

Rapid In-situ Hybridization for Dematiaceous Fungi Using a Broad-spectrum Oligonucleotide DNA Probe

Kathleen T. Montone, MD,* Virginia A. Livolsi, MD,* Donald C. Lanza, MD,†
Michael D. Feldman, MD, PhD,* David W. Kennedy, MD,‡ James Palmer, MD,‡
Alexander G. Chiu, MD,‡ and Irving Nachamkin, DrPH, MPH*

Abstract: Dematiaceous fungi are a diverse group of “darkly” pigmented fungi, which contain melanin in their cell walls and are commonly found in soil worldwide. Although morphology and histochemical stains may aid identification in tissue sections, these means for species identification are not specific. In-situ hybridization (ISH) for abundant fungal rRNA sequences may provide a means for detecting dematiaceous fungi. In this study, a 24-base synthetic biotin-labeled oligonucleotide probe targeting rRNA sequences of a variety of dematiaceous fungi was developed. This probe was tested on a cohort of 29 patients with culture-proven cases of dematiaceous fungal-associated rhinosinusitis (26 allergic fungal sinusitis, 2 fungal ball, and 1 acute invasive fungal sinusitis). Fungal cultures were positive for *Alternaria species* (10), *Bipolaris species* (5), *Curvularia species* (10), *Cladosporium species* (1), *Scedosporium prolificans* (1), *Scopulariopsis species* (1), and dematiaceous species, not otherwise specific (1). ISH showed positivity in fungal organisms in 24 of 29 specimens. ISH was negative in culture-proven examples of *Rhizopus species*, *Aspergillus species*, *Fusarium species*, *Paecilomyces species*, *Histoplasmosis capsulatum*, *Candida species*, and *Blastomyces dermatitidis*. ISH with a dematiaceous-specific fungal probe may be useful for differentiating dematiaceous fungi from other filamentous fungi in tissues, particularly those responsible for fungal rhinosinusitis.

Key Words: in situ hybridization, rRNA, fungi, dematiaceous, *Aspergillus*

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Dematiaceous fungi are a diverse group of “darkly” pigmented fungi, which contain melanin in their cell walls and are commonly found in soil worldwide. Initially believed to be nonpathogenic, it is now widely accepted that dematiaceous fungi cause a spectrum of

fungal-related diseases (phaeohyphomycoses) in immunocompromised and immunocompetent hosts including superficial and deep fungal infections, allergic conditions, and rarely disseminated disease.^{1–4}

Dematiaceous fungal hyphae are often variably pigmented, beaded, dilated, and fragmented with irregular septae formation, differing from the standard 45-degree branching and the regular septa formation of *Aspergillus species*.⁵ However, this histologic appearance is often not readily apparent in formalin-fixed, paraffin-embedded (FFPE) tissue. Fontana-Masson stain often highlights the melanin pigment in the dematiaceous fungal cell walls; however, other fungal pathogens including many types of *Aspergillus* and *Zygomycetes* also react with this histochemical stain.⁶ Therefore, if fungal cultures are negative or have not been performed, identification of a specific pathogen may be based solely on histologic appearances, which may not be reliable. However, with newly improved means of treating specific types of fungal infections, the specific fungal agent involved in a disease process is often important for appropriate patient management as not all invasive fungal infections respond in a manner similar to antifungal agents, particularly dematiaceous fungi some of which can be resistant to azoles.^{1–3}

All eukaryotic fungal organisms contain abundant, highly conserved, and species-specific rRNA sequences. Owing to their abundance and species specificity, rRNA sequences have been established as optimal targets for in-situ hybridization (ISH).^{7–9} ISH with site-specific oligonucleotide probes in FFPE tissues has shown that rRNA sequences are preserved in tissues and can be used to identify fungi in tissues.^{10–16} Using cultured, confirmed examples of fungal rhinosinusitis, a disease entity often associated with dematiaceous fungi, we report the use of an ISH assay to detect a variety of dematiaceous fungi in FFPE tissues.

MATERIALS AND METHODS

Patient Material

Twenty-nine FFPE specimens with histopathologically diagnosed fungal rhinosinusitis and culture positivity for dematiaceous fungal pathogens were included in the study. The specimens included 26 cases of allergic

From the Departments of *Pathology and Laboratory Medicine; †Otorhinolaryngology, University of Pennsylvania Medical Center, Philadelphia, PA; and ‡The Sinus & Nasal Institute of Florida, St Petersburg, FL.

The authors declare no conflict of interest.

Reprints: Kathleen T. Montone, MD, Department of Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, 3400 Spruce Street, 6 Founders, Philadelphia, PA (e-mail: kmontone@mail.med.upenn.edu).

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fungal rhinosinusitis (AFRS), 2 cases of fungal ball, and 1 case of acute invasive fungal rhinosinusitis. Fungal cultures were positive for *Alternaria species* (10), *Bipolaris species* (5), *Curvularia species* (10), *Cladosporium species* (1), *Scopulariopsis species* (1), *Scedosporium prolificans* (1), and dematiaceous species, not otherwise specific (1). Controls consisted of 33 FFPE culture-proven examples of *Aspergillus species* (11), *Fusarium species* (3), *Rhizopus species* (6), *Histoplasma capsulatum* (3), *Blastomyces dermatitidis* (3), *Paecilomyces* (4), and *Candida species* (3). Four micron tissue sections were placed on ProbeOn Plus slides for the ISH procedure.

Oligonucleotide Probe

A 24-base DNA probe with the following sequence: 5'-GGTATGACGTCTCCCCTATATGGC-3' targeting the fungal rRNA gene sequences of a variety of dematiaceous fungi¹⁷ was selected and then commercially synthesized, high-performance liquid chromatography purified, and the 3' end terminally biotin labeled with a single biotin-tetra-ethyleneglycol 15-atom spacer arm (Integrated DNA Technology). By GenBank BLAST analysis, this sequence was shown to have 100% homology to nucleic acid sequences of a variety of fungi in the phylum Ascomycota including *Curvularia species* and *Bipolaris species*, and in uncultured soil fungi as well.¹⁸ The lyophilized probe was reconstituted to a concentration of 1 µg/µL in TE buffer, pH 7.5 and was diluted to a final concentration of 1 µg/mL in a nonformamide-based probe diluent, the composition of which has been described earlier.¹⁹

ISH Procedure

ISH with the DNA probes was performed using modifications of earlier described methods using manual capillary action technology on the MicroProbe Staining System (Fisher Scientific).^{10,11} In brief, the tissue sections were dewaxed, cleared, and rehydrated. The tissues were then treated with a full-strength Pepsin solution (Diagnostic Biosystems) for 3 minutes at 105°C. As reported earlier, the short time of using pepsin at a high temperature does not seem to denature the effects of the enzyme and results in uniform ISH results.²⁰ The slides were washed with distilled water, the probe was applied to the slides, and the tissues were heated at 105°C for 4 minutes, cooled at room temperature for 1 minute, and then hybridized at 40°C for 1 hour. After hybridization, the tissues were washed with 0.2 × standard saline citrate 3 times at 3 minutes, each at 40°C. The hybrids were detected with prediluted streptavidin conjugated with alkaline phosphatase (Biogenex) for 20 minutes at room temperature followed by signal development with nitro-blue-tetrazolium chloride (NBT)/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (BCIP) chromogen (Roche) for 20 minutes at room temperature. The slides were washed with distilled water, coverslipped with Universal Mount (Diagnostic Biosystems), and examined by light microscopy. Positive signal was determined by

the presence of any fungal organisms staining a dark blue/purple color.

Negative controls consisted of omission of the probe from the hybridization solution and the paraffin-embedded tissue specimens that were culture positive for nondematiaceous fungi including *Rhizopus species*, *Aspergillus species*, *Fusarium species*, *Paecilomyces species*, *H. capsulatum*, *Candida species*, and *B. dermatitidis*.

RESULTS

The ISH assay was positive in fungal organisms in 24 of 29 specimens (Fig. 1). The 5 negative specimens were AFRS specimens and consisted of 2 specimens each of *Bipolaris species* and *Curvularia species* and 1 specimen of *Scedosporium prolificans*. Two of the negative specimens had undergone decalcification in an HCl-based decalcification solution before tissue processing, whereas the remaining 27 specimens including the remaining 2 negative cases had not been exposed to decalcification solutions. Decalcification in HCl-based solutions have been shown to reduce ISH for nucleic acids including rRNA.²¹ The dematiaceous fungal-specific probe did not hybridize to culture-positive examples of *Rhizopus species*, *Aspergillus species*, *Fusarium species*, *Paecilomyces species*, *H. capsulatum*, *Candida species*, and *B. dermatitidis* (Fig. 2).

DISCUSSION

The definitive diagnosis of tissue-based fungal infections often hinges on the results of fungal cultures. However, fungal cultures may not be performed if infection is unsuspected or culture results may be negative although histopathologic examination shows the presence of a fungal agent. Although there are certain histologic characteristics, which can be used to speciate fungal organisms, these characteristics may not be consistently evident in FFPE tissues. Recently, Sangoi et al²² showed misclassification of fungal pathogens in approximately 20% of patients with fungal infections diagnosed histologically in their institution, particularly filamentous fungi when compared with culture.

Dematiaceous fungi have particular characteristics, which can be used for species identification in the clinical microbiology laboratory.¹⁻³ Dematiaceous fungal hyphae are often beaded, dilated, fragmented, and contain melanin in their cell walls. However, these features are not specific and there are no definitive reliable criteria that can be used to identify these fungal species, particularly in tissue sections. In FFPE tissues, dematiaceous fungal organisms are often fragmented making diagnosis difficult especially in the inspissated mucin characteristics of AFRS in which organisms are often only evident by histochemical stains such as periodic acid-Schiff or silver stains, often fragmented, and may resemble *Aspergillus* particularly if they are not pigmented. Most dematiaceous fungi react with the Fontana-Masson stain for melanin but this stain is not specific and may react with other fungal organisms including *Aspergillus* and *Zygomycetes*, making definitive diagnosis of

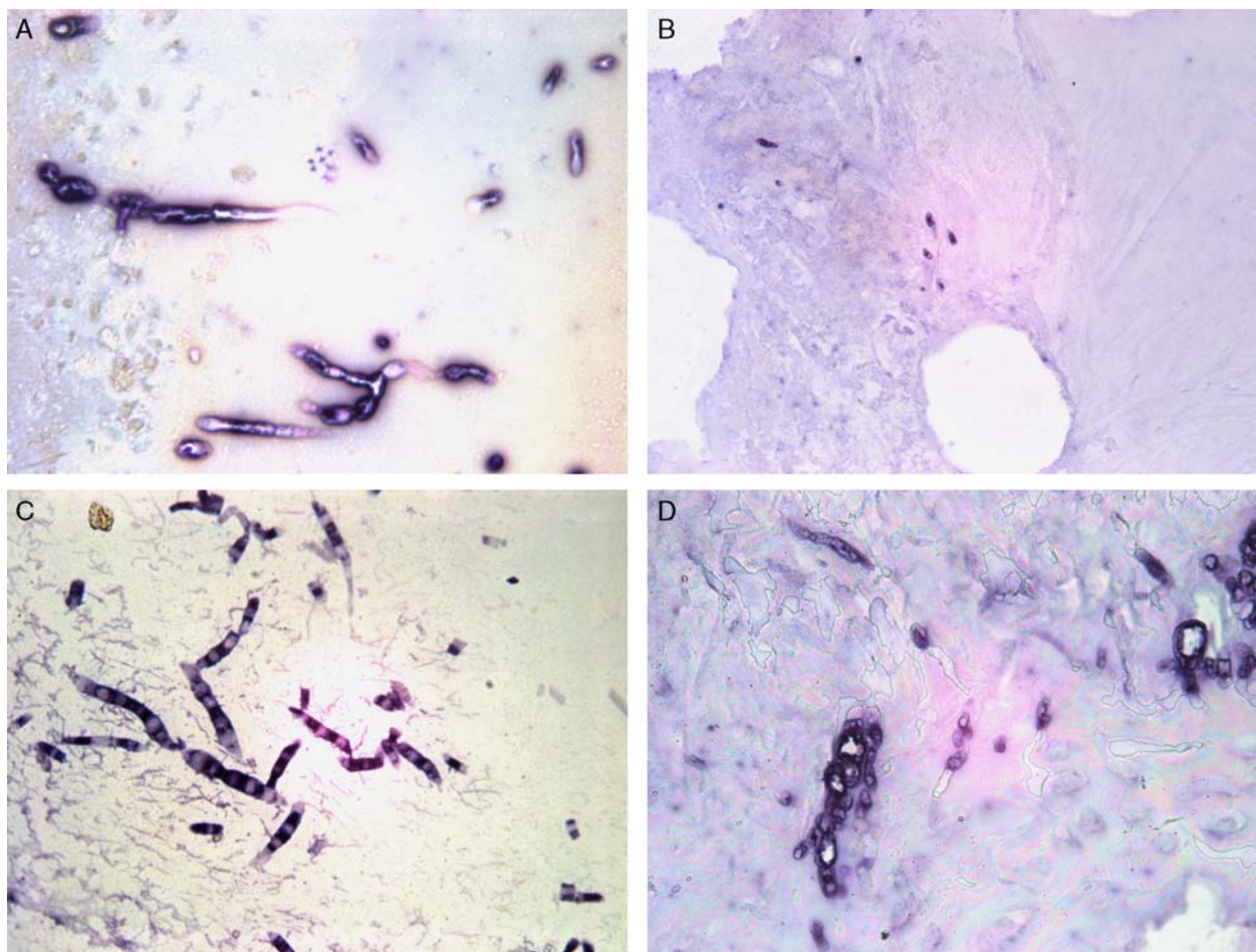


FIGURE 1. A, ISH with the dematiaceous fungal probe in a case of AFS culture positive for *Curvularia species* (NBT/BCIP, original magnification $\times 63$). B, ISH with the dematiaceous fungal probe in a case of AFRS culture positive for *Alternaria species*. Note the fragmented fungal forms (NBT/BCIP, original magnification $\times 40$). C, ISH with the dematiaceous fungal probe in a case of AFS culture positive for *Curvularia species* (NBT/BCIP, original magnification $\times 63$). D, ISH with the dematiaceous fungal probe in a case of acute invasive fungal sinusitis culture positive for *Alternaria species* (NBT/BCIP, original magnification $\times 63$). ISH indicates in-situ hybridization.

dematiaceous fungal infection difficult if fungal cultures are negative.⁶

In this study, we developed an ISH assay for a variety of dematiaceous fungal species, which are commonly isolated in sinonasal fungal disease. The 24-base oligonucleotide probe was selected to be complementary to rRNA gene sequences in the phylum Ascomycota, which contains *Curvularia species* and *Bipolaris species* and other known and unknown soil pathogens as well. The sequences were chosen as it was observed to be conserved among a variety of dematiaceous fungi by GeneBank analysis. This probe hybridized to multiple dematiaceous fungi including *Alternaria species*, *Bipolaris species*, *Curvularia species*, *Cladosporium species*, and *Scopulariopsis species*, and was negative in filamentous fungi such as *Aspergillus*, *Fusarium*, *Paecilomyces*, and *Rhizopus*. Although the probe was evaluated in sinonasal disease, the method may be applicable to dematiaceous fungal infections in other

tissue sites. However, as there are hundreds of species of dematiaceous fungi, it is highly likely that not all dematiaceous fungi will react with this single probe. It is also possible that owing to the broad nature of this probe that other fungal organisms that are nondematiaceous in nature may also react with this probe. However, as noted earlier there was no reactivity seen in a variety of known fungal organisms pathogenic in humans.

ISH targeting rRNA sequences has become a useful means for detecting a variety of specific fungal pathogens in tissues. This method can be performed rapidly and is useful for species identification if fungal cultures are not available. ISH using probes, which detect a variety of fungal pathogens will be useful for triaging tissue-based filamentous fungal infections such as those caused by *Aspergillus*, *Zygomycetes*, *Fusarium*, and dematiaceous fungi in which confirming cultures are not available.²³ As appropriate antifungal therapy often varies with the specific pathogen identified,^{1-3,24} it is often important

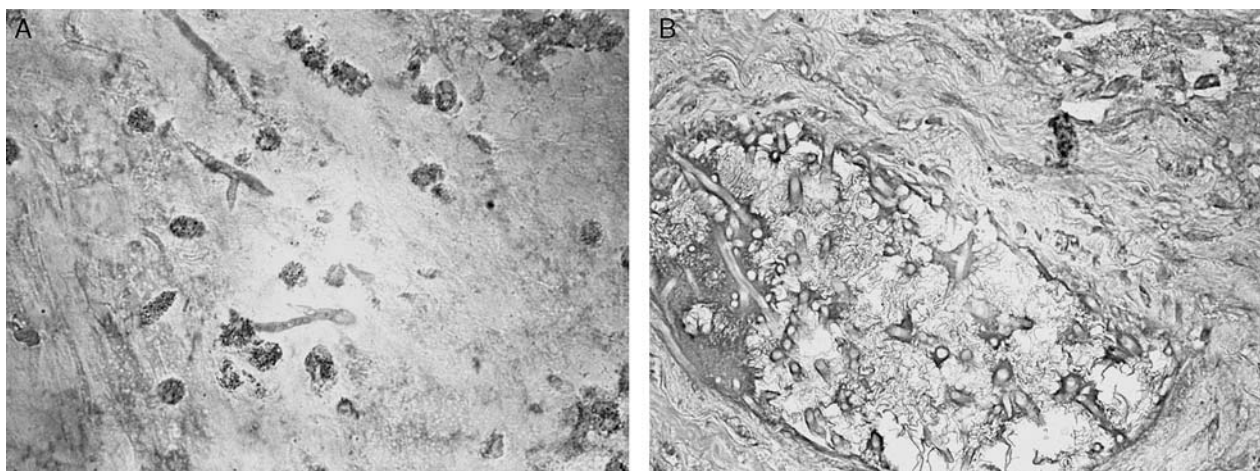


FIGURE 2. A, ISH with the dematiaceous fungal probe in a case of AFS culture positive for *Aspergillus* species. B, ISH with the dematiaceous fungal probe in a case of acute invasive fungal sinusitis culture positive for *Rhizopus* species. ISH indicates in-situ hybridization.

to accurately speciate fungal organisms as quickly as possible.

In summary, an ISH assay specific for a variety of dematiaceous fungi was developed. This assay may be useful along with other species-specific probes in the evaluation of fungal infections, particularly those involving the sinonasal tract when a fungal pathogen is histologically observed but fungal cultures are negative or have not been performed.

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