Immunohistochemical Detection of *Aspergillus* Species in Pediatric Tissue Samples

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**Key Words:** *Aspergillus*; Mab-WF-AF-1; Immunohistochemistry; Pediatric; Paraffin

**Abstract**

Definitive diagnosis of invasive aspergillosis often requires tissue samples for histologic evidence of fungal infection and culture confirmation of *Aspergillus* species. However, the culture frequently fails to isolate *Aspergillus* species. Alternative approaches to confirm *Aspergillus* infection use polymerase chain reaction, in situ hybridization, and immunohistochemical analysis on paraffin-embedded sections. These approaches are well characterized in animals and adult patients but not pediatric patients. We studied the immunoreactivity of a commercially available monoclonal antibody, Mab-WF-AF-1 (DAKO, Carpinteria, CA), on paraffin-embedded sections from 16 pediatric cases with invasive aspergillosis, of which 12 were proven by culture. Optimal immunoreactivity required microwave antigen retrieval using high pH; 5 other antigen retrieval approaches were unsuccessful. With optimization, the monoclonal antibody was strongly immunoreactive in all cases with staining of the *Aspergillus* cell wall, septa, and cytoplasm. Background was minimal with no cross-reactivity to *Candida albicans*. These findings demonstrate the usefulness of the Mab-WF-AF-1 antibody in pediatric tissues suspected of invasive aspergillosis.

*Aspergillus* species are a common cause of fungal infections in immunocompromised patients. Bone marrow transplant recipients are especially susceptible, with an incidence from 5% to 14%.1,2 The mortality associated with invasive aspergillosis is 85% in the pediatric population, with half of the patients dying within 29 days of the diagnosis.3 Early detection of invasive aspergillosis and prompt therapy can improve survival.4 However, aspergillosis frequently is not diagnosed until autopsy.5 Improvements in early diagnosis of invasive aspergillosis combined with the advances in antifungal drugs such as caspofungin and itraconazole6,7 hold great promise for altering the dismal outcome of this disease.

*Aspergillus* species do not readily grow in culture from blood cultures,8,9 and a diagnosis of invasive aspergillosis usually requires tissue biopsies for microbiological culture and histologic examination.9 In some circumstances, a fungal infection is not suspected or the specimen arrives fixed in formalin and, hence, no sample is submitted for culture. Even when submitted, up to 70% of the cultures fail to isolate *Aspergillus* species, despite the presence of characteristic septate hyphae on the tissue section.10 The explanation for the culture failure is unclear and includes sampling differences, previous antifungal therapy, and less than optimal microbiological culture conditions. Under these circumstances, the morphologic features of the fungus (septate hyphae and acute angle branching) have been used at our and other institutions11 to support a diagnosis of “consistent with *Aspergillus* species.” However, a definitive identification of Aspergillus infection cannot be made because other fungal organisms such as *Fusarium* species, *Scedosporium* species, and *Pseudallescheria boydii* have morphologic features similar to those of *Aspergillus* species. Approximately 3% to
17% of cases with histologically evident fungal infection with septate hyphae and acute angle branching represent infection by Fusarium or Scedosporium species and not Aspergillus species. Hence, additional confirmatory studies on tissue sections would be valuable for the definitive diagnosis of invasive aspergillosis.

Numerous methods have been used to confirm Aspergillus species in paraffin-embedded, fixed tissue sections. They include polymerase chain reaction (PCR) amplification for the Aspergillus 28S ribosomal RNA or galactomannan sequence in situ hybridization (ISH) for 5S or 18S ribosomal RNA, and immunohistochemical analysis using a monoclonal antibody against an uncharacterized Aspergillus antigen or the complex polysaccharide, galactomannan. These methods have been well characterized in animals and adult patients but not in pediatric patients.

The detection method for invasive aspergillosis in the adult population does not necessarily translate to the pediatric population. For example, antibodies against Aspergillus galactomannan are used in the enzyme-linked immunosorbent assay to detect circulating Aspergillus antigen in serum samples of patients with proven or suspected invasive aspergillosis. The specificity for this assay in the adult population is high, at 98.1% to 99.6%. In contrast, the specificity for this assay in the adult population does not necessarily translate to the pediatric population. For example, antibodies against Aspergillus galactomannan are used in the enzyme-linked immunosorbent assay to detect circulating Aspergillus antigen in serum samples of patients with proven or suspected invasive aspergillosis. The specificity for this assay in the adult population is high, at 98.1% to 99.6%. In contrast, the specificity for this assay in the pediatric population is significantly lower, at 47.6%, with even lower specificity in premature infants. The explanation for the low specificity is unclear but most likely represents cross-reaction to unknown circulating endogenous antigens present in the pediatric population. This raises the possibility that antibodies against galactomannan might have obscuring high background in immunohistochemical analysis of paraffin-embedded sections of pediatric tissue samples.

We studied the usefulness of the monoclonal antibody Mab-WF-AF-1 that is commercially available in the United States and has been well characterized in bovine invasive aspergillosis. This antibody, with proper antigen retrieval, detected Aspergillus species in paraffin-embedded sections of all examined pediatric cases with invasive aspergillosis.

Materials and Methods

Microbiological Cultures

By using sterile technique, tissue samples submitted from pathology or directly from the operating room were transported to microbiology. Specimens were inoculated onto Sabouraud dextrose agar, brain-heart infusion agar with chloramphenicol and cycloheximide, and Mycosel agar plates (Becton Dickinson BBL Microbiology Systems, Cockeysville, MD) and incubated at 30°C for 4 weeks. Plates were examined daily for growth, and molds such as Aspergillus were identified by macroscopic and microscopic morphologic features. Yeasts were identified by using the RapidID Yeast Plus system (Remel, Lenexa, KS).

Immunohistochemical Analysis

The monoclonal antibody Mab-WF-AF-1 was purchased from DAKO, Carpinteria, CA, and has been described. A mouse monoclonal antibody against Candida albicans was purchased from Chemicon International, Temecula, CA. Tissue samples were fixed in formalin for 6 to 72 hours, processed into paraffin blocks, and cut into 6-µm sections. Deparaffinized sections were treated with 3% hydrogen peroxide for 25 minutes, 3% horse serum (Vector Labs, Burlingame, CA) for 30 minutes, primary antibody against Aspergillus (1:200 dilution) or Candida (1:400 dilution) for 30 minutes, biotinylated secondary horse antimouse antibodies (Vector Labs) for 30 minutes, avidin-biotin complex (Vector Labs) for 30 minutes, and diaminobenzidine for 5 minutes (DAKO) and counterstained with Harris hematoxylin for 15 seconds. All reactions were performed at room temperature.

In some cases, antigen retrieval was performed before incubation with 3% horse serum using one of the following methods: (1) microwave for 10 minutes in Target retrieval solution high pH, pH 10.0 (DAKO); (2) microwave for 10 minutes in EDTA buffer, pH 8.0 (Zymed, San Francisco, CA); (3) microwave for 10 minutes in Antigen Unmasking Solution, pH 6.0 (Vector Labs); or (4) pepsin (2 mg/mL) digestion at 37°C for 10, 20, or 30 minutes.

Results

Autopsy and surgical pathology reports from 1999 to 2003 were screened for histologic identification of fungal infection with septate hyphae. These criteria were met in 16 cases. Twelve cases had culture isolation of Aspergillus species. Four cases had histologic evidence of Aspergillus infection but no Aspergillus species were isolated from culture. The patients were 8 males and 8 females ranging in age from 1 month to 18 years. Six cases were obtained from autopsies, and the remaining cases were surgical tissue biopsies. Most of the infected organs were lung, but numerous other organs also were represented. The culture-proven Aspergillus species included Aspergillus fumigatus, Aspergillus flavus, and Aspergillus niger. The H&E- and Gomori methenamine silver–stained sections of all cases were reviewed, and the presence of invasive fungus with septate hyphae was confirmed.
Representative cases with histologic evidence of *Aspergillus* infection were analyzed by paraffin immunohistochemical analysis using the Mab-WF-AF-1 antibody that most likely reacts against the *Aspergillus* antigen galactomannan.  

Three patterns of immunoreactivity were seen (Table 1, Image 1): strong immunoreactivity with staining of the fungus cell wall, septa, and cytoplasm.  

This last pattern of staining suggested that the Mab-WF-AF-1 detected the *Aspergillus* antigen, but the immunoreactivity was suboptimal. We hypothesized that the immunoreactivity could be increased by using antigen retrieval. To test this hypothesis, 4 antigen retrieval methods were used. Microwave treatment in high pH buffer strongly increased immunoreactivity without antigen retrieval (Table 1). The background was limited to the cytoplasm of pulmonary macrophages as previously reported.  

Previous studies demonstrated the specificity of the Mab-WF-AF-1 antibody with no immunoreactivity to a wide range of fungal organisms, including *C albicans*, the most common fungal infection in the immunocompromised patients. To demonstrate that this specificity was not altered by our antigen retrieval, paraffin immunohistochemical analysis was performed on cases with known *Candida* species infection. One case represented an autopsy case with morphologic evidence of both *Candida* and *Aspergillus* infections (Table 1). Premortem blood cultures isolated *C albicans* only. H&E- and Gomori methenamine silver–stained sections demonstrated fungal infections that consisted of both budding yeast and branching septate hyphae forms consistent with *Candida* and *Aspergillus* species, respectively (Image 2A, Image 2B, Image 2C, Image 2D). These findings strongly suggest that the specificity of the Mab-WF-AF-1 was not altered by the antigen retrieval method.

### Table 1

Summary of Pediatric Cases With Invasive Aspergillosis

<table>
<thead>
<tr>
<th>Sex/Age</th>
<th>Procedure</th>
<th>Tissue</th>
<th>Morphologic Findings</th>
<th>Culture Result</th>
<th>Immunohistochemical Result</th>
<th>Preretrieval</th>
<th>Postretrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/15 y</td>
<td>Biopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td>Aspergillus species</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>F/9 y</td>
<td>Biopsy</td>
<td>Nasopharynx</td>
<td>Septate hyphae and budding yeast</td>
<td><em>Candida albicans</em></td>
<td>Positive</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>M/11 y</td>
<td>Biopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td>No fungi isolated</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>F/5 y</td>
<td>Biopsy</td>
<td>Nasopharynx</td>
<td>Septate hyphae</td>
<td>No fungi isolated</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>F/3 y</td>
<td>Biopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td>No fungi isolated</td>
<td>Focal and weak</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>M/14 y</td>
<td>Autopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td><em>Aspergillus fumigatus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>F/2 mo</td>
<td>Autopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td><em>A fumigatus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>F/7 y</td>
<td>Autopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td><em>A fumigatus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>M/18 y</td>
<td>Biopsy</td>
<td>Lung, spleen, brain</td>
<td>Septate hyphae</td>
<td><em>A fumigatus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>F/7 y</td>
<td>Biopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td><em>A fumigatus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>M/18 y</td>
<td>Biopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td><em>A fumigatus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>M/3 y</td>
<td>Biopsy</td>
<td>Brain</td>
<td>Septate hyphae</td>
<td><em>A fumigatus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>F/3 y</td>
<td>Biopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td><em>A fumigatus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>F/11 y</td>
<td>Autopsy</td>
<td>Heart</td>
<td>Septate hyphae</td>
<td><em>Aspergillus flavus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>M/15 y</td>
<td>Biopsy</td>
<td>Lung</td>
<td>Septate hyphae</td>
<td><em>Aspergillus flavus</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>M/10 y</td>
<td>Biopsy</td>
<td>Ear</td>
<td>Septate hyphae</td>
<td><em>Aspergillus niger</em></td>
<td>ND</td>
<td>Positive</td>
<td></td>
</tr>
</tbody>
</table>

ND, not done.

Discussion

The diagnosis of invasive aspergillosis is hampered by the frequent inability to isolate *Aspergillus* species from blood or tissue samples and the isolation of contaminating or colonizing nonpathologic *Aspergillus* species.
The diagnosis also is hampered by other invasive fungal organisms that mimic the morphologic features of *Aspergillus* species. In 1 study, 17% of autopsy cases (4/23) and 3% of surgical cases (1/30) had histologically evident fungal infection with septate hyphae and acute 45° branching but cultured for *Fusarium* species or *Scedosporium apiospermum.* For these reasons, definitive diagnosis of invasive aspergillosis should be made by histologic and culture examination of tissue biopsy specimens or fine-needle aspiration samples.

Besides culture confirmation, other methods such as PCR, ISH, and immunohistochemical analysis have been used to confirm invasive aspergillosis in paraffin-embedded sections. In cases in which culture is not available or is delayed, these approaches can provide rapid turnaround time for diagnosis with high sensitivity and specificity. While these approaches have been well characterized in adult patients and animals, they have not been well characterized in the pediatric population.

We studied the usefulness of immunohistochemical analysis to detect invasive *Aspergillus* species in paraffin-embedded sections from pediatric patients by using the commercially available monoclonal antibody Mab-WF-AF-1. This antibody has been well characterized in...
bovine invasive aspergillosis and detects the *Aspergillus* species *A. fumigatus, A. flavus,* and *A. niger* without cross-reactivity to *Candida* species, *Fusarium solani,* Mucoraceae organisms, *Scedosporium* species, *Trichophyton* species, *Mortierella wolfii,* and many other fungal organisms. We found that this antibody was highly sensitive, detecting 16 of 16 cases of invasive aspergillosis. Optimal detection required antigen retrieval using microwave in high pH, similar to other monoclonal antibodies. This optimization seems not to affect specificity because the antibody was not immunoreactive against known cases of *Candida* infection.

Another monoclonal antibody, EB-A1, also is available commercially and has been shown to recognize invasive aspergillosis in paraffin-embedded sections in adults, with high specificity and sensitivity. This antibody is likely to have immunoreactivity similar to that of Mab-WF-AF-1 because both antibodies detect galactomannan. The EB-A1 antibody is commercially available in Europe but not in the United States. Therefore, we were unable to directly compare the 2 antibodies.

The sensitivities for the detection of invasive aspergillosis in culture-proven cases by PCR amplification, ISH, and immunohistochemical analysis are 88%, 12, 13

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**Image II** (cont) and specific but focal and weak immunoreactivity (*E, x*4,000; arrows point to the focal weak signals). **F,** The weak focal immunoreactivity seen in *E* was strongly enhanced with antigen retrieval using microwave in high pH buffer. **G** and **H,** Low-power magnifications of **E** and **F,** respectively, showed uniform enhancement of the immunoreactivity with antigen retrieval (*x*250). For product information, see the text.
Mab-WF-AF-1 antibody with antigen retrieval retained its specificity in regard to *Candida albicans*. A and B, Sections of 1 case revealed both budding yeast (left portion of each panel) and septate hyphae (right portion of each panel) fungal forms, consistent with *Candida* and *Aspergillus* species, respectively (A, H&E, ×4,000; B, Gomori methenamine silver, ×4,000). Paraffin immunohistochemical analysis using Mab-WF-AF-1 (C, ×4,000) and *Candida* (D, ×4,000) antibodies with antigen retrieval showed specific immunoreactivity for both antibodies.

88% to 100%, 11,14-16 and 89% to 94%, 17-20 respectively. Most of the undetected cases represent culture-proven cases without histologic detection of fungal infection, suggesting sampling differences or culture isolation of a contaminant. Another potential cause of decreased sensitivity is the loss of the ISH signal in necrotic fungal forms that has been attributed to inhibition of hybridization by necrotic tissue and degradation of nucleic acid.11 This potential cause is less likely to affect immunohistochemical analysis because this approach can detect remnants of *Aspergillus* organisms in the cytoplasm of histiocytes.18 In fact, immunohistochemical analysis potentially could reveal invasive aspergillosis that might not be recognized histologically.

Immunohistochemical analysis likely is more sensitive than culture of tissue samples. In adult and bovine tissues, immunohistochemical analysis confirmed invasive aspergillosis in many cases that had histologic evidence but the culture failed to isolate *Aspergillus* species.19,20 Four of our cases also represented histologically positive but culture-negative cases. The explanation for the inability to culture *Aspergillus* from tissue with invasive aspergillosis is unclear but has been reported in up to 70% of the histologically confirmed cases.10
A limitation of our study is that we were unable to determine the specificity of immunohistochemical analysis for invasive fungal organisms with morphologic features similar to those of *Aspergillus* species, such as *Fusarium* and *Scedosporium* species and *P. boydii* because of a lack of samples. We were able to identify only 1 culture-proven biopsy sample with *Fusarium* species during the last 15 years, and that biopsy sample was exhausted (data not shown). No culture-proven cases of *Scedosporium* species or *P. boydii* were identified. However, the original study showed that the Mab-WF-AF-1 antibody did not react against *F. solani*, *Scedosporium inflatum*, or the asexual form of *P. boydii*, *S. apiospermum*. This would suggest that immunohistochemical analysis with Mab-WF-AF-1 can differentiate between *Aspergillus* and other morphologically similar fungal forms. In contrast, EB-A1, the other commercially available monoclonal antibody against *Aspergillus* galactomannan, showed weak reactivity to *P. boydii*, suggesting a lower specificity than the Mab-WF-AF-1 antibody.

We report optimal conditions for detecting invasive aspergillosis in paraffin-embedded sections from pediatric patients using immunohistochemical analysis and the commercially available monoclonal antibody Mab-WF-AF-1. The immunoreactivity seems specific, with no evidence of obscuring background that hampers enzyme-linked immunosorbent assays for circulating *Aspergillus* antigen in serum samples from pediatric patients. Paraffin immunohistochemical analysis using the Mab-WF-AF-1 antibody should be useful for rapid confirmation of invasive aspergillosis, especially in cases in which culture is not available or delayed.

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References


