Immunoperoxidase staining for identification of *Aspergillus* species in routinely processed tissue sections

P E Verweij, F Smedts, T Poot, P Bult, J A A Hoogkamp-Korstanje, J F G M Meis

### Abstract

**Aims**—To evaluate the performance of an immunoperoxidase stain using the monoclonal antibody EB-A1 to detect *Aspergillus* species in formalin fixed, paraffin wax embedded tissue.  

**Methods**—The monoclonal antibody EB-A1 directed against galactomannan was used to detect *Aspergillus* species in 23 patients with suspected or confirmed invasive aspergillosis. Immunostaining was performed on formalin fixed, paraffin wax embedded tissue using the streptavidin-biotin method and compared with conventional haematoxylin and eosin, periodic acid-Schiff, and Gomori-Grocott stains. Results of immunostaining were semi-quantitatively analysed with regard to both intensity of staining and number of positively staining microorganisms. Tissue sections from 16 patients with confirmed invasive mycoses due to *Candida* species, *Apophysomyces elegans*, *Rhizopus oryzae*, *Pseudallescheria boydii* and *Histoplasma capsulatum* were used as controls.  

**Results**—In 19 (83%) of 23 cases invasive aspergillosis was confirmed by both histological examination and culture (18 *Aspergillus fumigatus* and one *A. flavus*). Immunoperoxidase stains were positive in 17 (89%) of 19 cases including one case of disseminated infection due to *A. flavus*. Furthermore, the immunoperoxidase stain was positive in a culture negative tissue section with histological evidence of mycelial development, indicating the presence of *Aspergillus* species. Some cross-reactivity was observed with the highly related fungus *P. boydii*, although the number of mycelial elements that stained was low.  

**Conclusions**—Immunoperoxidase staining using the monoclonal antibody EB-A1 performs well on routinely processed tissue sections and permits detection and generic identification of *Aspergillus* species, although it was no better than conventional histopathology in identifying the presence of an infection. An additional advantage is that the immunostain may help to provide an aetiological diagnosis when cultures remain negative.


**Keywords**: aspergillosis, galactomannan, immunoperoxidase.

*Aspergillus* species can cause severe pulmonary infections in immunocompromised patients. The ante-mortem diagnosis of invasive aspergillosis is difficult and, although new sensitive diagnostic methods are being evaluated, a definitive aetiological diagnosis can be made only by the demonstration of tissue invasion by fungal mycelium and a positive culture with morphological identification of *Aspergillus* species. The diagnosis of invasive aspergillosis by histological examination of tissue sections alone is not reliable, because numerous filamentous fungi cannot be differentiated from *Aspergillus* organisms with any of the commonly used fungal stains. A correct aetiological diagnosis, however, is important for the management of systemic mycoses and for the study of fungal epidemiology. Furthermore, the necessity for an aetiologic diagnosis will increase because new fungal pathogens, such as *Acremonium* and *Alternaria* species, have been reported to cause invasive infections in immunocompromised patients. To obtain a rapid and accurate generic identification, both poly-1 and monoclonal2–10 antibodies have been used for direct immunoperoxidase staining of tissue sections. Previous observations have suggested that the monoclonal antibody EB-A1 directed against galactomannan was useful in the diagnosis of infections resulting from *Aspergillus* species.11 In this study we evaluated the performance of this monoclonal antibody to detect and identify *Aspergillus* species in tissue sections obtained from patients with invasive mycoses.

**Methods**

Between 1989 and 1991 pathology and microbiology records from University Hospital Nijmegen were reviewed for consecutive necropsy cases with invasive aspergillosis. In each case identified the medical records were reviewed for clinical and radiological evidence of invasive aspergillosis. Tissue sections were also obtained from patients with confirmed invasive infections due to other filamentous fungi and *Candida* species, in order to evaluate the specificity of the immunoperoxidase stain.

All tissue specimens were fixed in 10% formalin solution, and processed to paraffin wax. Wax sections (4 μm) were cut for conventional stain techniques including haematoxylin and eosin, periodic acid-Schiff (PAS), Gomori's methenamine silver (GMS), and immunohistochemistry. Immunohistochemical staining was performed using the streptavidin-biotin-
peroxidase complex method. In short, sections were rehydrated, endogenous peroxidase activity was blocked (by incubation in 1% 
$\text{H}_2\text{O}_2$), after which sections were incubated for 20 minutes in normal goat serum (diluted 1 in 5 in phosphate buffered saline (PBS)). The slides were then incubated with 20 $\mu$g/ml of the primary antibody (EB-A1, Eco-Path Aspergil-
lus, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) for 60 minutes at room temperature. As a detection system, we used 
Multilink I (BioGenex, San Ramon, Californi-
a) followed by streptavidin-peroxidase 
d conjugate (BioGenex). As a chromogen, we 
used a diaminobenzidine solution containing 
0.65% imidazol. The staining was intensified 
by incubation in 0.5% CuSO\(_4\) and 0.9% NaCl 
for five minutes. Finally, sections were weakly 
counterstained with Mayer’s haematoxylin, 
dehydrated, and mounted. As a negative 
control, sections were incubated with PBS 
instead of the primary antibody.

Tissue sections were examined for the presence 
of the characteristic brown staining and 
semiquantitatively evaluated by estimating the 
number of stained mycelial elements in the fol-
lowing categories: no staining and only a few 
scattered positive elements (less than 5%) (−); 
staining in 5% to 25% of elements (+); staining 
in 25% to 50% (+ +); staining in 50% to 75% 
(+ + +); and staining in 75% to 100% 
(+ + + +). The intensity was graded on a scale of 
− to + + + : absence of staining (−); mild 
staining (+); moderate staining (+ +); and 
intense staining (+ + + +). Equivocal reactions 
were considered negative. Furthermore, any 
background staining including non-specific 
staining of cells or tissues was noted separately.

All slides were scored independently by three 
of the authors (PV, FS, JM).

### Results

A total of 40 tissue sections from 39 patients 
with either suspected or necropsy confirmed 
invasive fungal infection was studied. Twenty 
three patients had either confirmed or sus-
ppected infection with Aspergillus species. The 
clinical characteristics of these patients and the 
results of histology, culture and immunoperox-
dase staining are shown in Table 1. In 19 of 
23 patients there was evidence of disease based 
on histopathology and culture, including seven 
patients with disseminated infection (cases 1 to 
7). All tissue sections obtained from the 
patients with disseminated infection showed 
intense hyphal immunostaining (number/ 
intensity: + + + + / + + + +) (fig IA), including 
one patient with A. flavus infection (case 7). 
One patient (case 8) with acute myeloblastic 
leukaemia died of respiratory insufficiency, 
and invasive pulmonary aspergillosis was diagnosed 
at necropsy. The presence of Aspergillus species 
was confirmed by both culture and immu-
noperoxidase stain. In this patient histological 
examination of a pulmonary thrombus showed 
hyphal elements. Culture yielded Rhizopus 
microsporus variant rhizopodiformis and the 
immunostaining was negative. Another patient 
(case 11) with chronic myeloid leukaemia died 
of septicaemia and respiratory insufficiency. At 
necropsy a bronchopneumonia was diagnosed in 
the lower lobe of the left lung with mycelial 
elements demonstrated using the GMS stain. 
Although cultures remained negative, the 
immunostaining identified Aspergillus species. 
A total of 18 (90%) of 20 tissue sections with 
mycelial development stained positively by the 
immunoperoxidase method. In three patients 
(cases 21 to 23) bronchopneumonia was diag-
nosed at necropsy but, although culture of the 
lung tissue yielded A. fumigatus, mycelial

### Table 1 Clinical characteristics of 23 patients with invasive aspergillosis and results of histology, culture and immunohistochemistry

<table>
<thead>
<tr>
<th>Case No</th>
<th>Sex</th>
<th>Age</th>
<th>Underlying disease</th>
<th>Aspergillus infection</th>
<th>Histology</th>
<th>Culture</th>
<th>Immunoperoxidase (number/intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>41</td>
<td>Myelodysplastic syndrome</td>
<td>Disseminated</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>51</td>
<td>ALL</td>
<td>Disseminated</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>3</td>
<td>F/1.5</td>
<td>66</td>
<td>Pearson’s syndrome</td>
<td>Disseminated</td>
<td>Fungi +</td>
<td>A fumigatus, C. albicans</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>66</td>
<td>Grawitz’s tumour</td>
<td>Disseminated</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>72</td>
<td>AML</td>
<td>Disseminated</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>6</td>
<td>F/45</td>
<td></td>
<td>AML, BMT</td>
<td>Disseminated</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>7</td>
<td>F/29</td>
<td></td>
<td>SLE</td>
<td>Disseminated</td>
<td>Fungi +</td>
<td>A flavus</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>60</td>
<td>AML</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus, Rhizopus microsporus var. rhizopodiformis</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>9</td>
<td>F/15</td>
<td></td>
<td>Agammaglobulinaemia</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + + / + + +</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>58</td>
<td>ALL</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>51</td>
<td>CML</td>
<td>Lung</td>
<td>Fungi +</td>
<td>Negative</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>32</td>
<td>ALL</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>59</td>
<td>Adenocarcinoma lung</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>14</td>
<td>F/15</td>
<td></td>
<td>NHL, BMT</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>11</td>
<td>CGD</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>57</td>
<td>Hepatocellular carcinoma</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ / + / +</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>60</td>
<td>Myelodysplastic syndrome</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>58</td>
<td>AML</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>19</td>
<td>F/44</td>
<td></td>
<td>AML, BMT (2x)</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>29</td>
<td>AML</td>
<td>Lung</td>
<td>Fungi +</td>
<td>A fumigatus</td>
<td>+ + / + + +</td>
</tr>
<tr>
<td>21</td>
<td>M</td>
<td>61</td>
<td>HL</td>
<td>Lung</td>
<td>Fungi −</td>
<td>A fumigatus</td>
<td>−</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>55</td>
<td>Rheumatoid arthritis, kidney transplant</td>
<td>Lung</td>
<td>Fungi −</td>
<td>A fumigatus</td>
<td>−</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>50</td>
<td>AML</td>
<td>Lung</td>
<td>Fungi −</td>
<td>A fumigatus</td>
<td>−</td>
</tr>
</tbody>
</table>

**ALL = acute lymphoblastic leukaemia; AML = acute myeloblastic leukaemia; BMT = bone marrow transplantation; SLE = systemic lupus erythematosus; CML = chronic myeloid leukaemia; NHL = non-Hodgkin’s lymphoma; CGD = chronic granulomatous disease; HL = Hodgkin’s lymphoma.**
elements could not be detected by conventional fungal staining. Immunostaining was also absent in all three patients.

The cross-reactivity of the monoclonal antibody EB-A1 was tested by staining tissue sections obtained from 16 patients with non-Aspergillus mycoses (table 2), including 12 patients with histopathological and culture confirmed invasive candidiasis, a patient with pulmonary pseudallescheriasis, a patient with A. elegans osteomyelitis of the humeral bone, a patient with a Rhizopus oryzae bronchopneumonia, and a patient with a disseminated Histoplasma capsulatum infection. No cross-reactive immunostaining was observed with Candida species, A. elegans (fig 1B (−/−)), R. oryzae (fig 1C (−/−)) and H. capsulatum. Immunostaining was observed in P. boydii mycelial elements (fig 1D (+/+)), although most elements showed no reactivity. Background staining was observed to be mild in infections in which there was frank necrosis, and did not hamper the detection of the micro-organisms. In vital lung tissue cross-reactivity of the antibody was observed in epithelial cells lining the alveoli and in alveolar macrophages. Immunostaining in these specimens was sometimes quite intense, but mycelial elements were easily identified on the basis of their very different morphology. The immunostain was not better than conventional fungal staining for identifying the presence of an infection.

Discussion

An accurate identification of fungal elements in tissue sections using conventional fungal stains requires considerable knowledge of fungal morphology, and remains difficult when based on morphological features alone. This is particularly difficult for histopathologists who are usually not familiar with fungal morphology. The histological differentiation of Aspergillus species from Fusarium, Acremonium, Scopulariopsis, Trichoderma, Blastoschizomyces species and P. boydii is particularly difficult as these fungi may induce similar clinical features and exhibit filamentous development in host tissue. Although subtle morphological features, such as dichotomous branching at 45° of Aspergillus mycelium, may help to differentiate this fungus from other filamentous fungi, only a presumptive diagnosis can be made when cultures remain negative. Immunohistochemistry using mono- or polyclonal antibodies capable of

Table 2  Histological findings, microbiological identification, number of cases and sites of involvement of 16 control patients with invasive non-Aspergillus mycoses

<table>
<thead>
<tr>
<th>Histological examination</th>
<th>Culture</th>
<th>No of cases</th>
<th>Sites of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudo hyphae</td>
<td>Candida spp*</td>
<td>12</td>
<td>Disseminated; lung; tongue</td>
</tr>
<tr>
<td>Mycelial elements</td>
<td>T. gypseum*</td>
<td>2</td>
<td>Lung; humerus</td>
</tr>
<tr>
<td>Mycelial elements</td>
<td>Pseudallescheria boydii</td>
<td>1</td>
<td>Lung</td>
</tr>
<tr>
<td>Yeast form</td>
<td>Histoplasma capsulatum</td>
<td>1</td>
<td>Disseminated</td>
</tr>
</tbody>
</table>

*Including C. albicans (10 cases), C. tropica (one case), and C. parapsilosis (one case).
†Including Apophysomyces elegans and Rhizopus oryzae.
Identification of Aspergillus species in routinely processed tissue
detecting specific fungi in tissue sections is helpful in this respect.\textsuperscript{7-11} A drawback, however, is that only genera of fungi can be differentiatiated. An advantage of immunostaining is that formalin fixed, paraffin wax embedded sections are used and that the staining method is relatively easy to perform. In the present study immunoperoxidase staining with EB-A1 proved useful for the generic identification of Aspergillus species in tissue sections. The presence of Aspergillus organisms was confirmed in 17 of 19 culture positive tissue sections, and in one patient fungal mycelium was identified as Aspergillus species in a culture negative tissue section. Cross-reactivity with three Candida species, two Zygomycete species, and H. capsulatum was not observed.

An important factor in the evaluation of any immunohistochemical method is the estimation of background staining in relation to positive staining micro-organisms. We did note low levels of background staining in proteinaceous oedema fluid in the lungs and also sometimes in exudates as well as in alveolar macrophages. The levels of background staining were low and usually did not deduct identification of the fungi. In some cases, however, macrophages did show higher levels of immunostaining, reflecting the high concentrations of endogenous peroxidases within these cells; again this staining did not make fungal identification more difficult.

Immunofluorescence studies have shown that Aspergillus, Fusarium, and P. boydii are antigenically closely related.\textsuperscript{15} Previous observations indicated that the monoclonal antibody EB-A1 does not cross-react with Fusarium species,\textsuperscript{8} but the reactivity with P. boydii was not ascertained. Our results suggest that EB-A1 can also differentiate Aspergillus organisms from P. boydii. Although some cross-reactive staining occurred with the P. boydii hyphae, the intensity of immunostaining was low and most elements were not stained. The monoclonal antibody EB-A1 cross-reacts with Penicillium marneffei\textsuperscript{8-14} which has some immuno-dominant epitopes in the cell wall which are identical with those of Aspergillus species.\textsuperscript{16} Invasive infections with both Aspergillus species\textsuperscript{17} and P. marneffei\textsuperscript{18} have been reported in patients with AIDS, and therefore the monoclonal antibody EB-A1 cannot be used to differentiate these fungi. As the tissue form of P. marneffei develops as a yeast-like structure,\textsuperscript{19} differentiation from Aspergillus species can be made by histological examination with conventional fungal stains. However, histological examination should be interpreted with caution because elongated forms or filamentous development of P. marneffei have been reported to occur in the stratum corneum of the skin,\textsuperscript{20} in liver and lung tissue specimens of a patient with AIDS,\textsuperscript{21} and in pulmonary cavities.\textsuperscript{22}

In conclusion, immunoperoxidase staining with EB-A1 in formalin fixed and paraffin wax embedded tissue is very helpful in providing proof of an Aspergillus infection and excluding certain other fungal genera, but is not better than conventional histopathology in identifying the presence of an infection. The results should be interpreted within the whole clinical context of the patient, because false negative results and cross-reactivity with P. marneffei or P. boydii may occur.

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