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Evaluation of eight antibody tests and one antigen test for the diagnosis of invasive aspergillosis

Validierung von acht Antikörper-Nachweisen und einem Antigen-Nachweis zur Diagnose der invasiven Aspergillose

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Schlüsselwörter. *Aspergillus*, Aspergillosis, Serologie, Antikörper, Antigen.

Summary. Eight *Aspergillus* antibody detection assays – three indirect haemagglutination assays (IHA-LD, IHA-Roche, IHA-Fumouze), three enzyme immunoassays (EIA-IgG, EIA-IgM, EIA-IgA, DDV) and two complement fixation tests (CF-metabolic and CF-somatic, Virion – and one latex agglutination test (LAT) for *Aspergillus* galactomannan antigen detection (Sanofi Pasteur) were evaluated in 14 patients with proven invasive aspergillosis (a total of 47 serum samples and one cerebrospinal fluid sample) and in 68 selected control individuals (one selected serum sample each). For the antibody tests, sensitivity ranged from 14% to 36% and specificity from 72% to 99%. The antigen detection test had a sensitivity of 36% and a specificity of 100%. Currently commercially available antibody detection assays for the serodiagnosis of invasive aspergillosis are inadequate. The antigen detection test appears to be highly specific, but lacks sufficient sensitivity.

Zusammenfassung. Acht *Aspergillus*-Antikörpernachweis-Systeme, nämlich drei indirekte Hämagglutinationsteste (IHA-LD, IHA-Roche, IHA-Fumouze), drei Enzymimmunassays (EIA-IgG, EIA-IgM, EIA-IgA, DDV) und zwei Komplementbindungsreaktionen (CF-metabolic und CF-somatic, Virion) sowie ein Latex-Agglutinationstest zum Nachweis von *Aspergillus*-Galaktomannan-Antigen (Sanofi-Pasteur) wurden an insgesamt 14 Fällen bewiesener invasiver Aspergillose (47 Serumproben und ein Liquor) und einer Kontrollgruppe von 68 Probanden (je eine Serumprobe) bewertet. Bei diesen Antikörper-Nachweisen lag die Sensitivität zwischen 14% und 36% und die Spezifität zwischen 72% und 99%. Der Antigen-Nachweis zeigte eine Sensitivität von 36% und eine Spezifität von 100%. Die derzeit erhältlichen Antikörpernachweis-Systeme zur Serodiagnose der invasiven Aspergillose sind unbefriedigend. Der Antigennachweis ist hochspezifisch, doch ist auch seine Sensitivität unzureichend.

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Introduction

Invasive pulmonary or disseminated aspergillosis has a high mortality among patients with neutropenia or neutrophil dysfunction. Successful therapeutic strategies require an early definitive diagnosis, which is difficult to obtain. Effective serodiagnostic tests for invasive aspergillosis are needed [1–4].

Currently, a diverse panel of assay formats is in use worldwide. However, there is no national or international agreement on the validity of individual tests. General statements on insufficient antibody production in immunocompromised hosts prevail in the medical literature [5]. Antibody detection has been studied in patients suffering from allergic bronchopulmonary aspergillosis (ABPA) and aspergilloma, but only rarely in patients with invasive aspergillosis [1, 6–8]. Several previous studies (1) lack proven cases of invasive aspergillosis, (2) include fewer than 10 proven cases of invasive aspergillosis or (3) do not include

details of proven cases of invasive aspergillosis [1, 5, 9–12]. Patients recovering from recent open heart surgery and patients with multiple myeloma are prone to false-positive reactions in many antibody tests and have never been specifically included in such studies.

Thus, in this investigation, we provide detailed background information on 14 patients with proven aspergillosis with several serum samples each. In addition, selected control individuals include the clinically most relevant high-risk groups (e.g. bone marrow transplant recipients) as well as immunized patients (e.g. patients with cystic fibrosis). The aim of this study was to evaluate nine commercially available immunoassays – three haemagglutination tests, three enzyme immunoassays and two complement fixation tests – for the detection of circulating antibodies and one latex agglutination test for the detection of *Aspergillus* galactomannan antigen (Table 1).

Patients and methods

Fourteen patients with proven invasive aspergillosis, providing a total of 47 serum samples and one cerebrospinal fluid sample, were diagnosed in the area of Heidelberg, Germany, during 1990–93. In 11 cases the diagnosis was confirmed histopathologically by biopsy or autopsy. In 8 of these 11 cases the diagnosis was confirmed microbiologically, but in three cases fusariosis or pseudallescheriosis could not be excluded. In one case the diagnosis was confirmed by two independent blood cultures with growth of *Aspergillus fumigatus*. In two cases stringent histopathological or microbiological criteria for invasive aspergillosis were not met, although radiological findings, therapy response and further clinical data strongly suggested invasive aspergillosis (Table 2).

Controls included one selected serum sample from each of 68 individuals without evidence of aspergillosis, as follows: students and staff members ($n=15$), patients with leukaemia or lymphoma ($n=20$), patients with multiple myeloma ($n=3$), patients after open heart surgery ($n=10$), patients with cystic fibrosis ($n=8$) and patients with deep mycoses other than aspergillosis ($n=12$) (Table 3). Serum samples were either tested within 24 h after separation from the clot or kept at −20 °C before being tested. Several serum samples were available from each of the control individuals. Selection criteria for the one sample used for this evaluation included: (1) for the students and staff members – the sample with the largest volume; (2) for the patients with leukaemia, lymphoma and multiple myeloma – the first of samples drawn weekly under empiric intravenous antimycotic chemotherapy (amphotericin B, flucytosine, fluconazole, itraconazole, liposomal amphotericin B and/or combinations thereof) for fever of unknown origin; (3) for the patients with open heart surgery – a sample drawn 1–3 days after surgery, which is the period during which haemagglutination assays are prone to false-positive results; (4) for patients with cystic fibrosis – the sample with the largest volume; (5) for patients with deep mycoses other than aspergillosis – a sample drawn 2–4 weeks after onset of disease. Eight antibody detection assays (Tables 1 and 4) were performed according to the recommendations of the manufacturers.

The latex agglutination test from Sanofi Pasteur (Pastorex®) was used for *Aspergillus* galactomannan antigen detection and performed according to the recommendations of the manufacturer.

Sensitivity and specificity were calculated as follows:

$$\text{Sensitivity } (\%) = a/(a+b) \times 100$$

$$\text{Specificity } (\%) = d/(c+d) \times 100$$

where a is the number of patients with invasive aspergillosis and a positive test, b is the number of patients with aspergillosis and a negative test, c is the number of controls with a positive test and d is the number of controls with a negative test.

Each serum sample was tested with each assay once or up to three times, if there was sufficient sample volume available. If tests were repeated, results varied by one titre step at maximum. In these cases, the median titres of replicate examinations were evaluated. Positive and negative control sera included in each kit were tested with each experiment and yielded the specified results. Values above the cut-offs indicated by the manufacturers (Table 1) were designated positive.

Results

The technical performance of all tests was simple and appropriate for a routine clinical laboratory. The required time ranged from 15 min (LAT) to 20 h (CF-somatic/metabolic). The cost per tested serum sample was by far the lowest for the CF tests. The LAT and the IHA from Fumouze were particularly expensive (Table 1).

The general characteristics of the 14 patients are presented in Table 2, and the histopathological proof of invasive aspergillosis is documented for 10 of the 14 patients in Fig. 1. Underlying diseases/conditions include haematological malignancies ($n=9$), heart transplantation ($n=1$), liver transplantation ($n=1$), cystic fibrosis ($n=1$), AIDS

Table 1. Characterization of commercial serodiagnostic tests for invasive aspergillosis

Assay format*	Manufacturer†	Parameter detected	Type/ nature	Antigen‡/ antibody used for detection	Cut-off	Positive control	Tests per kit [volume of serum needed (µl)]	Time (h)	Cost per serum sample (DM) in 1993	Specificity	Sensitivity	Comment
IHA	LD	Antibody	IgM, IgG, IgA	58 kDa–AG, somatic AG	1:160	1:160	80 (5)	15	7.55	87	29	No pre-treatment
IHA	Roche	Antibody	IgM, (IgG)	Galactomannan	1:20	1:320	80 (50)	15	5.72	68	36	Pre-absorbed with sheep erythrocytes
IHA	Fumouze	Antibody	IgM, (IgG)	Metabolic AG	1:640	1:1280	17 (50)	2.5	15.56	88	36	Easy handling, fast, expensive
EIA												
IgG	DDV	Antibody	IgG	Somatic AG	60 EIU	100 EIU	96(10)	2	5.93	72	29	Quantitative, automated reading
IgM		Antibody	IgM		75 EIU	100 EIU	96(10)	2	5.93	91	14	
IgA		Antibody	IgA		40 EIU	100 EIU	96(10)	2	5.93	93	29	
CF												
som	Virion	Antibody	IgG, IGM	Somatic AG (30–50 kDa) metabolic AG	1:10	1:160	240 (100)	20	0.89	79	29	
met		Antibody	IgG, IgM	(30–50 kDa)	1:10	1:40	320 (100)	20	0.70	99	21	Lowest cost
LAT	Sanofi Pasteur	Antigen	Galacto-mannan	Rat monoclonal antibody (EB-A2) to galactomannan	None	1:5 (75 ng ml^{-1}§)	40 (300)	0.25	10.93	100	36	Rapid clearance, 100% specific, very fast

* IHA, indirect haemagglutinations assay; EIA, enzymeimmunoassay; CF, complement fixation; som, somatic; met, metabolic; LAT, latex agglutination test.
† LD, Labor Diagnostika, 46359 Heiden, Germany; Roche Serologie GmbH, 81929 Munich, Germany; Trignost Diagnostica (German distributer), 74889 Sinsheim und F-6700 Strasbourg, France (Original distributer: Fumouze Diagnostics, F-92600 Asnières, France); DDV Diagnostika, 35037 Marburg, Germany; Institut Virion, 97072 Würzburg, Germany; NV Sanofi Diagnostics Pasteur B-3600 Genk, Belgium.
‡ Antigen generally prepared from *Aspergillus fumigatus*.
§ Lower detection limit 15 ng ml^{-1}.

Table 2. Characteristics of patients with proven invasive aspergillosis

Patient no.	Age (years)	Sex	Underlying disease	*Aspergillus* species	Isolation from	Site of histological detection of *Aspergillus*	Neutrophils per mm³	Systemic antimycotic treatment	Dose (per day)	Duration of treatment (days)	Outcome
1	33	F	Non-Hodgkin's lymphoma	*A. fumigatus*	Tracheal secretion	Lungs, trachea	<1000	AMB	40 mg/60 mg	5/1	Death
								Liposomal AMB	200 mg	17	
2	53	M	Non-Hodgkin's lymphoma	Unknown	None	Lungs	80–300	Fluconazole	200 mg	7	Death
								AMB/5-FC	35 mg/4 × 2.5 g	9	
3	8	M	Cystic fibrosis	*A. fumigatus*	Blood culture, sputum	ND	9900–13 200	AMB/5-FC	1.0 mg kg^{-1}/4 × 625 mg	24	Survival
4	61	M	Multiple myeloma	*A. niger*	None	Nasal cavity	6300–6400	AMB	30 mg	28	Death
						Aspergillus heads		Itraconazole	400 mg	4	
5	74	M	AML	*A. fumigatus*, *A. terreus*	Sputum	Right lung	2600–7000	AMB	30 mg	28	Death
								5-FC/AMB	4 × 2.5 g/30 mg	7	
6	24	F	Hodgkin's disease, heart transplant	*A. fumigatus*	Axillary nodule	Axillary nodule	7000–27 000	Fluconazole	200 mg	90	Survival
7	56	M	ALL	*A. terreus*	Two blood cultures	Heart, kidneys, left suprarenal gland, brain	3600–6700	Itraconazole	400 mg	120	Death
								NA	NA	NA	
8	58	M	Liver transplant	*A. fumigatus*	Sputum	Heart, lungs, thyroid gland	6400–15 500	Fluconazole	200 mg	9	Death
								AMB/5-FC	45 mg/2 × 2.5 g	10/4	
9	52	M	AML	Unknown	None	Transbronchial biopsy: negative	120	AMB/5-FC	30–60 mg/4 × 2.5 g	47	Death
								Itraconazole	400 mg	60	
10	58	F	AML	*A. fumigatus*	None	Transbronchial biopsy: *Aspergillus* heads	100–2000	Fluconazole	400 mg	7	Death
								AMB/5-FC	50 mg/4 × 2.5 g	18/6	
11	8	M	ALL	Unknown	None	ND	100–400	AMB/5-FC	7.5 mg/4 × 130 mg	60/14	Death
12	56	M	Non-Hodgkin's lymphoma	Unknown	None	Sphenoidal sinus, heart, lungs	1300–7200	Fluconazole	200 mg	8	Death
13	56	M	AIDS	*A. fumigatus*	Sputum	Transbronchial biopsy	3100	Itraconazole, 5-FC/AMB	400 mg, 4 × 1.5 g/20 mg	30 300	Death
14	25	M	Testicular carcinoma	Unknown	None	Resected lung	8600	None (partial pneumonectomy)	NA	NA	Survival

AML, acute myelogenous leukaemia; ALL, acute lymphocytic leukaemia; AIDS, acquired immune deficiency syndrome; AMB, amphotericin B; 5-FC, 5-fluorocytosine; ND, not done; NA, not applicable.

Table 3. Frequency of false-positive *Aspergillus* serodiagnostic tests in selected control groups

Control group	Total no.	No. of males	Average age in years (range)	No. of false-positive individuals* (%)	No. of false-positive test results/ total number of tests (%)	
					Antibody tests ($n=8$)	Antigen test (LAT)
Students and staff members	15	6	26 (21–34)	6 (40.0)	9/120 (7.5)	0/15 (0.0)
Leukaemia or lymphoma†	20	12	33 (22–46	0 (0.0)	0/160 (0.0)	0/20 (0.0)
Immunocytoma	3	1	40 (28–48)	2 (66.7)	11/24 (45.8)	0/3 (0.0)
Cardiac surgery‡	10	8	58 (19–83)	9 (90.0)	26/80 (32.5)	0/10 (0.0)
Cystic fibrosis	8	4	16 (5–30)	7 (87.5)	23/64 (35.9)	ND
Deep mycoses other than aspergillosis§	12	6	41 (7–83)	3 (25.0)	8/96 (8.3)	0/12 (0.0)
Total controls	68	37	35 (5–83)	27 (39.7)	77/544 (14.2)	0/60 (0.0)

* One selected serum sample each. Individuals were considered false positive if at least one test (of nine) was positive.
† Bone marrow transplant recipients for acute lymphocytic leukaemia ($n=1$), acute myelogenous leukaemia ($n=3$), Hodgkin's lymphoma ($n=6$) and non-Hodgkin's lymphoma ($n=10$).
‡ Coronary bypass ($n=6$), atrial septum defect ($n=2$), valve replacement ($n=1$), pericardiotomy ($n=1$).
§ Candidosis ($n=9$), cryptococcosis ($n=2$) and zygomycosis ($n=1$).
LAT, latex agglutination test; ND, not done.

($n=1$) and testicular carcinoma ($n=1$). Actiological agents include *Aspergillus fumigatus* ($n=6$), *Aspergillus terreus* ($n=1$), *Aspergillus fumigatus* plus *Aspergillus terreus* ($n=1$) and *Aspergillus niger* ($n=1$). It is, however, essential for the understanding and correct interpretation of the test results to know the detailed circumstances under which the available serum samples were drawn. For this reason, short case reports, including therapy failure analyses, are presented.

Patient 1

A 33-year-old woman with a non-Hodgkin's lymphoma died from invasive pulmonary aspergillosis 35 days after autologous bone marrow transplantation with persistent leucopenia. Four serum samples, drawn on days 17, 16, 10 and 1, before death, failed to show any antibody response. The final serum sample was antigen positive with a titre of 1:1. Amphotericin B (40–60 mg day^{-1}), followed by liposomal amphotericin B (200 mg day^{-1}), was instituted too late in the course of the disease, and the dosage was increased too slowly, to prevent the fatal outcome.

Patient 2

A 53-year-old man with a non-Hodgkin's lymphoma died from invasive pulmonary aspergillosis with a leucocyte count ranging from 80 to 300 μl^{-1} after chemotherapy. Four serum samples, drawn on days 21, 16, 9 and 1 before death, were negative for anti-*Aspergillus* antibodies. The final serum sample was antigen positive with a titre of 1:2. The initial antimycotic chemotherapy was fluconazole, 200–400 mg day^{-1}, which is not efficient against *Aspergillus*. The correct chemotherapy, a combination of amphotericin B 35 mg day^{-1} and flucytosine 4×2.5 g day^{-1}, was administered only for the final 9 days.

Patient 3

An 8-year-old boy (19.3 kg body weight) with cystic fibrosis survived a 4-week episode of *Aspergillus fumgiatus* septicaemia with fever of 40 °C due to invasive pulmonary aspergillosis. Evidence for this diagnosis was provided by several positive sputum cultures, followed by two independent positive blood cultures. Systemic amphotericin B (1–1.8 mg kg^{-1} day^{-1}) plus flucytosine ($4 \times$ 625 mg day^{-1}) for 24 days successfully cured the infection. One serum sample, drawn 7 days after the second positive blood culture, was positive for anti-*Aspergillus* antibodies with three haemagglutination tests, two complement fixation tests and two enzyme immunoassays. The antigen test remained negative.

Patient 4

A 61-year-old man with multiple myeloma suffered from invasive maxillary sinusitis with a 2-cm perforation of the hard palate caused by *Aspergillus niger*, as shown by histological examination of a biopsy specimen. The patient died 2 months later at home from progression of his underlying disease. Antimycotic therapeutic strategies included systemic amphotericin B (30 mg day^{-1} for 4 weeks), followed by oral itraconazole (400 mg day^{-1}) after discharge from the hospital until death. An autopsy

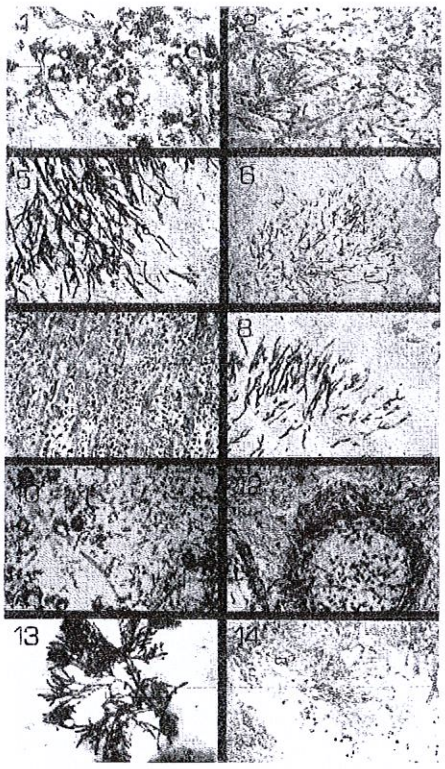

Figure 1. Histopathological evidence of invasive aspergillosis in biopsy specimens (pictures 6, 10 and 14), autopsy specimens (pictures 1, 2, 5, 7, 8 and 12) and a bronchial washing (picture 13), from 10 of the 14 study patients. Picture numbers correspond to the patient numbers in Table 3 and to the case numbers in the Results section. PAS, periodic acid–Schiff reagent; GMS, Grocott methenamine silver stain; HE, haematoxilyn and eosin stain. The original magnification of all pictures is 160×; that of picture 6 is 50×. *Patient 1*: Aerated part of the upper lobe of the right lung with numerous conidiophores and characteristic heads, which allow diagnosis of the species *Aspergillus fumigatus*. PAS. *Patient 2*: Mycelial growth in lung tissue. Frequent septa, uniform diameter and dichotomous branching at an angle of approximately 45° are characteristic of the genus *Aspergillus*. GMS plus eosin. *Patient 5*: *Aspergillus terreus* hyphae (species identification by culture) cause massive haemorrhage in the lung tissue. GMS. *Patient 6*: *Aspergillus fumigatus* hyphae thriving in a subcutaneous axillary nodule, little affected by an abortive defensive reaction. PAS. *Patient 7*: Myocardium with micronodular fungal infiltrates. Two blood cultures, drawn 3 days and 1 day before death from cardiac failure, grew *Aspergillus terreus*. Note the extraordinarily frequent septation of the hyphae. PAS. *Patient 8*: Typical centrifugal growth of *Aspergillus* hyphae within the heart tissue. GMS. *Patient 10*: Endobronchial biopsy of the dorsal segment of the right upper lobe of the lung, featuring *Aspergillus fumigatus* conidial heads. HE. *Patient 12*: Heart of the patient, who died from fungal endocarditis and myocarditis. *Aspergillus* thrombus in a cardiac vessel, with invasion of the vessel wall and the surrounding tissue. Note the poor cellular immune response. PAS. *Patient 13*: Bronchial washing from the lower lobe of the right lung, showing semi-invasive growth of *Aspergillus* hyphae. PAS. *Patient 14*: Lung tissue, resected for suspected metastasis of testicular carcinoma, reveals the true nature of the lesion: *Aspergillus fumigatus*. PAS.

was not performed. One serum sample drawn at the time of active sinusitis was negative with all assays tested.

Patient 5

A 74-year-old man (74 kg body weight) with acute myelogenous leukaemia survived a 2-month episode of invasive *Aspergillus fumigatus* pneumonia under systemic therapy with amphotericin B (30 mg day^{-1} for 4 weeks), but 6 months later succumbed to a relapse of invasive aspergillosis, in this case due to *Aspergillus terreus*, despite 4 weeks' combination therapy with amphotericin B (30 mg day^{-1}) and flucytosine (4×2.5 g day^{-1}). Leucocyte counts were 2600 μl^{-1} and 7000 μl^{-1} shortly before death. Between day 36 and day 8 before death, a significant fourfold increase in titre was detected with two haemagglutination tests (IHA-LD, 1:20 to 1:80; IHA-Fumouze, 1:160 to 1:640). All other antibody tests and the antigen test were negative.

Patient 6

A 24-year-old female heart transplant recipient (60 kg body weight) suffered from a meningitis of unknown aetiology 6 weeks after surgery. She recovered under fluconazole therapy (400 mg i.v. for 4 weeks, 200 mg orally for 3 more months). However, under this regimen, she developed two subcutaneous nodules in the right axilla (histologically and culturally *Aspergillus fumigatus*) and one more subcutaneous nodule above the right knee. Leucocyte counts ranged from 11 000 to 27 000 μl^{-1}. After 4 months' itraconazole therapy (400 mg day^{-1}), she eventually fully recovered. Six serum samples revealed a titre increase with the IHA-LD (1:40 to 1:320) between recovery from meningitis and the onset of disseminated aspergillosis. The first of three samples of cerebrospinal fluid (CSF) was positive for *Aspergillus* galactomannan antigen (titre 1:2). As the culture remained negative, a false-positive LAT in the CSF cannot be excluded.

Patient 7

A 56-year-old man with acute lymphocytic leukaemia succumbed to a disseminated *Aspergillus terreus* infection without the administration of any antimycotic chemotherapy. Blood cultures taken 3 days and 1 day before death were positive for *Aspergillus terreus* (with characteristic alcuriospores). Autopsy revealed *Aspergillus* mycelia in the heart, the brain, the left suprarenal gland and the kidneys. Four serum samples drawn 60, 59, 53 and 3

days before death failed to produce positive results with all test kits evaluated.

Patient 8

A 58-year-old man succumbed to a disseminated *Aspergillus fumigatus* infection (involving the heart, both lungs and the thyroid gland), 19 days after receiving a liver transplant. Antimycotic chemotherapy was inappropriate in the beginning (fluconazole, 400 mg day^{-1} for 10 days). The dosage of subsequent amphotericin B, 45 mg day^{-1} for 6 days, was far below the required quantity. Appropriate therapy was begun late after onset of the disease, with amphotericin B, 45 mg day^{-1}, plus flucytosine, 2×2.5 g day^{-1}, for the last 4 days. Four serum samples, drawn on days 19, 11, 3 and 1 before death revealed seven positive antibody tests, four of them on the day of liver transplantation (IHA-Roche, 1:40; EIA-IgM, 75 U ml^{-1}; CF-somatic, 1:160; CF-metabolic, 1:160), two on day 11 before death [IHA-LD, 1:160 (borderline positive); CF-somatic, 1:20] and one on days 3 and 1 before death (CF-somatic, 1:40). The antigen detection was borderline positive (titre 1:1) on days 11 and 1 before death.

Patient 9

A 52-year-old man with an acute myelogenous leukaemia and pulmonary infiltrates on chest radiographs responded well to systemic administration of amphotericin B (30–60 mg day^{-1}) plus flucytosine (4×2.5 g day^{-1}), which was continued for 10 weeks. However, during treatment with itraconazole (400 mg day^{-1}) overlapping for 1 month and continued for 3 more months, he finally succumbed to lung failure due to massive pulmonary infiltrates with leucocyte counts persisting below 100 μl^{-1}. A transbronchial biopsy was negative for invasive mycoses, an autopsy was not performed and cultures were negative. Five serum samples drawn on days 107, 101, 74, 9 and 2 before death revealed high titres of anti-*Aspergillus* antibodies in all assays evaluated (e.g. IHA-LD up to 1:20 000). However, no antigen was detectable in any serum sample by the LAT kit.

Patient 10

A 38-year-old woman with acute myelogenous leukaemia died from an invasive pulmonary aspergillosis. The diagnosis was confirmed by a transbronchial biopsy, which was positive for *Aspergillus fumigatus*. With persisting low neutrophil counts (300 to 100 μl^{-1}), antimycotic chemotherapy was not successful: fluconazole (400 mg day^{-1} for 7 days), followed by a combination of amphotericin B (50 mg day^{-1}) and flucytosine (4×2.5 g day^{-1}) for 6 days, and finally amphotericin B alone (50 mg day^{-1}) for 12 more days. Four serum samples, drawn on days 19, 12, 6 and 5 before death, were all negative for antibodies and antigen.

Patient 11

An 8-year-old boy (18 kg body weight) with acute lymphocytic leukaemia (neutrophil counts during therapy 200 to 500 μl^{-1}), had several 1.8 cm coin lesions on chest radiographs, which were reduced in size to 1.0 cm under combination therapy (7.5–15 mg of amphotericin B plus 4×130 mg of flucytosine for 4 months). However, during subsequent oral therapy with itraconazole (100 mg day^{-1}) for 2.5 months, the child relapsed and died after another month of combination therapy (20 mg of amphotericin B plus 4×600 mg of flucytosine). A biopsy was not taken, fungal cultures remained negative and an autopsy was not performed. Seven serum samples drawn on days 166, 146, 142, 131, 125, 8 and 1 before death revealed positive antibody results during the first pulmonary infection (IHA-LD, 1:320; IHA-Roche, 1:80; EIA-IgG, 80 U ml^{-1}; CF-somatic, 1:40), but negative antigen tests. During the fatal relapse, the IHA-LD became negative (<1:20), the IHA-Roche titre increased to 1:320, the EIA-IgG increased again to 89 U ml^{-1}, the CF-somatic increased again to 1:40, and the antigen test became positive with titres of 1:4 in the final two serum samples.

Patient 12

A 56-year-old man with a non-Hodgkin's lymphoma (68 kg body weight) died from disseminated aspergillosis with a neutropenia of 1300 leucocytes μl^{-1}. The antimycotic chemotherapy was intravenous fluconazole, 200 mg day^{-1}, for the final 8 days. An autopsy revealed *Aspergillus* mycelia in the lungs, the heart and the sphenoidal sinus. Three serum samples drawn on days 36, 28 and 8 before death were all negative.

Patient 13

A 56-year-old man with full blown AIDS (CD4-positive cells <100 μl^{-1}) died from invasive pulmonary aspergillosis with possible haematogenous dissemination to the liver. A bronchoalveolar lavage was positive for *Aspergillus fumigatus*. Administration of itraconazole (400 mg day^{-1}) and amphotericin B (20 mg day^{-1} plus flucytosine 4×1.5 g day^{-1}) did not prevent the fatal outcome.

One serum sample drawn 15 months before death, at a time when the pulmonary aspergillosis was still considered semi-invasive (Figure 1, patient 13), was negative with all tests.

Patient 14

A 25-year-old man with a testicular carcinoma underwent a pulmonary lobectomy for suspected metastasis. The lesion, however, turned out to be due to *Aspergillus* infection (Figure 1, patient 14). One serum sample, drawn 17 days after surgical removal of the pulmonary *Aspergillus* lesion, gave positive results in three antibody tests (IHA-Fumouze, 1:640; EIA-IgG, 90 U ml^{-1}; EIA-IgA, 72 U ml^{-1}), whereas the antigen test was negative at that time.

There was a total of 14.2% false-positive results (77 of 544 serological tests). Twenty-seven of 68 control individuals (39.7%) had at least one false-positive antibody test (Table 3).

In 9 of the 15 healthy students and staff members all nine tests were negative. One subject tested falsely positive in three tests (IHA-LD, 1:320; IHA-Roche, 1:40; and EIA IgG, 69 U ml^{-1}), another tested positive in two tests (EIA IgG, 65 U ml^{-1}; and CF-somatic, 1:20) and four more individuals tested falsely positive in one test (IHA-Roche, 1:40; IHA-Roche, 1:160; EIA IgG, 101 U ml^{-1}; and CF-somatic, 1:20).

Twenty patients with leukaemia or lymphoma after bone marrow transplantation did not yield a single false-positive result with any of the tests evaluated.

Serum samples from two of the three patients suffering from multiple myeloma reacted strongly in all antibody tests. In the case of the third patient the serum sample was negative in all assays.

Only one serum sample from the 10 patients who had had open heart surgery tested correctly negative in all tests. One patient's serum produced false-positive results in six tests and another patient's serum produced false-positive results in five tests, as did two patients' sera in four tests, two patients' sera in two tests and three patients' sera in one test.

The bronchopulmonary system of the eight patients with cystic fibrosis was colonized with *Staphylococcus aureus* ($n=5$), *Pseudomonas aeruginosa* ($n=4$) and *Candida albicans* ($n=3$). None of them was colonized with *Aspergillus* species or suffered from invasive aspergillosis or allergic bronchopulmonary aspergillosis. Serum samples from seven of these eight patients suffering from cystic fibrosis tested falsely positive in at least one of the antibody tests.

Among the 12 patients with proven deep-seated mycoses other than aspergillosis, six patients with candidosis, two with cryptococcosis, and one with zygomycosis did not show any false-positive *Aspergillus* test. Sera from three patients with *Candida* infection reacted with *Aspergillus* reagents.

The sensitivity of the antibody tests was generally low (14–36%) (Table 4). There was no significant difference between all eight antibody tests. The specificity was generally higher, reaching 99% with the complement fixation test on the basis of metabolic *Aspergillus fumigatus* antigen. The LAT had a sensitivity of 36% and a specificity of 100%.

Table 4 Diagnostic value of serological tests for invasive aspergillosis*

Assay format	Manufacturer	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Efficiency (%)
IHA	LD	29	87	31	85	77
IHA	Roche	36	68	19	84	62
IHA	Fumouze	36	88	38	87	79
EIA-IgG	DDV	29	72	17	83	65
EIA-IgM	DDV	14	91	25	84	78
EIA-IgA	DDV	29	93	44	86	82
CF-som	Virion	29	79	22	84	71
CF-met	Virion	21	99	75	86	85
LAT	Pasteur	36	100	100	86	87

* Based on 14 proven cases of invasive aspergillosis and 68 control individuals.
IHA, indirect haemagglutination assay; EIA, enzyme immunoassay; CF, complement fixation; som, somatic; met, metabolic; LAT, latex agglutination test; LD, latex agglutination test; Labor Diagnostik, D-16359 Heiden, Germany; Roche, Roche Serologie, D-81929 Munich, Germany; Fumouze, Fumouze Diagnostics, F-92600 Asnières, France; DDV, DDV Diagnosika, D-35037 Marburg, Germany; Virion=Institut Virion GmbH, D-97072 Würzburg, Germany; Pasteur, NV Sanofi Diagnostics Pasteur, B-3600 Genk, Belgium.

Discussion

Invasive fungal disease met the stringent histopathological and/or microbiological criteria required to confirm the diagnosis in 12 cases. Fusariosis or pseudalle scheriosis could not be excluded in three cases, although it appears far less likely than invasive aspergillosis. In two cases stringent criteria for proof of invasive aspergillosis could not be met, and these results must therefore be interpreted carefully. The value of both antibody and antigen detection assays was limited by a significant number of false-negative results in the group of patients with proven invasive aspergillosis. Likely reasons for the low sensitivity of all tests (14–36%), i.e. failure correctly to identify proven cases of aspergillosis, include (1) the limited number and poor timing of serum samples (cases 13 and 14), (2) possible limited cross-reactivity with *Aspergillus terreus* (case 7), (3) severe immune deficiency (cases 1, 2 and 10), (4) isolated maxillary sinus affection (case 4) and (5) unknown factors (case 6). False-negative results with the LAT for antigen detection may be due to (1) analysis of frozen samples in several cases, with a possible loss of activity, and (2) rapid clearance of the galactomannan antigen [13, 14]. In this investigation, false-positive results were a problem with antibody detection tests only.

One of the healthy individuals who tested falsely positively in three tests was a 30-year-old woman who was a long-term staff member of the mycology laboratory. There was no obvious explanation for the occasional false-positive results in another five students.

The lack of false-positive results in patients with leukaemia or lymphoma who underwent bone marrow transplantation and received early empiric antimycotic chemotherapy for fever of unknown origin is encouraging. This patient population represents one of the major high-risk groups for the development of invasive aspergillosis and needs to be screened with *Aspergillus* serodiagnostic tests. Serum samples from patients with multiple myeloma are known to interfere with many serodiagnostic tests.

Patients recovering from recent open heart surgery constitute a newly discovered group of patients with a high frequency of false-positive *Aspergillus* titres (haemagglutination tests in particular) for hitherto unknown reasons.

The respiratory tract of children suffering from cystic fibrosis is often colonized with *Aspergillus fumigatus* (up to 30%) [15], resulting in the production of specific antibodies.

The two patients with proven deep-seated candidosis whose serum samples reacted positive with four and three anti-*Aspergillus* antibody tests may have suffered from an undiagnosed coexisting infection with *Aspergillus*, which is quite common.

The sensitivity of antibody detection assays for the diagnosis of invasive aspergillosis ranges from 31% to 89% (average 66.3%) according to the current literature [1, 7, 8] (Table 5). The average sensitivity of eight antibody tests in this investigation was 27.9% (range 14–36%). The only prominent antibody detection assay was the complement fixation test, using metabolic antigen of *Aspergillus fumigatus*. Its sensitivity (99%) was higher than that of the other seven antibody detection tests, and the overall costs were lower for this assay. Using the combination of eight antibody tests in this study, an overall sensitivity of 50.0% was achieved.

The detection of antibodies against specific protein antigens of defined molecular size, e.g. against a 58-kDa antigen, has been claimed to promise more specificity for deep infection [8]. The

Table 5. Summary of recently published reports on the diagnostic value of antibody detection in human invasive aspergillosis

Reference	Assay format	Patients with invasive aspergillosis		Patients with positive results	Sensitivity (%)
		No.	Proven		
Culino *et al.* (cit. in [1])	IHA	32	No	26	81.3
Pinon *et al.* (cit. in [1])	ELIFA	9	No	7	77.8
Burnie & Matthews [7]	Western blot	50	Yes	26	52.0
Fratamico *et al.* [8]	Western blot	38	Yes	34	89.5
Kugel (doctoral thesis)	ELISA IgG	23 (64 sera)	No	54 sera	84.4
	ELISA IgA	23 (64 sera)	No	41 sera	64.1
	IHA	23 (64 sera)	No	20 sera	31.3
This study (see Table 4)	Eight tests	14	Yes	7	50.0

ELIFA, enzyme-linked immunofiltration assay; ELISA, enzyme-linked immunosorbent assay; IHA, indirect haemagglutination assay.

haemagglutination test from LD is the only commercially available *Aspergillus* antibody detection assay, based on a partially purified antigen (Table 1). However, there was no obvious advantage of this assay in this investigation. The other antigens, used as a basis for the antibody detection assays evaluated in this study, are mixed somatic or metabolic antigens (Table 1).

The LAT for *Aspergillus* antigen detection was the only test not giving false-positive results in this investigation. However, experience in the routine testing of a large number of serum samples in our laboratory suggests that positive results with a titre of 1:1 (i.e. agglutination of 40 µl supernatant of the pretreated serum sample with 10 µl of latex reagent) may be non-specific. This false-positive reaction, however, will not hold positive when the supernatant is diluted 1:1 with glycine buffer (pH 8.2) and then retested, i.e. the titre 1:2 will be negative. The reasons for this non-specific agglutination of undiluted supernatants are unknown. Positive LAT titres for *Aspergillus* galactomannan antigen with undiluted supernatant only (titre 1:1) must therefore be interpreted cautiously. In this study, no false-positive antigen tests resulting from contamination of serum samples with moulds were found [16].

Since no urine samples were available, we cannot comment on a previously described superiority of urine antigen testing over serum antigen testing [5]. However, in our hands, in contrast to observations by Ansorg *et al.* [5], there was no positive LAT antigen titre resulting from exposure to environmental, non-infectious *Aspergillus* antigens in any of the control individuals.

Five of 14 proven cases of invasive aspergillosis were correctly diagnosed with aid of the LAT: in each of cases 1 and 2, one serum sample reacted with a titre of 1:1 and 1:2 respectively. Both samples were drawn 1 day before death. In case 6, one sample of cerebrospinal fluid drawn at the beginning of meningitis produced a titre of 1:2, and in cases 8 and 11 two serum samples each reacted with titres of 1:1 (2×) and 1:4 (2×) respectively. The blood samples were drawn 11 days and 1 day before death in case 8 and 8 days and 1 day before death in case 11. This confirms previous observations on positive fungal antigen detection in serum samples taken from severely ill patients shortly before death, whereas negative results were obtained with antigen detection assays in early stages of fungal infections [4, 11]. Furthermore, our results are in agreement with previous findings that maximum titres in patients generally do not exceed values of 1:8 [5, 9, 11, 13, 16].

The low sensitivity of the *Aspergillus* galactomannan antigen detection assay found in this study falls into the range of values reported in 16 previous investigations of *Aspergillus* antigen detection in patients (sensitivity in this study 35.7% compared with an average of 64.3%; range 16.7–100.0%) (Table 6). Six of these investigations evaluated galactomannan antigen detection using

Table 6. Summary of recently published reports on the diagnostic value of antigen detection in human invasive aspergillosis

Reference no.	Assay format	Patients with invasive aspergillosis		Patients with positive results	Sensitivity (%)
		No.	Proven		
Reiss *et al.* (cit. in [1])	CIE	6	Yes	6	100
Schaffer *et al.* (cit. in [1])	RIA	3	Yes	3	100
Weiner *et al.* (cit. in [1])	RIA	15	Yes	10	67.0
Sabetta *et al.* (cit. in [1])	ELISA	19	Yes	11	58.0
Weiner *et al.* (cit. in [1])	RIA	7	Yes	4	57.1
Dupont *et al.* [10]	RIA	12	Yes	2	16.7
Talbot *et al.* (cit. in [1])	RIA	22	Yes	17	77.3
Wilson *et al.* (cit. in [1])	ELISA	9	Yes	8	89.0
Fujita *et al.* (cit. in [1])	RIA	12	Yes	2	16.7
Johnson *et al.* (cit. in [1])	BALISA	4	Yes	3	75.0
Dupont *et al.* [11]	LAT	15	Yes	14	93.3
Rogers *et al.* (cit. in [1])	ELISA	19	Yes	18	94.7
Burnie [12]	RPLA	50	Yes	15	29.4
Warnock *et al.* [9]	LAT	6	No	4	66.7
Hashigushi *et al.* (cit. in [1])	LAT	18	Yes	7	38.8
Ansorg *et al.* [5]	LAT	4	Yes	2	50.0
This study (see Table 4)	LAT	14	Yes	5	35.7

ALISA, biotin–avidin-linked immunosorbent assay; CIE, counterimmunoelectrophoresis; ELISA, enzyme-linked immunosorbent assay; LAT, latex agglutination test; RIA, radioimmunoassay, RPLA, reverse passive latex agglutination.

polyclonal rabbit antibodies [1] or monoclonal rat antibodies [5, 9, 11], the sensitivity of which is limited, in particular, by rapid clearance from the bloodstream [14]. Other authors detected protein antigens using assays based on culture filtrate antigens prepared from *Aspergillus fumigatus* [12].

In summary, these 16 investigations were based on serum samples from an average of 13.8 (range 3–50) proven cases of invasive aspergillosis. In contrast to 12 out of 14 cases in our study, criteria for proven invasive aspergillosis in several studies, included isolation of *Aspergillus* species from bronchoalveolar lavage in combination with clinical response to systemic antimycotic chemotherapy. The exact timing of serum samples with documented stages of invasive fungal disease has been performed only very rarely (e.g. in ref. 1). Higher sensitivities of antigen assays reported by other authors (e.g. in ref. 1) are due to a selection of cases and serum samples.

A failure analysis of systemic antimycotic chemotherapy in the 14 cases of invasive aspergillosis reported in our study results in the following conclusions. The mortality in this group was 78.6% (11/14). The three successfully treated patients were the 8-year-old boy with cystic fibrosis (case 3), the 24-year-old heart transplant recipient (case 6) and the 25-year-old patient with testicular carcinoma, who was lobectomized without antimycotic chemotherapy (case 14). Reasons for therapy failures include lack of antimycotic chemotherapy (case 7), inappropriate initial antimycotic chemotherapy (e.g. fluconazole in cases 2, 8, 10 and 12), failure to institute appropriate antimycotic chemotherapy in time (cases 1, 4 and 5) and persistent neutropenia or relapse of the underlying disease (cases 9, 11 and 13).

In conclusion, currently commercially available antibody detection assays are insufficient for the detection of invasive aspergillosis. The detection of circulating *Aspergillus* galactomannan antigen using the LAT does, indeed, confirm the diagnosis of an invasive aspergillosis. However, negative results do not exclude the existence of this disease, especially in the early stages.

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