

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

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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 semiquantitatively analysed with regard to both intensity of staining and number of positively staining micro-organisms. 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.

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Aspergillus species can cause severe pulmonary infections in immunocompromised patients.¹ The antemortem diagnosis of invasive aspergillosis is difficult and, although new sensitive diagnostic methods are being evaluated,²⁻⁴ 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 aetiological diagnosis will increase because new fungal pathogens, such as *Acremonium* and *Alternaria* species,^{5,6} have been reported to cause invasive infections in immunocompromised patients. To obtain a rapid and accurate generic identification, both poly-⁷ and monoclonal⁷⁻¹⁰ 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.⁸⁻¹¹ 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 methanamine silver (GMS), and immunohistochemistry. Immunohistochemical staining was performed using the streptavidin-biotin-

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

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

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.

peroxidase complex method. In short, sections were cut and rehydrated, endogenous peroxidase activity was blocked (by incubation in 1% H₂O₂), 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 µg/ml of the primary antibody (EB-A1, Eco-Path *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) for 60 minutes at room temperature. As a detection system, we used Multilink I (BioGenex, San Ramon, California, USA) followed by streptavidin-peroxidase conjugate (BioGenex). As a chromogen, we used a diaminobenzidine solution containing 0.65% imidazol. The staining was intensified by incubation in 0.5% CuSO₄ 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 following 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 suspected infection with *Aspergillus* species. The clinical characteristics of these patients and the results of histology, culture and immunoperoxidase 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 1A), 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 immunoperoxidase 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 diagnosed at necropsy but, although culture of the lung tissue yielded *A fumigatus*, mycelial

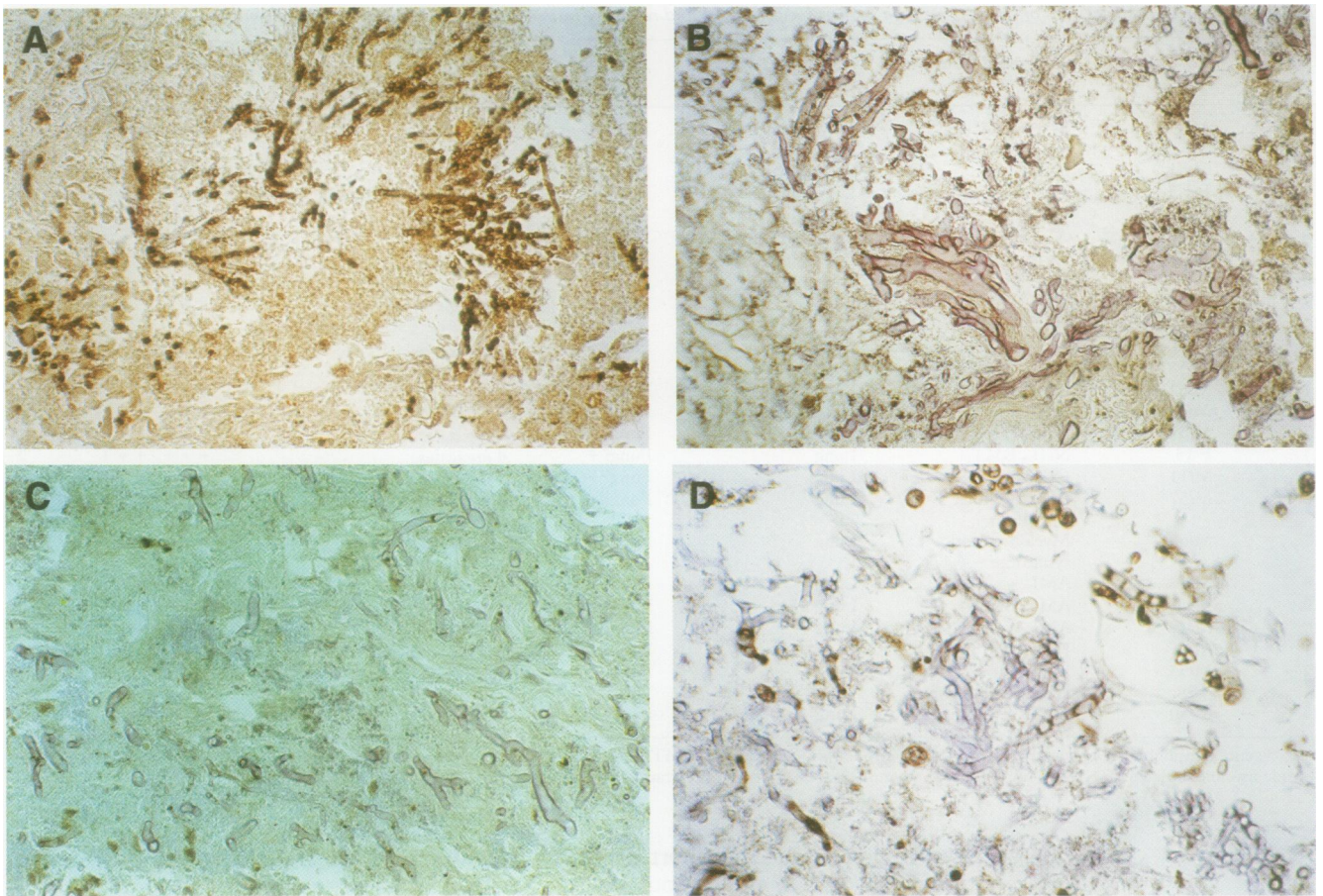


Figure 1 Immunostaining of formalin fixed, paraffin wax embedded tissue sections using the streptavidin-biotin method and the monoclonal antibody EB-A1. The sections were weakly counterstained with Mayer's haematoxylin. (A) *Aspergillus fumigatus* in lung tissue (+ + + + / + + +). (B) *Apophysomyces elegans* in necrotic bone tissue (- / -), counterstained with Mayer's haematoxylin. (C) *Rhizopus oryzae* in lung tissue. No immunostaining (- / -), counterstained with Mayer's haematoxylin highlights fungal elements. (D) *Pseudallescheria boydii* in lung tissue (+ / +); some fungal elements show cross-reactive immunostaining. (Original magnification $\times 400$.)

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,¹² a patient with *A. elegans* osteomyelitis of the humeral bone,¹³ 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

Histological examination	Culture	No of cases	Sites of infection
Pseudo hyphae	<i>Candida</i> spp*	12	Disseminated 6; lung 5; tongue 1
Mycelial elements	Zygomycetes†	2	Lung 1; humerus 1
Mycelial elements	<i>Pseudallescheria boydii</i>	1	Lung
Yeast form	<i>Histoplasma capsulatum</i>	1	Disseminated

*Including *C. albicans* (10 cases), *C. tropicalis* (one case), and *C. parapsilosis* (one case).

†Including *Apophysomyces elegans* and *Rhizopus oryzae*.

detecting specific fungi in tissue sections is helpful in this respect.^{7-11 14} A drawback, however, is that only genera of fungi can be differentiated. 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 positively 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 deter 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.¹⁵ Previous observations indicated that the monoclonal antibody EB-A1 does not cross-react with *Fusarium* species,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 marneffeii*^{8 14} which has some immunodominant epitopes in the cell wall which are identical with those of *Aspergillus* species.¹⁶ Invasive infections with both *Aspergillus* species¹⁷ and *P marneffeii*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 marneffeii* develops as a yeast-like structure,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 marneffeii* have been reported to occur in the stratum corneum of the skin,¹⁴ in liver and lung tissue specimens of a patient with AIDS,²⁰ and in pulmonary cavities.¹⁹

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 in the whole clinical context of the patient, because false negative results and cross-reactivity with *P marneffeii* and *P boydii* may occur.

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