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Commentary

Molecular Diagnostics for Invasive Fungal Infections

A Call for Refinement and Implementation

Gary W. Procop

From the Section of Clinical Microbiology, Department of Clinical Pathology, Cleveland Clinic, Cleveland, Ohio

Fungal infections are a silent epidemic. In contrast to the epidemics caused by RNA viruses such as influenza A and dengue, which are the material of headline news, the epidemic of fungal infections is played out quietly with few in attendance. Fungal infection affects immunocompromised hosts, patients hospitalized with severe underlying diseases (eg, acute myelogenous leukemia), those requiring complex surgical procedures (eg, trauma patients), and individuals who require support in intensive care units. And this epidemic continues to grow. The causes of this ever-increasing immunocompromised population include congenital and infectious causes of immunosuppression (e.g., the heretofore uncontrolled AIDS pandemic), the ongoing success of stem cell and solid organ transplantations, the need for chemotherapy and immunotherapy for the treatment of patients with cancer, and the use of new monoclonal antibody therapy for conditions such as rheumatoid arthritis and Crohn's disease, as well as a variety of other reasons.[1–4]

The types of fungal infection that occur in the patients described vary accordingly with the type of underlying disease of the host, as well as with their epidemiological exposure.[5] For example, patients with profound neutropenia are at heightened risk for invasive filamentous fungal infections, whereas patients in the intensive care unit who have numerous indwelling intravascular access catheters are at increased risk for infections by yeasts, such as *Candida* species. Many patients, however, have multiple risk factors (eg, patients with neutropenia often also have indwelling intravascular catheters).

Although *Aspergillus fumigatus* and *Candida albicans* are the most common causes of invasive filamentous fungal infections and yeast-related infections in these patients, there is such a wide variety of opportunistic fungal pathogens that it is impossible to determine which type of fungus may be the specific cause of an infection in an individual patient based on clinical findings and risk factors alone. For the laboratory detection of these organisms, we have relied on age-old techniques, which have a variety of limitations. These techniques include histopathology and other forms of direct examination, and culture. Although histopathology is useful in confirming the presence of many types of fungal infection, by the time the patient requires surgery the disease has usually progressed and has often deeply infiltrated the tissues. In addition, even the most highly skilled infectious disease pathologist has limitations in evaluating the morphological differentiation of many fungi that cause invasive disease.[6] For example, although a surgical pathologist can differentiate the hyphae of zygomycetes from hyaline septate molds, it is not possible, in most instances, to differentiate the type of zygomycete present or to definitively differentiate the types of hyaline septate molds (eg, *Aspergillus* versus *Fusarium* versus *Pseudallescheria boydii*) that may be responsible for the infection.[6] Likewise, although *Cryptococcus neoformans* may be definitively identified in histological sections through the demonstration of the capsule using histochemical stains, the yeast of the *Candida* genus, apart from *Candida glabrata,* cannot be differentiated from one another on a morphological basis alone.[6]

Culture, which is sometimes erroneously considered definitive evidence of infection, also has a number of limitations. Culture may be too insensitive for the detection of fungal pathogens in some instances. In a well-controlled animal model study wherein neutropenic rabbits were given histologically confirmed pulmonary aspergillosis, Francesconi et al[7] determined that the most sensitive methods of detecting the presence of the pathogen in the bronchoalveolar lavage fluid were galactomannan and a quantitative *Aspergillus*-specific PCR, with sensitivities of 100 and 80%, respectively; in contrast, the sensitivity of culture was only 46%. In this study, the sensitivities of galactomannan, quantitative PCR, and culture fell to 92, 50, and 16%, respectively, after antifungal therapy.[7] In addition to false-negative cultures, issues with false-positive results also exist. Fungal agar plates of clinical

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Address reprint requests to Gary W. Procop, M.D., M.S., Clinical Pathology/L11 Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195.

specimens may be easily contaminated with ubiquitous fungal spores, or transient fungal microbiota that do not represent the etiological agent of disease. Perfect et al,[8] in a multicenter review, demonstrated that most *Aspergillus* culture isolates from nonsterile body sites did not represent disease. Although the likelihood that a culture that was positive for *Aspergillus* actually represented true disease increased for patients in high-risk groups, such as bone marrow transplant recipients and those with hematological cancers,[8] they concluded that better diagnostic tests are necessary.

The specific determination of the type of fungus causing the infection in the immunocompromised host is critical, because the taxonomic designation of the organism provides useful information regarding the types of antifungal agents that may be most useful in treating the patient.[5] Unlike bacteria, the antifungal profile of fungi is largely stable; however, when patients are receiving long-term, suppressive antifungal therapy, even usually susceptible fungi may develop resistance (eg, fluconazole-resistant *C. albicans* recovered from patients who have received fluconazole for an extended period of time).[9–11] The challenge, therefore, is essentially a diagnostic one, and the words of a patriarch of American medicine, Sir William Osler, M.D., ring as true today as they did on the day they were spoken: "There are three phases of treatment, diagnosis, diagnosis, diagnosis."

Although we have learned much from the pioneers of transplant-related infection diagnostics, the detection of fungi in the bloodstream of patients at risk will probably prove to be considerably more complicated than determining cytomegaloviral, Epstein-Barr viral, or BK viral loads. Whereas a particular virus is targeted by a particular quantitative molecular assay, any one of numerous fungi may be responsible for an infection in an appropriate host, and the successful assay or assays devised will need to account for this. It is of limited value to use an assay that detects only a single pathogen, even if that pathogen is among the most frequently encountered (eg, *A. fumigatus* or *C. albicans*), because the patient may actually be dying of fusariosis or cryptococcosis. Successful approaches will probably involve either a multiplex or broad-range PCR approach. Another challenge that the molecular diagnostician will have to address with these agents that is not a problem with transplant-associated viruses is environmental contamination. The fungi that cause infections in this patient population are either commonly found in the environment (eg, *Rhizopus* is a common bread mold) or are part of the normal endogenous microbiota of the patient (eg, *Candida* species). This prevalence could lead to reagent and specimen contamination, which potentially could result in false-positive reactions if qualitative assays were used.[12] Although it remains to be determined in clinical practice, the quantitative nature of rapid cycle PCR may possibly be useful for differentiating low-level background contamination from fungal loads that are demonstrated to be significant through well-controlled studies. Cuenca-Estrella et al[13] have demonstrated such a finding using an *Aspergillus*-specific PCR. Similar applications have also been demonstrated in an animal model.[14]

Mandviwala et al[15] have approached many of these issues in this issue of *The Journal of Molecular Diagnostics.* They have used broad-range PCR with ITS1 and ITS2 primers, which amplify the most common fungal pathogens and have tested this assay using a battery composed of the most commonly isolated medically important *Candida* species, which is an excellent start. The amplicon was detected with a third-generation, high-saturating dsDNA binding dye and monitored in a rapid cycle format, which affords the opportunity to determine the amount of organism present (ie, the fungal load). In contrast to using either traditional (Sanger) sequencing or pyrosequencing to determine the identity of the fungus present, this group used high resolution melt curve analysis to determine the *Candida* species present. They first built a library of high resolution melt curves that were generated from well-characterized *Candida* species and then compared the high resolution melt curves generated from an unknown with this library to generate a match, much the same way in which an unknown DNA sequence is submitted for a BLAST search, albeit with a different level of discrimination at the nucleotide base level. The authors are complemented for simultaneously addressing many of the issues that will probably prove important in the detection of a variety of potential pathogens (ie, a broad-range approach): a means to determine fungal load (ie, a way to differentiate low-level background contamination from medically important values) and a simple and direct method of species level differentiation, which will have a direct impact on therapy. In addition, such an approach is likely to prove cost-effective, given that it does not require fluorophore-labeled probes or more expensive postamplification analysis, such as DNA sequencing or microarray hybridization.

It is beyond debate that improved diagnostic assays are needed for the early diagnosis of patients with invasive fungal infections. Although antigen-based assays (ie, galactomannan and β-glucan) hold promise when used appropriately, nucleic acid molecular diagnostic assays will probably be serious contenders as optimal assays for these applications. The ability to determine the identity of the infecting species, whether it is derived from a multiplex or a broad-range approach, is the differential advantage of nucleic acid testing compared with antigen-based assays. The article from Mandviwala et al,[15] as well as others in the literature, has demonstrated the feasibility and the potential promise of improved diagnostics for patients with fungal infections. We need, however, to take the next steps to refine these assays, use them clinically in a routine manner to fulfill this promise, and work with our clinical colleagues to help curtail this silent epidemic.

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