Unique cases	35
Autopsy	12 (34.3)
Nonautopsy	23 (65.7)
Fungal burden	
3	26 (59.1)
2	10 (22.7)
1	8 (18.2)
Histopathology	
Septate hyphae	18 (40.9)
Pauciseptate hyphae	15 (34.1)
Hyphae (nondescript)	4 (9.1)
Spherules/endospores	1 (2.3)
Budding yeast/pseudohyphae	4 (9.1)
Mixed	2 (4.5)
Cultures ordered	23 (52.3)
Positive	12 (52.2)
Negative	11 (47.8)
Age of block, years	
<1	17 (38.6)
1–2	22 (50.0)
>2	5 (11.4)

Specimens that had positive fungal staining were selected from the past 3 years. The characteristics, including types of cases, fungal burden, observed histopathology, fungal culture status, and age of block, were included. Fungal burden was defined as follows: 1, <50; 2, 50 to 100; and 3, >100 fungal elements per slide.

high pathogenicity. If RipSeq and AmpliSeer identified the same genus but different species, CLCbio (Qiagen, Hilden, Germany) Amplicon-based OTU Clustering workflow was used to manually analyze the data and the clustered reads were searched by National Center for Biotechnology Information Blast to confirm the species. Because of the nonsterile nature of FFPE samples, common skin and environmental contaminants were occasionally detected, usually below our thresholds. These contaminants were excluded from data analysis (Supplemental Table S1) because of their established nonpathogenicity to humans based on the literature.[13–18]

Comparison of Histopathology and ITS Amplicon-Based NGS Results

After case selection, a second surgical pathologist (G.A.F.), who was blinded from the original case information and NGS results, performed an independent scoring and reporting of fungal morphology and provided a list of suspected organisms that are most consistent with the observed morphology. The following characteristics were noted: presence of hyphal septations; branching angle of hyphae; pigmentation; presence of budding yeasts; presence of pseudohyphae; and presence of both budding yeast and hyphal forms. An agreement was called when at least one organism detected by NGS matched the organism(s) suspected by the surgical pathologist.

Results

Characterization of Selected Cases and Specimens

A total of 44 specimens from 35 patients were included in this study, including both autopsy ($n = 12$) and nonautopsy cases ($n = 23$), as well as varying degrees of fungal burden (Table 1) from multiple anatomic sites (Table 2). The selected FFPE blocks were positive for the following fungal morphologies: septate hyphae ($n = 18$, 40.9%), pauciseptate hyphae ($n = 15$, 34.1%), nondescript hyphae ($n = 4$, 9.1%), spherules/endospores ($n = 1$, 2.3%), budding yeast/pseudohyphae ($n = 4$, 9.1%), and mixed morphology ($n = 2$, 4.5%) (Table 1), representing the spectrum of fungal infections typically seen in the patient population at University of California, Los Angeles.

Performance of the *ITS* Amplicon Analysis by NGS

Sequences acquired per sample averaged 828,191 (13,150 to 3,152,567) raw reads and 539,792 (6,865 to 2,485,181) processed reads (defined by reads that passed all quality control filters and were assigned a taxonomic classification), with a mean of 59.5% of reads processed (4.5% to 92.4%) (Supplemental Table S2). The percentage of reads processed (passing all quality control criteria) can depend on several factors, including the quantity and quality of input microbial DNA, the quality of the sequencing, and the PCR conditions. Low biomass samples generally result in lower values because of the stochastic nature of the library preparation and sequencing, but all values should be interpreted in the context of what is expected given the input sample and experimental conditions. Our observed mean value of 59.5% is well within the expected range of fungal applications given the wide variety of input samples. A similar study using the *ITS1* region reported rarefaction at <25% of total input reads.[19]

In this study, reads mapped to the identified organism ranged from 254 to 2,253,413 (mean, 376,372). As a minimum read cutoff for any dimorphic fungi was not observed, *Histoplasma capsulatum* (254 reads) was the only result with <1000 reads (Supplemental Table S3). In total, NGS identified 20 unique species across the 54 total organisms detected, with 96.3% ($n = 52$) identified to the species level and 3.7% ($n = 2$) with split speciation (*Curvularia affinis/tsudae* and *Aureobasidium pullulans/namibiae*) (Supplemental Table S3), including 40 molds, 10 yeasts, and 4 dimorphic fungi. *Aspergillus fumigatus* was the most commonly detected fungus ($n = 13$), followed by *Rhizopus oryzae* ($n = 7$) and *Rhizopus microsporus* ($n = 6$). The most common contaminants detected were *Penicillium* spp, *Mycosphaerella* spp, and *Malassezia* spp (Supplemental Table S1).

Table 2 Anatomic Sources of FFPE Tissue

Source	Organ system	Specimens, *n* (*N* = 44 total)
Cardiovascular/circulatory (*n* = 5)	Heart	2
	Aorta	1
	Pericardium	1
	Femoral artery	1
Gastrointestinal (*n* = 2)	Ileum	1
	Fistula	1
Genitourinary/endocrine (*n* = 3)	Bladder	1
	Adrenal	1
	Kidney	1
Head and neck (*n* = 13)	Cheek	1
	Esophagus	1
	Nasal cavity	1
	Orbit	1
	Palate	1
	Sinus	7
	Skull base	1
Lower respiratory (*n* = 16)	Lung	16
Skin (*n* = 5)	Arm	2
	Abdomen	1
	Leg	1
	Neck	1

Specimens were categorized into the anatomic source and organ system.
FFPE, formalin fixed, paraffin embedded.

Comparison of Histopathology to NGS

The histopathologic morphology corresponded to the organisms identified by NGS, with 100% (44/44) concordance. For septate hyphae with acute angle branching, NGS identified *Aspergillus* spp, *Alternaria* spp, *Aureobasidium* spp, *Curvularia* spp, *Fusarium* spp, *Scedosporium* spp, and *Trichosporon* spp. Broad pauciseptate hyphae corresponded with *Mucor* spp, *Rhizomucor* spp, and *Rhizopus* spp, whereas budding yeast corresponded with *Candida* spp and *Histoplasma* spp. The single case of spherules and endospores was identified by NGS as *Coccidioides posadasii* (Table 3 and Supplemental Table S4). However, in eight cases, NGS identified more than one organism with all of the additional organisms matching the described morphology except for the addition of *Candida* spp in three specimens. One was confirmed by culture; the second was from a site with established *Candida* colonization (sinus); and the third was from an autopsy sample in which *Candida* may have been a skin flora contaminant (Supplemental Table S4).

When comparing NGS results with suspected organisms by the surgical pathologist, 41 of 44 (93.2%) agreed. One discrepant case was an ileum sample suspected to have *Candida* spp by the surgical pathologist but the *ITS* amplicon result revealed *H. capsulatum* (Supplemental Table S4). In another case, *A. pullulans/namibiae* was detected by NGS in a lung specimen, but the surgical pathologist suspected a group of fungi, including *Aspergillus*, *Fusarium*, *Scedosporium*, *Trichoderma*, *Paecilomyces*, *Scopulariopsis*, *Acremonium*, *Schizophyllum*, *Phaeoacremonium*, or *Trichosporon*, collectively designated as the A-T group organisms. In the third case, a skull base lesion was suspected to have an A-T group organism or *Candida*, but instead NGS detected *C. affinis/tsudae*. In all three cases, the organism had the same morphology as the suspected organism.

Although we initially sought to compare fungal serology results with our sequencing results, we discovered that appropriate fungal serology, aside from the two dimorphic fungi cases (*Coccidioides* and *Histoplasma*), was not ordered in the vast majority of cases (*Aspergillus*, *Candida*,

Table 3 Histopathologic Morphology Compared with NGS Results

Histopathology observation	NGS identification (genus)	Samples, *n*	NGS identification (species)
Septate hyphae with acute angle branching	*Alternaria* spp	3	*A. alternata* (*n* = 2), *A. metachromatica* (*n* = 1)
	Aspergillus	14	*A. fumigatus* (*n* = 13), *A. tubingensis* (*n* = 1)
	Aureobasidium spp	1	*A. pullulans/namibiae* (*n* = 1)
	Curvularia spp	1	*C. affinis/tsudae* (*n* = 1)
	Fusarium spp	4	*F. solani* (*n* = 4)
	Scedosporium spp	1	*S. boydii* (*n* = 1)
	Trichosporon spp	1	*T. asahii* (*n* = 1)
Broad pauciseptate hyphae	*Mucor* spp	1	*M. circinelloides* (*n* = 1)
	Rhizomucor spp	2	*R. miehei* (*n* = 1), *R. pusillus* (*n* = 1)
	Rhizopus spp	13	*R. microsporus* (*n* = 6), *R. arrhizus* (*n* = 7)
Budding yeast	*Candida* spp	9	*C. orthopsilosis* (*n* = 1), *C. albicans* (*n* = 5), *C. glabrata* (*n* = 2), *C. parapsilosis* (*n* = 1)
	Histoplasma spp	3	*H. capsulatum* (*n* = 3)
Spherules and endospores	*Coccidioides* spp	1	*C. posadasii* (*n* = 1)

The observed histopathologic morphology was divided into four categories: i) septate hyphae with acute angle branching, ii) broad pauciseptate hyphae, iii) budding yeast, and iv) spherules and endospores. The sequencing results for each category are provided with corresponding number of samples and speciation.
NGS, next-generation sequencing.

Fusarium, and *Trichosporon*) or was not relevant to the identified organisms (eg, β-D-glucan is not typically found in cases with *Mucor*, *Rhizopus*, and *Rhizomucor*) (Supplemental Table S5). When comparing with culture results, only 52.3% (23/44) of FFPE blocks had a corresponding fresh tissue submitted for fungal culture; and of those fungal cultures ordered, only 52.2% (12/23) were positive, leading to only a 27.3% (12/44) fungal culture positive rate in all FFPE specimens (Table 1 and Supplemental Table S6).

Discussion

Herein, we described the test development of an *ITS* amplicon-based NGS for identification of fungi in FFPE tissues. Fungal organisms were identified in a variety of anatomic sites with varying fungal burdens. Although degradation of fungal DNA in FFPE is known to affect molecular testing,[20] fungal DNA has been successfully detected in blocks for up to 7 years.[15] In this study, *ITS* amplicon-based NGS testing could successfully detect fungi in FFPE blocks up to 3 years old. Samples were also tested with various fungal burdens. Fungi with <50 organisms seen in a slide were identified and species-level identification was obtained with processed reads as low as 6,865. The lowest fungal burden was estimated to be 10 organisms per slide. This emphasizes the ability of *ITS* amplicon-based NGS to detect organisms even when fungal burden and read count are low. In one case of *H. capsulatum*, just 254 high-quality sequence reads provided a correct identification. The ability of *ITS* amplicon-based NGS to detect low amounts of fungal DNA is promising for solving challenging and insidious fungal infection cases. Moreover, NGS identified 20 unique organisms of 44 samples, demonstrating its ability for unbiased broad-range fungal pathogen detection and high specificity. This is particularly important as there is a wide variety of common and unusual opportunistic fungal pathogens that may cause devastating infections in the immunocompromised patient population.

One-hundred percent concordance was observed between the *ITS* amplicon-based NGS results and the morphology described by an experienced surgical pathologist. When comparing NGS results with suspected organism group, 41 of 44 (93.2%) agreed. In practice, pathologists vary in their morphology reporting policy and experience, with some reporting genus-level identification based on morphology alone. One retrospective study found that 21% of pathology cases were misidentified when compared with culture results, with *Rhizopus*, *Coccidioides*, *Histoplasma*, *Scedosporium*, *Fusarium*, and *Aspergillus* spp being among those misidentified.[9] Errors can occur as a result of morphologic mimics and inappropriate or inconsistent terminology usage,[21] which can be circumvented by implementing standard reporting of morphology rather than attempting organism identification. Furthermore, pathogen-associated inflammatory response observed in tissue may be altered and hyphae may be degenerated, which may lead to differences in selected staining and affect the screening and characterization of microorganisms.[3,22] In this study, even a highly experienced pathologist was only able to provide a definitive group of organisms in 32 of 44 (73%) samples, whereas the *ITS* amplicon-based NGS test provided definitive species-level fungal identification in 100% of cases, further highlighting the advantage of NGS.

On retrospective chart review, two cases representatively demonstrated the clinical utility of the *ITS* amplicon-based NGS test. In the first case, a pediatric patient with a history of pre−B-cell acute lymphoblastic leukemia presented with violaceous targetoid lesions on extremities and disseminated fungal disease confirmed by computed tomography scan. A skin biopsy was collected, and the pathologic examination noted numerous septate hyphae consistent with *Aspergillus* spp. This observation was further supported by a positive galactomannan enzyme immunoassay while all fungal cultures remained negative. However, the NGS test revealed *Rhizomucor miehei*, a completely different mold requiring different antifungal treatment. This result was confirmed by a reference laboratory using Sanger sequencing. In the second case, tissues from multiple gastrointestinal sources were taken from a geriatric patient, where the pathologic report noted budding yeast consistent with *Candida* spp. This observation led to an interpretation by the infectious disease clinicians as normal gut flora contamination, and the patient was discharged without treatment. The NGS test revealed *H. capsulatum*, which was confirmed by the same reference laboratory using Sanger sequencing. No organisms were recovered by fungal culture in either case. In both cases, the NGS results would have changed the diagnosis to a more aggressive fungal infection and lead to appropriate antifungal treatment. We are in the process of assay validation and implementation in the clinical laboratory setting. Once implemented, we plan to further assess the real-time clinical impact of this NGS assay.

The reported *ITS* amplicon-based NGS assay improved detection of polymicrobial fungal infections, which is particularly important in immunocompromised[21] and burn[23] patients. Of 44 specimens, the NGS assay detected greater than one organism in eight specimens (18.2%), whereas histopathologic examination identified more than one organism in only two of the specimens (4.5%). This is not surprising as the typical morphologies of the organisms are similar, making it nearly impossible to differentiate based on pathologic examination alone. In one case, two yeasts (*Candida glabrata* and *Candida albicans*), which require different empirical antifungal treatments and are indistinguishable by histopathology, were both identified by the *ITS* amplicon-based NGS assay.

Complementing the literature,[3,7,8] fungal culture was only ordered for 52.3% of the specimens and recovery of fungi was low (52.2%) for those with submitted specimens,

making the overall fungal culture-positive rate <30%. *Mucorales* was detected in 34.1% of our specimens and are delicate molds that are often killed during tissue processing before fungal culture.[24] Moreover, the lack of submitted fungal cultures has long been problematic for diagnosis as biopsies are often shared between histology and microbiology, with numerous tests and stains ordered, reducing the amount of tissue available for fungal culture. The reported *ITS* amplicon-based NGS assay uses only four slices of FFPE tissue, thus representing a more attractive method for low-yield specimens.

Although NGS technologies are more sensitive than the conventional methods, contamination can be a challenging issue. FFPE is not processed in a sterile manner, allowing for introduction of environmental contaminants. In this study, *Malassezia* spp and *Penicillium* spp were identified as the most commonly encountered contaminants, which is consistent with other studies.[15,25] To reduce noise from environmental contaminants, we established a stringent bioinformatic filtering system, which drastically reduced environmental contaminants being identified as true positives. Others have used an SD above the mean read approach for defining significance.[25] Because of the potential contamination issues, NGS results should always be interpreted in conjunction with histopathology, fungal culture, and clinical presentation to confirm a diagnosis.

The biggest limitation of this study, as well as with any *ITS* amplicon-based NGS in general, is that the current fungal databases are incomplete and less developed than the bacterial databases. To address this concern, two separate bioinformatics platforms using two different databases were used to achieve the most accurate fungal identification. Pathogenomix RIPSEQ utilizes the GenBank Fungal 5 database, whereas Three Coin Analytics AmpliSeer uses the UNITE database. The UNITE database is an online database that targets the *ITS* region specifically,[26] which is the region amplified by our primers. This database is routinely updated and curated, allowing for continual improvements, tracking of changes, and revised nomenclature.[26] On the other hand, GenBank is a broader database covering fungal and nonfungal organisms. Caution should be exercised with any database as sequence identification may not be 100% accurate. With GenBank, an estimated 20% of fungal sequences are incorrectly annotated because of nonupdated taxonomic names, unpublished sequences, and partially named sequences.[27] Multiple fungal databases should be compared to avoid misidentification by a single database. Any species-level discrepancies were addressed using the CLCbio Amplicon-based OTU Clustering workflow and then manually searched the clustered reads using National Center for Biotechnology Information Blast to confirm the species identification.

Different bioinformatic pipelines were found to require different cutoffs to remove false positives while ensuring sensitivity, as described in a study investigating NGS for corneal infection identification.[28] Both user-friendly platforms generated comparable results. RipSeq provided a cleaner result and was conducive for per-sample analysis, whereas AmpliSeer was slightly more sensitive and preferred for per-run analysis. The ability to compare reads of each organism across runs allowed us to assess for sample spillover and contamination more easily. Our bioinformatics workflow consists of the following steps: compare sequences on a per-run analysis to remove environmental contaminants and spillover from samples with high number of reads; and compare sequences on a per-sample basis to confirm a true-positive result, as detailed in *Materials and Methods*. With this workflow, analysis takes roughly 3 to 4 hours per sequencing run of 12 to 18 samples. As NGS continues to increase in use in the clinical laboratory, the fungal databases will likely improve as a result of increased sequencing data becoming publicly available.

NGS-based metagenomic sequencing has already shown promise for bacterial pathogen detection in fresh samples.[29] Pan-fungal PCR, followed by Sanger sequencing assays, has also been available for more than a decade.[11,30–32] However, there are no fungal-specific NGS assays commercially available for FFPE tissues currently. With fungal-stain positive FFPE samples, a targeted approach would be preferred to reduce cost and increase specificity. FFPE tissues are the ideal specimens for pursuing unbiased fungal-targeting NGS as histopathology can provide an initial guide for fungal positivity, allowing for selection of samples that would be most appropriate for *ITS* amplicon sequencing. Because of the increased sensitivity of NGS assays, false-positive results due to contamination are a general concern. Therefore, enhancing the NGS test specificity by improving the pretest probability is critical. The assay developed in our study used a targeted approach to specifically amplify fungal sequences, thus avoiding the interference of nonfungal DNA contaminants. In addition, the specimen acceptance criteria are set, requiring all FFPE samples to have fungal elements visually present by histopathologic examination, which further enhances the specificity of the test. Histopathology is readily available and widely used, making it an easy initial screen for relevant samples, and provides a reference for NGS results. By comparing organism identification with expected morphology, a stronger support for diagnosis is generated. This also encourages collaboration and open discussion between surgical pathology and microbiology divisions, and ultimately improves patient care.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2020.06.017*.

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