Rapid Method for Detection of Aspergillus 5S Ribosomal RNA Using a Genus-specific Oligonucleotide Probe

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Ribosomal RNA (rRNA) is present in all prokaryotic and eukaryotic cells. Although sequences are conserved, variations in nucleic acid composition are known to be species specific. Formalin or Bouin's fixed, paraffin-embedded tissue specimens from 17 cases of culture proven Aspergillus sp infections were studied by a rapid (<30 minutes) in situ hybridization procedure using a biotinylated oligonucleotide DNA probe complementary to nucleic acids 1-22 of Aspergillus sp 5S rRNA sequence. Positivity was noted within fungal organisms in all 17 cases and was identified in both hyphal forms and within fruiting bodies. Signal was weak or absent within the center of Aspergillus abscess cavities with increasing signal located toward the periphery of the cavity, suggesting that rRNA in situ hybridization may detect viable fungal forms. In situ hybridization with the oligonucleotide probe on tissues containing several different organisms including Candida sp, Histoplasma capsulatum, Cryptococcus neoformans, Pneumocystis carinii, Pseudallescheria boydii, Fusarium sp, and Mucor were negative. Use of site specific oligonucleotide probes specific for a variety of rRNA sequences may aid in the diagnosis of several medically important bacterial, fungal, and protozoal pathogens. (Key words: Aspergillus; Ribosomal RNA; In situ hybridization; Oligonucleotide probes; Biotin) Am J Clin Pathol 1995;103:48-51.

All prokaryotic and eukaryotic cells contain ribosomal RNA (rRNA) sequences that are necessary for protein synthesis, although the exact function of these nucleic acid sequences is currently unknown. Ribosomal RNA is highly conserved and sequence analysis is important for phylogenetically classifying organisms.1-3 Whereas rRNA consists predominantly of conserved sequences, variable species specific regions also are encountered.1-3 Ribosomal RNA analysis is now used in clinical laboratory settings for diagnosing a variety of infectious pathogens, and in addition rRNA sequence analysis is useful for identifying and classifying unknown organisms.4-6 Because rRNA is present in high copy numbers in individual cells, it is an optimal target for in situ hybridization assays.5,10 The clinical importance of identifying infectious organisms in routine tissue and cytologic preparations is obvious because often cultures are not obtained at the time of specimen collection, or a particular organism is not suspected. This study describes a rapid in situ hybridization assay to detect fungal organisms using a genus-specific probe for Aspergillus rRNA sequences.

The described hybridization protocol is performed in Bouin's or formalin-fixed, paraffin-embedded tissues in less than 30 minutes.

MATERIALS AND METHODS

Aspergillus sp Oligonucleotide Probe

A 22-base oligonucleotide complementary to nucleic acids 1-22 of Aspergillus sp 5S rRNA sequence11 was selected, commercially synthesized and terminally biotin-labeled with 6 biotin moieties (Research Genetics, Huntsville AL).12 The sequence was shown to have 100% homology to A nidulans, A flavus, A niger and Penicillium chrysogenum 5S rRNA sequences by GenBank analysis (BLAST search).13 The lyophilized finished product was reconstituted to a 1 μg/μL solution in Tris-EDTA, pH 7.4 and was stored in 10 μL aliquots at −20 °C before dilution to 200 ng/mL for use in the hybridization reaction.

Tissue Specimens

Seventeen formalin or Bouin's fixed, paraffin-embedded tissue specimens with histologic evidence of septate fungi consistent with Aspergillus sp, which were subsequently proven by fungal cultures were studied. These included examples of A niger (1 case), A flavus (5 cases), A fumigatus (11 cases), and Aspergillus sp (not flavus or fumigatus) (1 case). The specimens represented examples of invasive aspergillosis (12 specimens), localized aspergillosomas (2 specimens), and sinonasal allergic Aspergillus sinusitis (3 specimens). The tissues studied included examples of Aspergillus infection in lung (11 cases), heart, lung, and thyroid (1 case), and sinonasal tract (5 cases).
All specimens contained septate fungal forms consistent with *Aspergillus* that were demonstrated by hematoxylin-and-eosin stains and by silver-methenamine stains. For a control group, 18 examples of tissues containing other infectious agents were used. These included examples of infections by *Candida* species (5 cases), *Cryptococcus neoformans* (3 cases), *Pneumocystis carinii* (5 cases), *Histoplasma capsulatum* (3 cases), *Pseudallescheria boydii* (1 case), and *Fusarium* sp (1 case). In addition, five tissue specimens which contained fungal organisms consistent with *Zygomycetes* (*Mucor*) were also studied. Twenty different normal autopsy and surgical tissue specimens without evidence of fungal infection were also used as negative controls. These included skin, thyroid, lung, kidney, liver, heart, lymph node, colon, and stomach. All tissues were fixed between 2 and 48 hours. No precautions were taken to inhibit endogenous or exogenous RNases.

**In situ Hybridization**

**In situ** hybridization was performed using manual capillary action technology on the MicroProbe Staining System (Fisher Scientific, Pittsburgh, PA) with significant modifications of previously described methods. Briefly, 5 μ sections were cut from the paraffin blocks, floated on a protein-free water bath, placed on ProbeOn Plus slides (Fisher Scientific) and air dried. The slides were placed in the MicroProbe slide holder so as to make a 130 μ gap and all of the subsequent reagents were placed on and drained from the slides by capillary action. The tissues were deparaffinized using a non-xylene based reagent (AutoDewaxer, Research Genetics) for 3 minutes at 105 °C. The tissues were rapidly cleared (AutoAlcohol, Research Genetics), rehydrated (Universal buffer, Research Genetics), and digested with stable pepsin solution (Research Genetics), which was used at full strength (2.5 mg/mL) for 3 minutes at 105 °C. The oligonucleotide probe was diluted to 200 ng/mL in a non-formamide based cocktail (Research Genetics), the composition of which has been described previously. The probe solution was applied to the slides, and the tissues were heated at 105 °C for 2 minutes to denature any secondary rRNA structures, cooled at room temperature for 30 seconds, and the tissue target and probe were allowed to hybridize for 8 minutes at 40 °C. Following hybridization, the tissues were washed with 0.5X SSC for 1 minute at room temperature. The biotinylated hybrids were detected with streptavidin-horseradish peroxidase (Research Genetics) and signal generation was with stable diaminobenzidine (Research Genetics). The tissues were washed with distilled water, counterstained with hematoxylin (Research Genetics) and cover slipped with Universal Mount (Research Genetics). The entire procedure took less than 30 minutes to complete.

**RESULTS**

The **in situ** hybridization assay for *Aspergillus* rRNA produced strong positive staining within the fungal organisms in all 17 cases of proven *Aspergillus* infection. The staining pattern was predominantly granular within the organisms. Positive signal was noted within hyphal forms and within fruiting bodies when present (Figs. 1 and 2). In all cases, the majority of organisms stained, except in those with extensive tissue necrosis or within the center of *Aspergillus* abscess cavities. In these cases, positivity was seen mainly in organisms along the leading edge of infection and in those organisms invading tissue structures (Fig. 3). Organisms within the center of the abscess cavities showed little or no staining. **In situ** hybridization with the rRNA probe on examples of infections by *Candida* sp, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Pneumocystis carinii*, *Pseudallescheria boydii*, and *Fusarium* sp were negative. In **in situ** hybridization on the five cases of fungal infections with histopathology consistent with *Zygomycetes* (*Mucor*) were also negative. No precautions were taken to inhibit endogenous or exogenous RNases.

**DISCUSSION**

Techniques for rapidly identifying infectious pathogens in anatomic pathology substrates are of great interest and importance because of the increasing number of patients encountered whose immune systems are compromised secondary to organ transplantation, malignancy, or AIDS. Clinical tests that can accurately diagnose infectious agents in fixed tissue specimens are desirable because often organisms may not grow in culture or a particular pathogen is not suspected. As improved and effective chemotherapeutic agents against bacteria, fungi, and protozoans are developed, accurate pathogen identification may not only be desirable, but a necessity for improved patient management.

Ribosomal RNA analysis is a widely used means for identifying bacteria, fungi, and protozoans. Whereas rRNA sequences are conserved to the extent that they aid in phylogenetic classifications, variations within the nucleic acid sequences are known to be species specific, and sequence analysis permits accurate speciation of infectious agents. Species identification by rRNA:DNA solution hybridization is available in many clinical microbiology laboratories. More recently, **in situ** hybridization with oligonucleotide DNA probes for specific rRNA sequences has been used to accurately identify a variety of pathogens in fixed tissues specimens.

The **in situ** hybridization protocol employed in this study took less than 30 minutes to complete from initial dewaxing to final counterstaining. Use of this method in combination with a variety of site specific oligonucleotide probes may be useful for the rapid detection of several medically important bacterial, fungal, or protozoan pathogens. These techniques may be beneficial for organisms that are not easily cultured, for those organisms that grow slowly in culture, and when organisms are obviously seen with histochemical stains, but cultures are negative or not performed. We have used methods similar to those described in this study to develop single oligonucleotide probes for *Pneumocystis carinii* 5S rRNA, *Toxoplasma gondii* 18S rRNA, and *Pseudomonas aeruginosa* 16S rRNA.

Ribosomal RNA is an optimal target for **in situ** hybridization in all prokaryotic and eukaryotic cells because of its great abundance that permits rapid hybridization reactions. In addition, rRNA in **in situ** hybridization may not only identify specific organisms, it may help determine an organism’s viability. This is in contrast to histochemical and immunohistochemical stains that may detect organisms that are no longer viable. Normal levels of rRNA can be seen in cells for up to 48 hours after cell death, after which levels rapidly decline. This may ex-
FIG. 1. (Top) *In situ* hybridization for *Aspergillus* rRNA within lung from a patient with disseminated aspergillosis tissue showing intense staining with the probe in hyphal forms (diaminobenzidine, hematoxylin counterstain; magnification ×132).

FIG. 2. (Middle) *In situ* hybridization for *Aspergillus* rRNA showing intense staining within fruiting body (diaminobenzidine, hematoxylin counterstain; magnification ×198).

FIG. 3. (Bottom) *In situ* hybridization for *Aspergillus* rRNA in a fungal microabscess showing positive staining toward the abscess periphery where it invades blood vessels and little or know staining toward the center of the microabscess. This finding suggests that the rRNA assay may aid in the detection of viable fungal forms (diaminobenzidine, hematoxylin counterstain; magnification ×33).

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Aspergillus, a mold, can be identified using oligonucleotide probes. However, because of their rarity, only two of these cases have been analyzed so far. The use of this probe as a diagnostic test for Aspergillus awaits further testing on specimens with septate fungi similar in histologic appearance to Aspergillus. The authors thank Jim Hudson and Kel Locklar of Research Genetics for their excellent work on synthesizing the oligonucleotide probes used in this study.

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REFERENCES