

Advances in the serological diagnosis of invasive *Aspergillus* infections in patients with haematological disorders

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Summary

A reliable diagnosis of invasive aspergillosis in patients with haematological malignancies is seldom achieved antemortem. Conventional laboratory diagnostic methods are insensitive and time-consuming, resulting in late diagnosis and treatment and contributing to unacceptably high mortality. As a result, routine antifungal prophylaxis and early empirical treatment have been recommended. However, overtreatment associated with these strategies results in increased toxicity and cost. The use of sensitive and rapid non-culture-based diagnostic assays, such as detection of *Aspergillus* antigens (galactomannan, β -D-glucan) or detection of genomic DNA sequences may allow a shift in emphasis from empirical to pre-emptive therapy, especially when substantiated by suggestive radiological findings. These new tools may be used to confirm a presumed diagnosis of invasive aspergillosis, or, when used to screen high-risk patients, may identify an infection at the early stage of disease. The excellent negative predictive value of these assays should convince clinicians to withhold antifungal therapy in persistently febrile neutropenic patients with no other signs of fungal infection. On the other hand, consecutive positive results in a high-risk population should at least trigger a complete diagnostic work-up. This review will focus on the diagnostic utility as well as on the pitfalls of serial screening for the presence of circulating fungal antigens in haematology patients.

Key words: Galactomannan, β -D-glucan, aspergillosis, neutropenia, diagnosis, pre-emptive.

Introduction

The incidence of deep fungal infections has increased over the last two decades, with the majority of these infections occurring in patients with haematological malignancies. To date, invasive fungal infection is a major direct or contributory cause of death in patients with acute leukaemia/myelodysplastic syndrome who undergo intensive chemotherapeutic treatment.¹ Invasive fungal infections are also the major cause of infectious mortality in haematopoietic stem cell transplant recipients, not only during the early neutropenic

phase, but also later when graft-vs.-host disease (GVHD) develops.² In addition, recent treatment strategies that result in prolonged impairment of B and T cell-mediated immunity (e.g. monoclonal antibodies such as rituximab or alemtuzumab; purine analogues) have created new populations at risk, including patients with low-grade haematological malignancies.³

Species of the *Aspergillus* family account for a substantial number of these infections, and in particular pulmonary infection with *Aspergillus fumigatus* has emerged notably in the developed world. A recent Italian cohort study found that *Aspergillus* accounted for 90% of all mould infections in patients with haematological malignancies.⁴ Recent work from the Seattle group confirmed the 10–20% incidence of invasive aspergillosis in allogeneic stem cell transplant recipients.⁵

The spectrum of disease manifestations is heterogeneous and determined by the inoculating dosage, the

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Accepted for publication 18 December 2006

ability of the host to resist infection at both local and systemic levels, and the virulence of the organism. Some manifestations are characterised by chronic growth of *Aspergillus* in preformed and poorly drained spaces (e.g. remnants of previous infections) and are non-invasive. However, if the immune defence system of the target organ is weakened, inhaled conidia will germinate and produce hyphae that invade the surrounding tissue, leading to the development of life-threatening disease. Eventually, invasion and destruction of the underlying tissue will lead to involvement of adjacent organs by continuous growth and to haematogenous dissemination.⁶

The increased incidence of invasive aspergillosis is explained by the rapid expansion of populations at risk, by the growing awareness of aspergillosis among clinicians, and possibly by the widespread prophylactic use of limited-spectrum triazoles. In addition, a continuous increase can be anticipated, basically because the number of people at risk is expected to grow.

1 Allogeneic haematopoietic stem cell transplantation is now a routine procedure for the treatment of a vast number of malignancies and immunological disorders. In addition, non-myceloablative procedures are being pioneered for a wide range of cancers, including solid tumours; their main objective was to induce a graft-vs.-malignancy effect, thereby putting patients at risk for opportunistic fungal infections.

2 Cord blood transplants and haplo-identical transplants are performed more frequently, but are complicated by longer periods of neutropenia and prolonged severe immunodeficiency, respectively.

3 A battery of immunomodulatory monoclonal antibodies has been licensed recently and has shifted the spectrum of patients at risk towards non-classical populations (e.g. low-grade myelodysplastic syndrome).

4 The use of T-cell immune suppressant purine analogues and antibodies is not longer confined to the treatment of low-grade malignancies; these agents are also used in cytotoxic regimens for acute leukaemia/myelodysplastic syndromes and for the treatment of GvHD.

5 The increased use of high-dose corticosteroids.

Although the diagnosis of an invasive fungal infection can be straightforward (e.g. cryptococcal meningitis), it remains problematic and challenging in the case of invasive aspergillosis.⁷ As a result, infections are often far advanced at the time of diagnostic confirmation (if ever), and overall outcome remains poor despite the availability of new antifungal agents. The most recent studies have demonstrated successful outcomes of approximately 50–70% 3 months after infection,

though <30–40% for allogeneic stem cell transplant recipients and leukaemia patients with uncontrolled underlying disease or persisting neutropenia.⁸ Despite the best current practices, mortality rates remain unacceptably high at 30–50%.⁹

For this reason, emphasis has been placed on developing tests (serological methods and detection of fungal DNA by polymerase chain reaction techniques) that will either confirm diagnosis in suspected patients or provide early clues of infection when applied as a screening tool. Serology (or the detection of species-specific antibodies or antigens) is a desirable primary or ancillary mode to facilitate diagnosis of infections that are caused by difficult-to-culture pathogens or that affect body sites that are difficult to sample, and can be used to monitor progression of the infection and/or response to therapy. The ideal marker for diagnosing an invasive fungal infection is present early in the infectious process, associated with infection rather than colonisation, is conserved within the fungal species of interest, does not cross-react with human or other microbial antigens, is easy to perform, and can be standardised and validated.¹⁰ This study will review the advances in the serodiagnosis of invasive aspergillosis in haematology patients.

Detection of galactofuranose-antigens

Traditionally, demonstration of specific antibodies has been required to establish the diagnosis of chronic pulmonary aspergillosis. However, the detection of circulating anti-*Aspergillus* antibodies is not useful for the diagnosis of acute invasive aspergillosis in neutropenic patients and haematopoietic stem cell transplant recipients, mainly because these populations are not capable of mounting an adequate or timely antibody response.^{11,12} Whether antibody detection may be useful for other risk groups has not been carefully validated, but at least in one report, antibody detection contributed to the diagnosis in lung transplant recipients.¹³

The low sensitivity and low predictive value of antibody detection have prompted the development of immunoassays for the detection of circulating fungal antigens. *Aspergillus* antigenaemia was first detected and characterised in the late-1970s by investigators from the Centre for Disease Control, Atlanta, Georgia.¹⁴ After immunising healthy rabbits with serum of an experimentally infected rabbit with disseminated aspergillosis, they demonstrated that the resultant immune serum formed an immunoprecipitate with an antigen present in an extract of *Aspergillus* hyphae. The same

antigen was also detected in serum and urine of patients with invasive aspergillosis.¹⁵ Physicochemical analysis indicated that this immunoreactive component was galactomannan, a heat-stable heteropolysaccharide present in the cell wall of most *Aspergillus* and *Penicillium* species. The molecule has an estimated molecular mass of 25–75 kDa and comprises a non-immunogenic mannan core that is heavily substituted with side chains of varying lengths containing immunodominant galactofuranosyl units.^{16–18} *In vitro*, the antigen can be released as pure polysaccharide, but the number of epitopes varies between genera and strains, as well as with the conditions used for its extraction and purification. In addition, several other fungal glycoproteins also contain immunogenetic galactofuranose units. Therefore, Mennink-Kersten *et al.* proposed the term ‘galactofuranose-antigens’ for all molecules that could be detected by galactofuranose-specific immunoassays, including galactomannan.¹⁹

Refinement of immunodiagnosis led to the development of more sensitive radioimmunoassays (RIA) and enzyme immunoassays (EIA). Published experience with RIAs to detect *Aspergillus* galactomannan in serum, urine and/or bronchoalveolar lavage fluid in experimental disseminated aspergillosis and in invasive disease in patients looked promising but came largely from a single medical centre.^{20–24} In a blinded study in patients with haematological malignancies, the sensitivity of the RIA was approximately 75%, the specificity 90%, the positive predictive value 82% and the negative predictive value 85%. Antigen was detected before fungal disease was suspected in 30% of case patients and before microbiological documentation in 46%.²⁴ As expected, testing of bronchoalveolar lavage fluid increased the sensitivity, especially in patients with extensive pulmonary involvement. In contrast, others found much lower sensitivities in lethally infected rabbits and in diseased patients and found urine to be a better diagnostic source than serum.²⁵ However, RIA has never gained widespread acceptance due to its limitations, including the inability to detect antigen concentrations in the low ng ml⁻¹ range, the short shelf life of radioisotopes, and the lengthy procedure which makes the technique less suitable for routine clinical use.

The results of specific EIAs, often with a lower limit of detection – as little as 10 ng of galactomannan per millilitre of serum – were in general comparable with RIAs, both in animals and patients with proven and suspected disease.^{26–28} Figures of sensitivity quoted in the published research vary but are generally rather low. Moreover, studies on serial samples from single

patients have demonstrated the transient nature of antigenaemia. These fluctuations were explained by urinary excretion and by mannose receptor-mediated clearance of galactomannan by hepatic Kupfer cells.²⁹ It was hypothesised that an increased efficiency would only be obtained by regular screening using immunoassays with highly specific and sensitive antibodies. The production and purification of monoclonal antibodies and the creation of standardised and reproducible assays were considered necessary steps towards better immunodiagnosis.

In Europe, a latex agglutination test using beads sensitised with the monoclonal antigalactomannan antibody EB-A2 (PastorexTM *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) became commercially available in the early-1990s. The antibody was produced by immunising rats with a mycelial extract of *A. fumigatus* and recognised the β-(1 → 5)-linked galactofuranose epitopes of galactomannan. The assay detected purified antigen at concentrations as low as 15 ng ml⁻¹ from several *Aspergillus* species, including *A. fumigatus*, *A. flavus*, *A. niger*, *A. terreus* and *A. versicolor*. In addition, cross-reaction with exoantigens from several other moulds was noted, including potential laboratory contaminants such as *Penicillium chrysogenum*, *Cladosporium herbarum*, *Acremonium* spp. and *Alternaria alternata*, as well as rare fungal pathogens such as *Fusarium oxysporum*, *Wangiella dermatitidis*, *Rhodotorula rubra* and *Penicillium marneffe*.^{30,31} When evaluated in a guinea-pig model of invasive aspergillosis, sensitivity was 97.6% and specificity was 96.2%.³² These encouraging initial results were also confirmed in human sera by Dupont *et al.*; the kit was positive in 13 of 15 proven cases and 17 of 18 patients with a high probability of invasive aspergillosis.³³ However, subsequent evaluations in larger series of neutropenic patients yielded disappointing results: clinical sensitivity was usually low (approximately 30%) and circulating antigen was detected only in the late stages of disease, negating the primary objective of developing fungal diagnostic methods.^{34–41}

In the early-1990s, serodiagnosis for invasive aspergillosis faced many challenges.^{34–41}

1 Although highly specific in (mainly) neutropenic patients, detection of galactomannan in serum and urine was only moderately sensitive and not reliable at all in non-invasive presentations of *Aspergillus* infections.

2 The temporal relationship of a positive assay and early symptoms of disease or initiation of treatment was not accurately assessed. Antigenaemia preceded clinical diagnosis or antifungal therapy in some patients, but

failed in many others to diagnose disease before other conventional means or start of empiric therapy, making the test clinically redundant.

3 Single sera tested often negative so that serial sampling was required. However, an optimal sampling frequency was not determined.

4 Antigen levels in body fluids correlated with the extent of fungal involvement and serological response correlated well with clinical response, but the use of galactomannan as a prognostic marker or its use in therapeutic monitoring had never been properly addressed.

5 Reproducibility was suboptimal.

Most importantly, the observed and reported variability in the performance of all these assays often resulted from methodological flaws that undermined virtually all clinical studies.

Factors that influence the performance of immunodiagnostic tests

An accurate assessment of a new diagnostic test relies on the presence of a gold standard criterion which allows discrimination of patients with the infection and those without the disease. Ideally there should be no other difference between both study groups that may influence the tests performance.⁴² Because the histopathological confirmation of fungal tissue invasion is still the reference diagnostic test for invasive aspergillosis, the ideal study population would be made up of patients at risk, who, at autopsy, are determined to have or not to have aspergillosis. Such a stringent criterion would confirm or rule out the presence of disease in both study groups and circumvent the problem of misclassification bias. However, solely relying on such undisputable definitions of cases and controls introduces a new bias by omitting a large segment of the relevant study population from the analysis (i.e. all episodes that cannot be adjudicated unambiguously), whereas these new diagnostic assays are often exactly developed for a better classification of these 'mild or difficult-to-classify' cases. It is well recognised that case-control studies (especially when using healthy volunteers as control group) cause overestimations of sensitivity and of specificity. According to a meta-analysis by Lijmer *et al.*, the use of a case-control design was the most important deviation from the ideal study design on diagnostic tests.⁴³ In addition, an autopsy-controlled design inevitably selects for more severe manifestations of aspergillosis. Therefore, stratification of patients according to their probability of disease seems to be a more realistic approach, although the true status of

disease of many study patients remains unknown in the absence of tissue specimens. In this setting, the performance of any diagnostic test will remain sub-optimally defined and will dramatically be influenced by the proposed case definitions. This methodological shortcoming could at least partly explain the broad range of sensitivities and specificities that was observed in one of the early RIA studies, reflecting the extreme premise that all patients with 'indeterminate' disease were either all 'case patients' or all 'control patients'.²⁴ Besides, as often is the case in diagnostic studies with an invasive gold standard, the majority of indeterminate and control patients are 'verified' by a softer negative reference standard, such as clinical judgement or evolution during follow up. This introduces new biases of partial verification and use of a different reference standard. Once again, applying different reference standards may over- or under-estimate the performance of a diagnostic test, because the strategy fails to identify both false-negative and true-negative test results.⁴⁴ Unfortunately, to date there exists no firm solution to this problem.

A critical appraisal of the test applicability relies on a detailed description of the population under study and the disease characteristics.⁴⁵ Patient populations at risk for invasive aspergillosis vary with regard to their underlying disease and the net state of immunosuppression; both factors influence the clinical presentation of aspergillosis, the production of antibodies, and the release and clearance of extracellular antigen. Differences in test performance may frequently be explained by differences in the spectrum of severity of disease as well as in the spectrum of differential diagnoses. The case-mix of severity of illness affects overall sensitivity (at a constant cut-off), whereas specificity depends on the spectrum of alternative or confounding diagnoses. Assuming that two separate studies are conducted on the accuracy of *Aspergillus* antigen detection, one in the intensive care unit, the other in the leukaemia and stem cell transplantation unit, and that both studies admit the same number of consecutive cases, very different sensitivities and specificities may be reported due to mismatches in disease severity (e.g. neutropenic vs. non-neutropenic) and alternative diagnoses (e.g. postsurgical inflammatory response, viral infections), respectively.

In addition to characteristics of study design, flaws related to methods of data collection and data reporting may undermine test accuracy. As opposed to retrospective studies that use a small number of samples (usually frozen sera) from patients known to have had the disease under study, prospective studies use samples obtained at predetermined intervals from a suitable

number of patients at risk. The larger number of available samples in the latter studies will falsely boost the test's sensitivity, especially if antigenaemia is transient and/or if samples are collected closely to the time of clinical diagnosis or death (disease progression bias). Ideally, patients at risk should be monitored serially during the period of highest risk.⁴²

When analysing dichotomous outcomes (e.g. aspergillosis vs. no aspergillosis) and predictor assays (seropositive vs. seronegative), statistical performance is derived from 2×2 contingency tables. However, very few serodiagnostic assays function perfectly well with the use of strictly positive and negative results; especially when using quantitative assays (e.g. reporting an OD index ratio) there is often an overlap in the distribution of serological values between diseased and non-diseased patients resulting in a 'gray area' of indeterminate or equivocal values. If so, the choice of the cut-off merits careful consideration: biasing of the threshold towards lower values will improve sensitivity at the cost of lesser specificity (overdiagnosis), whereas the reverse will enhance specificity but compromise sensitivity (missed diagnosis).⁴⁶ From a clinician's point of view, neither sensitivity nor specificity but rather the probability that a patient with a given test

result is actually infected or not (the predictive value) is much more informative. However, all predictive values very much depend on the prevalence of disease in the (study) population; if the infection is uncommon, false-positive tests will occur more likely in truly uninfected patients, despite a very sensitive and specific assay. Therefore, studies that include a large number of patients at low risk for aspergillosis (e.g. autologous peripheral blood stem cell transplants or solid tumour patients) will report low positive predictive values. As a rule of thumb, a test usually provides valuable information if the pretest probability exceeds 10%. Using likelihood ratios is an elegant way of getting around the dependence of predictive values on the prevalence of infection, but it remains subject to other biases (Table 1). In daily clinical practice, predictive values or likelihood ratios are integrated with a doctors' assessment of pretest probability, resulting in an individualised post-test probability. In patients with a high pretest probability, a positive result will be considered clinically important; vice versa, in patients with a low pretest probability, seropositivity will probably be discarded or one will seek confirmation by alternative means (e.g. additional imaging).⁴⁵

Author	Year	Likelihood ratio of proven/probable IA		
		Positive GM test result (LHR ⁺)	Negative GM test result (LHR ⁻)	Diagnostic odds ratio (LHR ⁺ /LHR ⁻)
Bretagne <i>et al.</i> [50]	1997	4.34	–	–
Bretagne <i>et al.</i> [51]	1998	3.54	0.28	12.64
Machetti <i>et al.</i> [39]	1998	4.41	0.30	14.7
Maertens <i>et al.</i> [52]	1999	18.6	0.07	265.71
Sulahian <i>et al.</i> [57]	2001	11	0.24	45.83
Ulusakarya <i>et al.</i> [55]	2000	13.8	0.32	43.12
Herbrecht <i>et al.</i> [59]	2002	4.57	0.73	6.26
Maertens <i>et al.</i> [58]	2002	10.62	0.16	66.37
Becker <i>et al.</i> [67]	2003	2.87	0.64	4.48
Platelia package insert [74]	2003	7.36	0.21	35
Jarque <i>et al.</i> [68]	2003	22.33	0.34	65.67
Moragues <i>et al.</i> [69]	2003	25	0.96	26.04
Pazos [70]	2003	44	0.12	366.66
Pinel <i>et al.</i> [71]	2003	25	0.96	26.04
Buchheidt <i>et al.</i> [72]	2004	33	0.67	49.25
Maertens <i>et al.</i> [61]	2004	48.5	0.03	1616.66
Marr <i>et al.</i> [66]	2004	2.07	0.62	3.33
Rovira <i>et al.</i> [60]	2004	22.33	0.34	65.67
Yoo <i>et al.</i> [73]	2005	3.90	0.17	22.94

Table 1 Comparison of different studies of galactomannan detection by PlateliaTM Aspergillus (excluding studies with non-haematological patients)

The likelihood ratio for a positive test result (LHR⁺) is calculated as sensitivity divided by (1 – specificity). The likelihood ratio for a negative test result (LHR⁻) is calculated as (1 – sensitivity) divided by specificity. The diagnostic odds ratio is calculated as (sensitivity × specificity) divided by [(1 – sensitivity) × (1 – specificity)] or as LHR⁺ divided by LHR⁻. Potentially useful tests tend to have diagnostic odds ratios well above 20.⁴⁵

Finally, technical aspects such as collection and storage of serum samples, pretreatment of sera and optimal dissociation of immune complexes, laboratory experience as well as presence of interfering substances (such as antibiotic or antifungal agents) may influence the performance. Fortunately, many of these immunoassays lend themselves to automation, allowing better execution of the test (including improved positive and negative control values, wider dynamic range of test values, fewer undecided results, improved intra- and inter-run reproducibility) at reduced technician time and lesser use of consumables.

The sandwich ELISA, a reliable assay

In recent years, increased experience has been gained with the Platelia™ *Aspergillus* (Bio-Rad Laboratories, Marnes-La-Coquette, France and Bio-Rad Laboratories, Hercules, CA, USA), a commercially available sandwich ELISA technique that employs the rat monoclonal antibody EB-A2. By using the antibody both as captor and detector antibody, the detection limit could be lowered to 0.5–1.0 ng of galactomannan per millilitre of serum, resulting in an earlier detection than the latex agglutination assay.⁴⁷ Available in Europe since the mid-1990s, this sandwich immunoassay was cleared by the Food and Drug Administration (FDA) for diagnostic use in the USA in 2003.⁴⁸

The diagnostic potential of this immunoenzymatic assay has been assessed in many studies.^{36,37,39,49–74} A major concern is the fact that the *sensitivity* for proven and probable invasive aspergillosis varies between 25% and 100%. However, a direct comparison of the results of these different studies is not easy, as many factors relating to the host and the study design and methodology influence the performance characteristics of the test (for example, see Table 2).

Type and severity of immunodeficiency.

To date, studies that have reported high sensitivity primarily included haematology patients at risk of invasive aspergillosis, such as those with acute leukaemia, myelodysplastic syndrome and those undergoing allogeneic stem cell transplant or treatment for GvHD. In contrast, studies that have enrolled large numbers of low-risk neutropenic episodes (such as autologous transplants and treatment for solid tumours, lymphoma and non-malignant disorders)⁵⁹ and non-neutropenic immunosuppressed patients (e.g. organ transplant recipients and critically ill patients)^{63–65} have invariably reported low sensitivity (15–30%). This observation is in

line with recently recognised differences in pathogenesis and disease progression of aspergillosis between animals with and without neutropenia.^{75–77} In neutropenic animals, pulmonary disease is characterised by intensive hyphal growth, high fungal burden and angioinvasion. This usually results in high galactomannan concentrations, both in the lungs and in the serum.⁷⁷ In contrast, in corticosteroid-immunosuppressed animals, pulmonary inflammation is the main cause of disease progression. The fungal burden is low with little angioinvasion, resulting in galactomannan levels close to the detection threshold.⁷⁷ In addition, circulating galactomannan levels may be lower in non-neutropenic patients as their ability to clear fungal antigens by macrophage mannose receptors remains unimpaired.

Timing and intensity of sampling.

Most prospective studies have tested for the presence of circulating galactomannan via serial screening (once or twice weekly) throughout the entire period of increased risk (neutropenia, use of immunosuppressive agents and steroids). However, a French study primarily addressed the diagnostic potential of 'targeted' galactomannan detection (up to three consecutive days) in patients with neutropenic fever of unknown origin or with clinically suspected pulmonary infection.⁵⁹ This conceptual difference inevitably results in prominent differences in the mean number and timing of measurements and affects the performance of the assay. For example, the mean number of samples per episode in the study by Maertens *et al.* (11.2 per episode)⁵⁶ differs greatly from that in the study of Herbrecht *et al.* (4.1 per episode).⁵⁹

Disease manifestation of invasive aspergillosis.

Invasive aspergillosis is not a single disease but a continuous spectrum of manifestations with distinct histological and radiological appearances that are based primarily on the immune status of the host. The spectrum ranges from classical angioinvasive disease in the severely immunosuppressed patient to hypersensitivity reactions such as allergic bronchopulmonary aspergillosis in the hyperreactive patient.⁶ Clearly, leakage of galactomannan into the circulation will differ in different disease manifestations. For example, the presence of haemorrhagic infarctions due to angioinvasion may facilitate leakage of antigens, whereas locally invasive (e.g. *Aspergillus* tracheobronchitis in lung transplant recipients)⁶⁴ or encapsulated manifestations (such as abscesses)⁷⁸ may hamper the release of galactomannan.

	Leuven, Belgium ⁵⁶	Strasbourg, France ⁵⁹
Number of study episodes	362	797
Age of study population, <i>N</i> (%)		
≥18 years	362 (100)	749 (94)
<18 years	0	48 (6)
Study population	Haematology	Oncology and haematology
Underlying disorder, <i>N</i> (%)		
Acute leukaemia	165 (45.5)	289 (36.3)
Myelodysplastic syndrome	52 (14.3)	32 (4)
Lymphoma/myeloma	0	242 (30.3)
Solid tumours	0	133 (16.7)
Other	33 (9)	101 (12.6)
Allogeneic stem cell transplants	112 (30.9)	146 (18.3)
Autologous stem cell transplants	0	224 (28.1)
Frequency of sampling	Twice weekly	Variable ¹
Mean number of sera/study episode	11.2	4.1
Cut-off for positivity	OD ≥1.0	OD ≥1.5
Definition of a positive episode	Two consecutive positive samples	A single positive sample
EORTC/MSG case definition		
Proven invasive aspergillosis	30	31
Probable invasive aspergillosis	9	67
Possible fungal infection	54	55
Antifungal prophylaxis	Itraconazole	Not specified
Empirical antibacterial therapy	Cefepime or meropenem	β-Lactam + aminoglycoside
Overall performance of the EIA (%)		
Sensitivity (proven and probable)	89.7	31.6
Sensitivity including possible cases	41.9	29.4
Specificity	98.1	94.8
Positive predictive value	87.5	57.7
Negative predictive value	98.4	84.9

¹Sampling strategy – clinical presentation: fever of unknown origin, suspected pulmonary infection or suspected extrapulmonary aspergillosis (up to three consecutive days). Weekly sampling in transplant recipients⁵⁹

Case definition of invasive aspergillosis.

Given the rigid gold standard definition of proven invasive aspergillosis, stringent criteria should be used in the analysis of sensitivity, specificity and predictive values and should preferably include the histopathological control of positive and negative test results. After verifying 71 clinically highly suspect haematology patients by autopsy and culture, the sensitivity and specificity of serial ELISA monitoring was 92.6% and 95.4%, respectively.⁵² However, autopsy-based evaluations are biased towards selecting manifestations of *Aspergillus* disease with greater organ burden and antigenaemia and omit many unverified, though relevant study episodes. Stratifying study episodes or patients according to their probability of disease while using standardised diagnostic criteria overcomes at least partly these methodological obstacles.⁴² The recently

proposed European Organization for Research and Treatment of Cancer/Mycosis Study Group set of research-oriented consensus criteria for defining degrees of diagnostic certainty (proven, probable and possible) has improved the uniform classification of patients with invasive aspergillosis.⁷⁹ Nevertheless, despite the use of these definitions in most recent studies, variable sensitivities have been reported, largely due to the addition of 'possible' cases to 'proven and probable' cases. 'Possible' infection is usually defined by clinico-radiological findings without microbiological confirmation. Numerous studies have concluded that the inclusion of 'possible' infections in the case definition underestimates the sensitivity; they should therefore be omitted from the sensitivity analysis.

The *specificity* of the assay largely depends on the definition of a positive result. We previously

Table 2 Differences in patient characteristics and study design in two prospective studies

demonstrated a specificity of 98.8% if two consecutive results were required (index ≥ 1.0) vs. 85.4% if only one was required for classification as true-positive.⁵⁶ However, false-positive results have been reported in up to 20% of cases, even in studies requiring consecutive positive samples. The cut-off for positivity is the second major variable that affects specificity. When launched in Europe almost a decade ago, the manufacturer recommended a cut-off serum ratio of 1.5, whereas an index between 1.0 and 1.5 was considered indeterminate.⁷⁴ In their analysis of reproducibility of the test, Verweij *et al.* already suggested to lower the threshold for negative samples from 1.0 to 0.8 and for positive samples from 1.5 to 1.0.⁸⁰ Meanwhile, many European investigators have gradually lowered the index cut-off to 1.0 or less, especially in an attempt to make an earlier diagnosis.^{56,59,61} More recently, the performance of the Platelia™ Aspergillus was also assessed in the USA and was found to be both sensitive (81%) and specific (89%) at a low cut-off. Therefore, in May 2003, the FDA cleared the test as an aid in the diagnosis of invasive aspergillosis in cancer patients, accepting an index cut-off for positivity of ≥ 0.5 .⁴⁸ This latter cut-off has recently also been accepted in Europe based on receiver-operating characteristic (ROC) analyses from two major centres.⁸¹

The problem of unique false-positive samples at a predefined ('static') cut-off can be handled by retesting the original sample (as requested by the FDA) or by requiring a subsequent positive sample ('dynamic' cut-off) for that patient. However, given the gradual rise in antigenaemia in proven and probable cases of IA – as opposed to the stable or fluctuant results in false positivity – a 'dynamic' threshold seems clinically more meaningful. The demonstration of two sequential sera with an OD ≥ 0.5 increases the specificity and the positive predictive value of the assay to 98.6% and the clinical efficiency to 98%.⁶¹ Thus, an increasing ratio above 0.5 strongly indicates the presence of invasive aspergillosis and should lead to an additional diagnostic work-up.

The incidence of *false-positive results* varies from approximately 5% in adults to as much as 83% in premature infants.¹⁹ Different causes are summarised here.

1 At least in neonates, false-positivity can be partly explained by gastrointestinal translocation of galactomannan.⁸² Besides, galactomannan is widely distributed throughout nature, has been detected in food and drinks, and is commonly found in human faeces. Smaller galactomannan molecules might translocate in patients with impaired integrity of their intestinal

mucosa, such as neonates. An identical mechanism probably applies to cancer patients with therapy-induced mucositis and might explain the higher occurrence of false-positive results in allogeneic hematopoietic stem cell transplant (HSCT) recipients during the first weeks after conditioning, although others have failed to demonstrate this.⁵⁸ Recently, the group of Nijmegen hypothesised that the origin of persistent antigenaemia in neonates (unlikely to have been exposed to contaminated foods) without invasive aspergillosis might be due to the presence of a lipoteichoic acid of *Bifidobacterium* sp., a major coloniser of the neonatal gut. This molecule shares epitopes with galactomannan that can be recognised by the EB-A2 monoclonal antibody used in the EIA.^{83,84}

2 The intravenous administration of the antibiotic piperacillin-tazobactam^{85–92} and the intravenous and oral formulation of amoxicillin-clavulanate^{92,93} has been shown to result in false-positive EIA reactivity. The precise origin for this reactivity remains unknown. However, piperacillin and tazobactam are semi-synthetic drugs derived from natural compounds produced by fungi of the *Penicillium* species. Because this latter species contains galactomannan in the cell wall, it has been hypothesised that detectable levels of antigen may be carried through the production process into batches for therapeutic use.

3 Cyclophosphamide and its metabolites have been suspected of causing false-positive results. However, we were unable to detect false-positivity in a series of transplant patients conditioned with a cyclophosphamide-containing regimen.⁵⁸

4 False EIA reactivity has occasionally been observed in patients infected with fungal organisms that share cross-reacting epitopes with *Aspergillus* species, such as *Penicillium* sp.⁹⁴ Fortunately, many of these pathogens are very rare in humans. False-positive assays have been found in neutropenic patients with bacteraemia caused by *Escherichia coli*, staphylococci, enterococci, *Corynebacterium jeikeium* and *Pseudomonas* species;⁹⁴ however, exoantigens from these organisms were not reactive in the assay and others failed to confirm this observation.^{56,59}

5 In liver transplant recipients, patients with underlying autoimmune liver disease were more likely to have false-positive tests.⁶⁵ This observation is in line with the occurrence of false reactivity in other conditions associated with autoimmune phenomena, such as chronic GvHD.⁹⁵ In that same study, patients on dialysis were also more likely to have false-positive assays.⁶⁵

6 *Aspergillus* colonisation does not seem to cause false-positive results.

7 Finally, miscellaneous other causes of false-positivity have been reported, such as the presence of glucopyranose in cotton swabs, laboratory contamination and technical problems.

The occurrence of *false-negative results* in cases of proven invasive aspergillosis is intriguing. Major variables affecting false-negativity include the presence of anti-*Aspergillus* antibodies and the prophylactic and empiric use of mould-active antifungal agents.

Contrary to the widely accepted belief that immunosuppressed patients are not able to mount a significant antibody response, Herbrecht *et al.* detected anti-*Aspergillus* antibodies in one-third of the patients with possible, probable and proven invasive aspergillosis at the onset of infection and demonstrated a higher sensitivity in patients without neutralising antibodies (64% for proven and probable infection) than in patients with anti-*Aspergillus* antibodies (37%).⁵⁹ However, a large group of this study population was not severely immunosuppressed and was probably immunised before the development of invasive disease.

The impact of the prophylactic or empirical use of mould-active agents appears to be much more problematic. Animal studies have shown that the prophylactic use of azoles with anti-*Aspergillus* activity (such as itraconazole, voriconazole and posaconazole) reduces the maximum concentration of antigen in the blood, resulting in a delayed detection of galactomannan.⁹⁶ Similarly, exposure to amphotericin B decreases levels of antigenaemia in neutropenic animals, probably due to reduced hyphal growth.⁹⁷ However, in several prospective studies patients received antifungal prophylaxis with itraconazole. In addition, empirical antifungal therapy is considered standard of care for the treatment of antibiotic-resistant or relapsing neutropenic fever and has been prescribed in many studies.⁹⁸ These confounding factors may have hampered the interpretation of those studies. As recently evidenced by Marr *et al.* sensitivity of the assay is highest in patients who do not receive preventative mould-active agents.⁹⁹ Clearly, the impact on test performance will further depend on the frequency of sampling and on the cut-off value for positivity.

Galactomannan detection leads to earlier diagnosis

The survival rate of patients with invasive aspergillosis varies from <10% to more than 60–70%, and depends on the clinical presentation of the infection and the status of the underlying immunodeficiency. Unfortunately, the case-fatality rate remains especially high in neutropenic patients and allogeneic stem cell transplant

recipients. It is hypothesised that earlier diagnosis, followed by earlier therapy, could reverse this dismal outcome. This is also reflected by the better outcome in probable disease compared with proven disease.¹⁰⁰ However, the classical clinical and microbiological criteria that trigger a diagnostic work-up have low predictive value, are poorly sensitive, or become positive late in the course of the disease.¹⁰¹

Serial monitoring for *Aspergillus* antigenaemia facilitates the early diagnosis of invasive aspergillosis in haematology patients. In a series of allogeneic stem cell transplant recipients, galactomannan detection performed better than any of the other triggers, including unexplained fever, development of new pulmonary infiltrates, positive culture for *Aspergillus* species and abnormalities seen on computerised tomography (CT). Antigenaemia preceded diagnosis on the basis of radiological examination or *Aspergillus* isolation by 8 and 9 days in 80% and 89% of patients, respectively, and preceded therapy in 83% of patients. Detection of galactomannan was especially useful in patients receiving steroid therapy or when coexisting conditions masked the diagnosis of invasive aspergillosis.⁵⁸ Sulahian *et al.* also reported that antigen detection preceded radiological evidence by more than 1 week in 65% of cases.⁵⁷ Of course, the likelihood of an early diagnosis will be influenced by the frequency of sampling and by the threshold for positivity. For instance, using a cut-off of 0.5, Marr *et al.* demonstrated test positivity approximately 1–2 weeks before clinical onset and diagnosis, respectively, whereas no benefit was observed with a higher cut-off.⁶⁶ Finally, in a prospective study of daily galactomannan monitoring, antigenaemia was the primary trigger for performing additional radiological investigation, preceding all the other classical triggers.¹⁰²

Galactomannan as a surrogate marker of therapeutic response

Doctors who manage patients with invasive aspergillosis have no reliable early tools to assess the effectiveness of antifungal therapy; clinical and even radiological assessment of treatment efficacy is often inconclusive until more than 2 weeks of treatment.¹⁰³ Because the level of antigenaemia is proportional to the fungal load in tissue, one can expect that the kinetics of antigenaemia would be a relevant marker of therapeutic response. Several authors have described declining serum levels in patients who responded to antifungal therapy or a gradual increase or reappearance in those who were refractory or relapsed.^{24,50,56,57}

However, considerable debate remains with regard to the utility of the Platelia™ *Aspergillus* in monitoring the progress of infection. Indeed, only one study has systematically analysed the kinetics of antigen serum levels and the relationship to outcome.¹⁰⁴ An increase in the OD index value of 1.0 over the baseline value at day 7 of treatment was predictive of treatment failure, potentially justifying a modification of antifungal treatment.

More recently, animal models and one case report have suggested that treatment with echinocandin antifungals (glucan synthesis inhibitors) results in persistent galactomannan antigenaemia,¹⁰⁵ or even in a paradoxical increase in antigenaemia,¹⁰⁶ in the setting of clinical and/or radiographical improvement. However, a *post hoc* analysis of the caspofungin invasive aspergillosis study suggested that patients do not appear to display similar paradoxical findings; four of five patients with a favourable response had a negative antigen titre by the end of treatment, whereas 10 of 12 patients who manifested no improvement in antigen titres had clinical and radiographic persistence or progression of the infection.¹⁰⁷ Although these first assessments look promising, the use of Platelia™ *Aspergillus* as a therapeutic monitoring tool warrants further evaluation.

Detection of (1,3)- β -D-glucan

Glucans are (1,3)- β -D-linked polymers of glucose. As part of the outer cell wall of most pathogenic fungi (exceptions are the Zygomycetes and *Cryptococcus* species), glucans are ubiquitous in the environment and have previously been used as a marker of fungal biomass. Glucans are not produced by humans, but are released or actively secreted into the blood of patients with fungal infections.

(1,3)- β -D-Glucan can be detected in plasma by three commercialised assays: two kinetic chromogenic assays, including Fungitell™ (Associates of Cape Cod Inc., East Falmouth, MA, USA; previously GlucateLL™) and Fungi-Tec G™ (Seikagaku Kogyo Corporation, Tokyo, Japan) and one kinetic turbidimetric assay, Wako-WB003 (Wako Pure Chemical Industries, Osaka, Japan). All assays are based on the ability of horseshoe crab hemolymph to clot in response to glucan. Upon contact with trace amounts of (1,3)- β -D-glucan (as low as 1 pg ml⁻¹), horseshoe crab (*Tachypleus tridentatus* or *Limulus polyphemus* depending on the assay) amoebocytes degranulate and release zymogens that become active serine proteases (so-called factor G). This active factor G triggers the activation of a pro-clotting enzyme. In the chromogenic assays, the activated clotting enzyme

cleaves *p*-nitroanilide from a chromogenic substrate peptide. This released *p*-nitroanilide is determined at absorbance at 405 nm. In the turbidimetric assay, (1,3)- β -D-glucan triggers a gelation reaction of factor G, which in turn increases the turbidity of the reaction mixture. The chromogenic assays differ in their affinity to the glucan compounds; this difference can be compensated by an adjustment of the cut-off value.¹⁰⁰ These tests are already widely used in Japan and the Fungitell™ assay has recently been approved by the FDA as an adjunct for the diagnosis of invasive fungal infections in the USA.

Following a promising evaluation in a small series of patients with proven *Candida* and *Aspergillus* infections,¹⁰⁸ the clinical usefulness of the FungiTec G™ test for diagnosing invasive fungal infections was examined in a series of 202 febrile episodes in patients with haematological malignancies.¹⁰⁹ Using a cut-off of 20 pg ml⁻¹, 37 of 41 episodes of proven fungal infections had positive results (sensitivity of 90%) and all of 59 episodes of non-fungal origin tested negative (specificity of 100%). In the absence of a sensitive gold standard to further discriminate fungal from non-fungal disease, and considering the remaining 102 episodes as non-fungal, the negative predictive value was 97% and the positive predictive value was only 59%. However, in a subsequent prospective study of neutropenic patients, sensitivity for detecting invasive aspergillosis was only 63% in a patient-based analysis. In addition, suggestive CT scan findings preceded a positive assay by 11.5 days.¹¹⁰

More recently, American investigators assessed the utility of the Fungitell™ (GlucateLL™) kit at a cut-off of 60 pg ml⁻¹. In a single-centre study in 283 evaluable patients with acute leukaemia and myelodysplastic syndrome, the negative predictive value of twice weekly sampling was 100%. Sensitivity was also 100% if one positive assay was considered a truly positive result. Of note, test results were not influenced by the prophylactic or empiric use of antifungals.¹¹¹

However, experience with these assays remains limited (especially in Europe) and most factors that influence the evaluation of galactomannan detection also apply to the detection of glucan, including the inadequate use of a case-control design, the heterogeneity of the study population, the spectrum of alternative diagnoses, the number of collected serum samples and the cut-off for positivity.^{112,113} In addition, the test is expensive, the use of endotoxin-free, glucan-free glassware is required, and a number of factors can lead to false-positive readings, including the use of albumin or immunoglobulins, exposure to

glucan-containing gauze, the use of antibiotics and haemodialysis.^{114,115}

Antigen detection in non-serum specimens

Although none of these serodiagnostic assays has been validated for the detection of antigen in specimens other than serum or plasma, other body fluids and specimens are being tested with increased frequency. Recently, this topic has been extensively reviewed for galactomannan.¹¹⁶ Galactomannan detection in urine, BAL fluid and cerebrospinal fluid seems a promising diagnostic tool but requires additional prospective confirmation as available data rely on the results of small series and case reports which are all compromised by a variety of methodological shortcomings.

Combining serological methods

A Japanese group monitored the levels of *Aspergillus* DNA, galactomannan and β -D-glucan (by kinetic assay) at weekly intervals in haematology patients who were at risk of invasive aspergillosis, and evaluated the diagnostic potentials by using ROC analyses. As a result, the ROC curve for the galactomannan assay (cut-off of 0.6) was better than those for the other two tests.¹¹⁷ Clearly, the jury is still out, but in view of the species specificity of the galactomannan assay and the changing fungal epidemiology in some haematology clinics,¹¹⁸ further research should focus on a combination of tests in order to identify false-positive and false-negative reactions by each test.¹¹⁹

Conclusion

The management of invasive aspergillosis has been hampered by the inability to diagnose these infections as definite diagnosis invariably centres on histological identification of *Aspergillus* hyphae in tissue. Because early effective therapy is critical for successful control of established disease, most practitioners tend to rely on generalised prophylaxis and early empirical (over)treatment. A better identification of high-risk patients and employment of these new diagnostic tools may allow an earlier and more targeted therapeutic approach. Indeed, the commercially available immunoassays for the detection of galactomannan and β -D-glucan appear promising as these fungal antigens can be detected in the blood of infected patients before conventional methods, often before the onset of clinical symptoms. Serial screening for these antigens in conjunction with sensitive imaging techniques (such as thoracic CT

scanning) may be used to guide the initiation of antifungal therapy in neutropenic patients and haematopoietic stem cell transplant recipients. Besides, the excellent negative predictive value of these new tools may convince clinicians to withhold antifungal therapy in persistently febrile neutropenic patients with no other signs of fungal infection, whereas consecutive positive results in a high-risk population warrant further diagnostic work-up. However, whether such 'pre-emptive strategies' will finally improve patient outcome and whether they will prove to be cost-effective remain to be investigated in carefully designed randomised-clinical trials.

References

- 1 Perea S, Patterson TF. Invasive *Aspergillus* infections in hematologic malignancy patients. *Semin Respir Infect* 2002; **17**: 99–105.
- 2 Cordonnier C, Ribaud P, Herbrecht R *et al.* Prognostic factors for death due to invasive aspergillosis after hematopoietic stem cell transplantation: a 1-year retrospective study of consecutive patients at French transplantation centers. *Clin Infect Dis* 2006; **42**: 955–63.
- 3 Blijlevens NM, Donnelly JP, de Pauw BE. Microbiologic consequences of new approaches to managing hematologic malignancies. *Rev Clin Exp Hematol* 2005; **9**: E2.
- 4 Pagano L, Caira M, Candoni A *et al.* The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. *Haematologica* 2006; **91**: 1068–75.
- 5 Marr KA, Carter Ra, Crippa F *et al.* Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. *Clin Infect Dis* 2002; **34**: 909–17.
- 6 Hope WW, Walsh TJ, Denning DW. The invasive and saprophytic syndromes due to *Aspergillus* spp. *Med Mycol* 2005; **43** (Suppl. 1): S207–38.
- 7 Stevens DA. Diagnosis of fungal infections: current status. *J Antimicrob Chemother* 2002; **49** (Suppl. 1): S11–9.
- 8 Herbrecht R, Denning DW, Patterson TF *et al.* Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N Engl J Med* 2002; **347**: 408–15.
- 9 Kontoyiannis DP, Bodey GP. Invasive aspergillosis in 2002: an update. *Eur J Clin Microbiol Infect Dis* 2002; **21**: 161–72.
- 10 Yeo SF, Wong B. Current status of nonculture methods for diagnosis of invasive fungal infections. *Clin Microbiol Rev* 2002; **15**: 465–84.
- 11 Young R, Bennett JE. Invasive aspergillosis: absence of detectable antibody response. *Am Rev Respir Dis* 1971; **104**: 710–6.

- 12 Bardana EJ, Gerber JD, Craig S, Cianciulli FD. The general and specific humoral immune response to pulmonary aspergillosis. *Am Rev Respir Dis* 1975; **112**: 799–805.
- 13 Tomee JFC, Mannes GPM, van der Bij W *et al.* Serodiagnosis and monitoring of *Aspergillus* infections after lung transplantation. *Ann Intern Med* 1996; **125**: 197–201.
- 14 Lehmann PF, Reiss E. Invasive aspergillosis: antiserum for circulating antigen produced after immunization with serum from infected rabbits. *Infect Immun* 1978; **20**: 570–2.
- 15 Reiss E, Lehmann PF. Galactomannan antigenemia in invasive aspergillosis. *Infect Immun* 1979; **25**: 357–65.
- 16 Bennett JE, Bhattacharjee AK, Glaudemans CPJ. Galactofuranosyl groups are immunodominant in *Aspergillus fumigatus* galactomannan. *Mol Immun* 1985; **22**: 251–4.
- 17 Latgé JP, Kobayashi H, Debeauvais JP *et al.* Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. *Infect Immun* 1994; **62**: 5424–33.
- 18 Morelle W, Bernard M, Debeauvais JP, Buitrago M, Tabouret M, Latgé JP. Galactomannoproteins of *Aspergillus fumigatus*. *Eukaryot Cell* 2005; **4**: 1308–16.
- 19 Mennink-Kersten MASH, Donnelly JP, Verweij PE. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. *Lancet Infect Dis* 2004; **4**: 349–57.
- 20 Weiner MH. Antigenemia detected by radioimmunoassay in systemic aspergillosis. *Ann Intern Med* 1980; **92**: 793–6.
- 21 Andrew CP, Weiner MH. Immunodiagnosis of invasive pulmonary aspergillosis in rabbits. Fungal antigen detected by radioimmunoassay in bronchoalveolar lavage fluid. *Am Rev Respir Dis* 1981; **124**: 60–4.
- 22 Andrew CP, Weiner MH. *Aspergillus* antigen detection in bronchoalveolar lavage fluid from patients with invasive aspergillosis and aspergilloma. *Am J Med* 1982; **73**: 372–80.
- 23 Weiner MH, Talbot GH, Gerson SL, Filice G, Cassileth PA. Antigen detection in the diagnosis of invasive aspergillosis. Utility in controlled, blinded trials. *Ann Intern Med* 1983; **99**: 777–82.
- 24 Talbot GH, Weiner MH, Gerson SL, Provencher M, Hurwitz S. Serodiagnosis of invasive aspergillosis in patients with hematologic malignancy: validation of the *Aspergillus fumigatus* antigen radioimmunoassay. *J Infect Dis* 1987; **155**: 12–27.
- 25 Dupont B, Huber M, Kim SJ, Bennett JE. Galactomannan antigenemia and antigenuria in aspergillosis: studies in patients and experimentally infected rabbits. *J Infect Dis* 1987; **155**: 1–11.
- 26 Sabetta JR, Minitier P, Andriole VT. The diagnosis of invasive aspergillosis by an enzyme-linked immunosorbent assay for circulating antigen. *J Infect Dis* 1985; **152**: 946–53.
- 27 De Repentigny L, Boushira M, Ste-Marie L, Bosisio G. Detection of galactomannan antigenemia by enzyme immunoassay in experimental invasive aspergillosis. *J Clin Microbiol* 1987; **25**: 863–7.
- 28 Wilson EV, Hearn VM, Mackenzie DWR. Evaluation of a test to detect circulating *Aspergillus fumigatus* antigen in a survey of immunocompromised patients with proven or suspected invasive disease. *J Med Vet Mycol* 1987; **25**: 365–74.
- 29 Bennett JE, Friedman MM, Dupont B. Receptor-mediated clearance of *Aspergillus* galactomannan. *J Infect Dis* 1987; **155**: 1005–10.
- 30 Stynen D, Safarti J, Goris A *et al.* Rat monoclonal antibodies against *Aspergillus* galactomannan. *Infect Immun* 1992; **60**: 2237–45.
- 31 Swanink CMA, Meis JFGM, Rijs AJMM, Donnelly JP, Verweij PE. Specificity of a sandwich enzyme-linked immunosorbent assay for detecting *Aspergillus* galactomannan. *J Clin Microbiol* 1997; **35**: 257–60.
- 32 Van Cutsem J, Meulemans L, Van Gerven F, Stynen D. Detection of circulating galactomannan by Pastorex *Aspergillus* in experimental invasive aspergillosis. *Mycoses* 1990; **33**: 61–9.
- 33 Dupont B, Improvisi L, Provost F. galactomannane dans les aspergilloses invasives humaines et animales avec un test au latex. *Bull Soc Fr Mycol Med* 1990; **19**: 35–41.
- 34 Rogers T, Haynes K, Barnes R. Value of antigen detection in predicting invasive pulmonary aspergillosis. *Lancet* 1990; **336**: 1210–3.
- 35 Haynes K, Rogers T. Retrospective evaluation of a latex agglutination test for diagnosis of invasive aspergillosis in immunocompromised patients. *Eur J Clin Microbiol Infect Dis* 1994; **13**: 670–4.
- 36 Verweij PE, Stynen D, Rijs AJMM, De Pauw BE, Hoogkamp-Korstanje JAA, Meis JFGM. Sandwich enzyme-linked immunosorbent assay compared with Pastorex latex agglutination test for diagnosing invasive aspergillosis in immunocompromised patients. *J Clin Microbiol* 1995; **33**: 1912–4.
- 37 Sulahian A, Tabouret M, Ribaud P *et al.* Comparison of enzyme immunoassay and latex agglutination test detection of galactomannan in the diagnosis of invasive aspergillosis. *Eur J Clin Microbiol Infect Dis* 1996; **15**: 139–45.
- 38 Kappe R, Schulze-Berge A, Sonntag HG. Evaluation of eight antibody tests and one antigen test for the diagnosis of invasive aspergillosis. *Mycoses* 1996; **39**: 13–23.
- 39 Machetti M, Feasi M, Mordini N *et al.* Comparison of an enzyme immunoassay and a latex agglutination system for the diagnosis of invasive aspergillosis in bone marrow transplant recipients. *Bone Marrow Transplant* 1998; **21**: 917–21.
- 40 Herrmann J, Gugel A, Freidank H, Bertz H, Finke J. *Aspergillus* antigen detection: comparison of a new sandwich ELISA and the latex agglutination test in

- patients with histologically proven invasive aspergillosis. *Mycoses* 1998; **41** (Suppl. 1): S83–5.
- 41 Kappe R, Schulze-Berge A. New cause for false-positive results with the Pastorex Aspergillus antigen latex agglutination test. *J Clin Microbiol* 1993; **31**: 2489–90.
- 42 De Repentigny L. Serodiagnosis of candidiasis, aspergillosis, and cryptococcosis. *Clin Infect Dis* 1992; **14** (Suppl. 1): S11–22.
- 43 Lijmer J, Mol BW, Heisterkamp S *et al.* Empirical evidence of design-related bias in studies of diagnostic test. *JAMA* 1999; **282**: 1061–6.
- 44 Whiting P, Rutjes AWS, Reitsma JB *et al.* Sources of variation and bias in studies of diagnostic accuracy. A systematic review. *Ann Intern Med* 2004; **140**: 189–202.
- 45 Fischer JE, Bachmann LM, Jaeschke R. A readers' guide to the interpretation of diagnostic test properties: clinical example of sepsis. *Intensive Care Med* 2003; **29**: 1043–51.
- 46 Cimolai N. Serodiagnosis for bacterial infections. In: Cimolai N (ed.), *Laboratory Diagnosis of Bacterial Infections*. New York, Basel: Marcel Dekker, Inc., 2001: 55–82.
- 47 Stynen D, Goris A, Sarfati J, Latgé JP. A new sensitive sandwich ELISA to detect galactofuran in patients with invasive aspergillosis. *J Clin Microbiol* 1995; **33**: 497–500.
- 48 Wheat LJ. Rapid diagnosis of invasive aspergillosis by antigen detection. *Transplant Infect Dis* 2003; **5**: 158–66.
- 49 Rohrlich P, Sarfati J, Mariani P *et al.* Prospective sandwich enzyme-linked immunosorbent assay for serum galactomannan: early predictive value and clinical use in invasive aspergillosis. *Pediatr Infect Dis* 1996; **15**: 232–7.
- 50 Bretagne S, Marmorat-Khuong A, Kuentz M, Latgé JP, Bart-Delabesse E, Cordonnier C. Serum *Aspergillus* galactomannan antigen testing by sandwich ELISA: practical use in neutropenic patients. *J Infect* 1997; **35**: 7–15.
- 51 Bretagne S, Costa JM, Bart-Delabesse E, Dhedin N, Rieux C, Cordonnier C. Comparison of serum galactomannan antigen detection and competitive polymerase chain reaction for diagnosing invasive aspergillosis. *Clin Infect Dis* 1998; **26**: 1407–12.
- 52 Maertens J, Verhaegen J, Demuynck H *et al.* Autopsy-controlled prospective evaluation of serial screening for circulating galactomannan by a sandwich enzyme-linked immunosorbent assay for hematological patients at risk for invasive aspergillosis. *J Clin Microbiol* 1999; **37**: 3223–8.
- 53 Maesaki S, Kawamura S, Hashiguchi K *et al.* Evaluation of sandwich ELISA galactomannan test in samples of positive LA test and positive *Aspergillus* antibody. *Intern Med* 1999; **38**: 948–50.
- 54 Williamson ECM, Oliver DA, Johnson EM, Foot ABM, Marks DI, Warnock DW. *Aspergillus* antigen testing in bone marrow transplant recipients. *J Clin Pathol* 2000; **53**: 362–6.
- 55 Ulusakarya A, Chachaty E, Vantelon JM *et al.* Surveillance of *Aspergillus* galactomannan antigenemia for invasive aspergillosis by enzyme-linked immunosorbent assay in neutropenic patients treated for hematological malignancies. *Hematol J* 2000; **1**: 111–6.
- 56 Maertens J, Verhaegen J, Lagrou K, Van Eldere J, Boogaerts M. Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. *Blood* 2001; **97**: 1604–10.
- 57 Sulahian A, Boutboul F, Ribaud P, Leblanc T, Lacroix C, Derouin F. Value of antigen detection using an enzyme immunoassay in the diagnosis and prediction of invasive aspergillosis in two adult and pediatric haematology units during a 4-year prospective study. *Cancer* 2001; **91**: 311–8.
- 58 Maertens J, Van Eldere J, Verhaegen J, Verbeken E, Verschakelen J, Boogaerts M. Use of circulating galactomannan screening for early diagnosis of invasive aspergillosis in allogeneic stem cell transplant recipients. *J Infect Dis* 2002; **186**: 1297–306.
- 59 Herbrecht R, Letscher-Bru V, Oprea C *et al.* *Aspergillus* galactomannan detection in the diagnosis of invasive aspergillosis in cancer patients. *J Clin Oncol* 2002; **20**: 1898–906.
- 60 Rovira M, Jimenez M, De la Bellacasa JP *et al.* Detection of *Aspergillus* galactomannan by enzyme immunosorbent assay in recipients of allogeneic hematopoietic stem cell transplantation: a prospective study. *Transplantation* 2004; **77**: 1260–4.
- 61 Maertens J, Theunissen K, Verbeken E *et al.* Prospective clinical evaluation of lower cut-offs for galactomannan detection in adult neutropenic patients and hematological stem cell transplant recipients. *Br J Haematol* 2004; **126**: 852–60.
- 62 Pereira CN, Del Nero G, Lacaz CS, Machado CM. The contribution of galactomannan detection in the diagnosis of invasive aspergillosis in bone marrow transplant recipients. *Mycopathologica* 2005; **159**: 487–93.
- 63 Fortun J, Martin-Davila P, Alvarez M *et al.* for the Ramon y Cajal Hospital's Liver Transplant Group. *Aspergillus* antigenemia sandwich-enzyme immunoassay test as a serodiagnostic method for invasive aspergillosis in liver transplant recipients. *Transplantation* 2001; **71**: 145–9.
- 64 Husain S, Kwak EJ, Obman A *et al.* Prospective assessment of Platelia™ *Aspergillus* galactomannan antigen for the diagnosis of invasive aspergillosis in lung transplant recipients. *Am J Transplant* 2004; **4**: 796–802.
- 65 xzKwak EJ, Husain S, Obman A *et al.* Efficacy of galactomannan antigen in the Platelia *Aspergillus* enzyme immunoassay for diagnosis of invasive aspergillosis in liver transplant recipients. *J Clin Microbiol* 2004; **42**: 435–8.
- 66 Marr KA, Balajee SA, McLaughlin L, Tabouret M, Bentsen C, Walsh TJ. Detection of galactomannan antigenemia by enzyme immunoassay for the diagnosis of

- invasive aspergillosis: variables that affect performance. *J Infect Dis* 2004; **190**: 641–9.
- 67 Becker MJ, Lugtenburg EJ, Cornelissen JJ, van der Schee C, Hoogsteden HC, de Marie S. Galactomannan detection in CT-based broncho-alveolar lavage fluid and serum in haematological patients at risk for invasive pulmonary aspergillosis. *Br J Haematol* 2003; **121**: 448–57.
 - 68 Jarque I, Andreu R, Salavert M *et al.* Value of *Aspergillus* galactomannan antigen detection in the diagnosis and follow-up of invasive aspergillosis in hematological patients (Spanish). *Rev Iberoam Micol* 2003; **20**: 116–8.
 - 69 Moragues MD, Amutio E, Garcia-Ruiz JC, Ponton J. Usefulness of galactomannan detection in the diagnosis and follow-up of hematological patients with invasive aspergillosis (Spanish). *Rev Iberoam Micol* 2003; **20**: 103–10.
 - 70 Pazos C, del Palacio A. Early diagnosis of invasive aspergillosis in neutropenic patients with bi-weekly serial screening of circulating galactomannan by Platelia *Aspergillus* (Spanish). *Rev Iberoam Micol* 2003; **20**: 99–102.
 - 71 Pinel C, Fricker-Hidalgo H, Lebeau B *et al.* Detection of circulating *Aspergillus fumigatus* galactomannan: value and limits of the Platelia test for diagnosing invasive aspergillosis. *J Clin Microbiol* 2003; **41**: 2184–6.
 - 72 Buchheidt D, Hummel M, Schleiermacher D *et al.* Prospective clinical evaluation of a LightCycler-mediated polymerase chain reaction assay, a nested-PCR assay and a galactomannan enzyme-linked immunosorbent assay for detection of invasive aspergillosis in neutropenic cancer patients and haematological stem cell transplant recipients. *Br J Haematol* 2004; **125**: 196–202.
 - 73 Yoo JH, Choi JH, Choi SM *et al.* Application of nucleic acid sequence-based amplification for diagnosis of and monitoring the clinical course of invasive aspergillosis in patients with hematologic diseases. *Clin Infect Dis* 2005; **40**: 392–8.
 - 74 Platelia [package insert]. Bio-Rad, Marnes-La-Coquette.
 - 75 Berenguer J, Allende MC, Lee JW *et al.* Pathogenesis of pulmonary aspergillosis. Granulocytopenia versus cyclosporin and methyl-prednisolone-induced immunosuppression. *Am J Respir Crit Care Med* 1995; **152**: 1079–86.
 - 76 Stephens-Romero SD, Mednick AJ, Feldmesser M. The pathogenesis of fatal outcome in murine pulmonary aspergillosis depends on the neutrophil depletion strategy. *Infect Immun* 2005; **73**: 114–25.
 - 77 Balloy V, Huerre M, Latgé JP, Chignard M. Differences in patterns of infection and inflammation for corticosteroid treatment and chemotherapy in experimental invasive pulmonary aspergillosis. *Infect Immun* 2005; **73**: 494–503.
 - 78 Verweij PE, Weemaes CM, Curfs JHAJ, Bretagne S, Meis JFGM. Failure to detect circulating *Aspergillus* markers in a patients with chronic granulomatous disease and invasive aspergillosis. *J Clin Microbiol* 2000; **38**: 3900–1.
 - 79 Ascioğlu S, Rex JH, De Pauw B *et al.* on behalf of the Invasive Fungal infections Cooperative Group of the European Organization for Research and Treatment of Cancer and Mycoses Study group of the National Institute of Allergy and Infectious Diseases. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis* 2002; **34**: 7–14.
 - 80 Verweij PE, Erjavec Z, Sluiter W *et al.* Detection of antigen in sera of patients with invasive aspergillosis: intra- and interlaboratory reproducibility. The Dutch Interuniversity Working Party for Invasive Mycoses. *J Clin Microbiol* 1998; **36**: 1612–6.
 - 81 Verweij P, Masson C, Klont R, Heinen C, Crepin B, Maertens J. *Optimisation of the Cut-off Value of the Platelia™ Aspergillus ELISA*. 16th European Congress of Clinical Microbiology and Infectious Diseases, Nic, 2006.
 - 82 Siemann M, Koch-Dorfler M, Gaude M. False-positive results in premature infants with the Platelia *Aspergillus* sandwich enzyme-linked immunosorbent assay. *Mycoses* 1998; **41**: 373–7.
 - 83 Mennink-Kersten MASH, Klont RR, Warris A, Op den Camp HJM, Verweij PE. Bifidobacterium lipoteichoic acid and false reactivity in *Aspergillus* antigen detection. *Lancet* 2004; **363**: 325–7.
 - 84 Mennink-Kersten MASH, Ruegebrink D, Klont RR *et al.* Bifidobacterial lipoglycan as a new cause for false-positive Platelia *Aspergillus* enzyme-linked immunosorbent assay reactivity. *J Clin Microbiol* 2005; **43**: 3925–31.
 - 85 Sulahian A, Touratier S, Ribaud P. False positive test for *Aspergillus* antigenemia related to concomitant administration of piperacillin and tazobactam. *N Engl J Med* 2003; **349**: 2366–7.
 - 86 Viscoli C, Machetti M, Cappellano P *et al.* False-positive galactomannan Platelia *Aspergillus* test results for patients receiving piperacillin-tazobactam. *Clin Infect Dis* 2004; **38**: 913–6.
 - 87 Adam O, Aupérin A, Wilquin F, Bourhis JH, Gachot B, Chachaty E. Treatment with piperacillin-tazobactam and false-positive *Aspergillus* galactomannan antigen test results for patients with hematological malignancies. *Clin Infect Dis* 2004; **38**: 917–20.
 - 88 Singh N, Obman A, Husain S, Aspinall S, Mietzner S, Stout JE. Reactivity of Platelia *Aspergillus* galactomannan antigen with piperacillin-tazobactam: clinical implications based on achievable concentrations in serum. *Antimicrob Agents Chemother* 2004; **48**: 1989–92.
 - 89 Walsh TJ, Shoham S, Petraitiene R *et al.* Detection of galactomannan antigenemia in patients receiving piperacillin-tazobactam and correlations in vitro, in vivo, and clinical properties of the drug-antigen interaction. *J Clin Microbiol* 2004; **42**: 4744–8.
 - 90 Penack O, Schwartz S, Thiel E, Blau IW. Lack of evidence that false-positive *Aspergillus* galactomannan antigen test

- results are due to treatment with piperacillin-tazobactam. *Clin Infect Dis* 2004; **39**: 1401–2.
- 91 Wu DH. Platelia *Aspergillus* assay and potential cross-reaction. *Clin Infect Dis* 2004; **39**: 1402.
 - 92 Adam O, Aupérin A, Wilquin F, Bourhis JH, Gachot B, Chachaty E. Reply to Penack *et al.* and Wu. *Clin Infect Dis* 2004; **39**: 1402.
 - 93 Mattei D, Rapezzi D, Mordini N *et al.* False-positive *Aspergillus* galactomannan enzyme-linked immunosorbent assay results in vivo during amoxicillin-clavulanic acid treatment. *J Clin Microbiol* 2004; **42**: 5362–3.
 - 94 Maertens J, Theunissen K, Verhoef G, Van Eldere J. False-positive *Aspergillus* galactomannan antigen test results. *Clin Infect Dis* 2004; **39**: 289–90.
 - 95 Hamaki T, Kami M, Kanda Y *et al.* False-positive results of *Aspergillus* enzyme-linked immunosorbent assay in patients with chronic graft-versus-host disease after allogeneic bone marrow transplantation. *Bone Marrow Transplant* 2001; **28**: 633–4.
 - 96 Petraitiene R, Petraitis V, Groll AH *et al.* Antifungal activity and pharmacokinetics of posaconazole (SCH 56592) in treatment and prevention of experimental invasive pulmonary aspergillosis: correlation with galactomannan antigenemia. *Antimicrob Agents Chemother* 2001; **45**: 857–69.
 - 97 Francis P, Lee JW, Hoffman A *et al.* Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: the potential role of bronchoalveolar D-mannitol and serum galactomannan as markers of infection. *J Infect Dis* 1994; **169**: 356–68.
 - 98 Martino R, Viscoli C. Empirical antifungal therapy in patients with neutropenia and persistent or recurrent fever of unknown origin. *Br J Haematol* 2005; **132**: 138–54.
 - 99 Marr KA, Laverdiere M, Gugel A, Leisenring W. Antifungal therapy decreases sensitivity of the *Aspergillus* galactomannan enzyme immunoassay. *Clin Infect Dis* 2005; **40**: 1762–9.
 - 100 Hope WW, Denning DW. Invasive aspergillosis: current and future challenges in diagnosis and therapy. *Clin Microbiol Infect* 2004; **10**: 2–4.
 - 101 Hope WW, Walsh T, Denning DW. Laboratory diagnosis of invasive aspergillosis. *Lancet Infect Dis* 2005; **5**: 609–22.
 - 102 Maertens J, Theunissen K, Verhoef G *et al.* Galactomannan and computed tomography-based pre-emptive antifungal therapy in neutropenic patients at high risk for invasive fungal infection: a prospective feasibility study. *Clin Infect Dis* 2005; **41**: 1242–50.
 - 103 Caillot D, Couaillier JF, Bernard A *et al.* Increasing volume and changing characteristics of invasive pulmonary aspergillosis on sequential thoracic computed tomography scans in patients with neutropenia. *J Clin Oncol* 2001; **19**: 253–9.
 - 104 Boutboul F, Albert C, Leblanc T *et al.* Invasive aspergillosis in allogeneic stem cell transplant recipients: increasing antigenemia is associated with progressive disease. *Clin Infect Dis* 2002; **34**: 939–43.
 - 105 Scotter JM, Chambers ST. Comparison of galactomannan detection, PCR-enzyme-linked immunosorbent assay, and real-time PCR for diagnosis of invasive aspergillosis in a neutropenic rat model and effect of caspofungin acetate. *Clin Diagn Lab Immunol* 2005; **12**: 1322–7.
 - 106 Klont R, Ruegebrink D, Mennink-Kersten M *et al.* Paradoxical increase in circulating *Aspergillus* antigen during treatment with caspofungin in a patient with pulmonary aspergillosis. *Clin Infect Dis* 2006; **43**: e23–5.
 - 107 Maertens J, Glasmacher A, Selleslag D *et al.* Evaluation of serum sandwich enzyme-linked immunosorbent assay for circulating galactomannan during caspofungin therapy: results from the caspofungin invasive aspergillosis study. *Clin Infect Dis* 2005; **41**: e9–e14.
 - 108 Miyazaki T, Kohno S, Mitsutake K *et al.* Plasma (1→3)-beta-D-glucan and fungal antigenemia in patients with candidemia, aspergillosis, and cryptococcosis. *J Clin Microbiol* 1995; **33**: 3115–8.
 - 109 Obayashi T, Yoshida M, Mori T *et al.* Plasma (1→3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. *Lancet* 1995; **345**: 17–20.
 - 110 Kami M, Tanaka Y, Kanda Y *et al.* Computed tomographic scan of the chest, latex agglutination test and plasma (1AE3)-beta-D-glucan assay in early diagnosis of invasive pulmonary aspergillosis: a prospective study of 215 patients. *Haematologica* 2000; **85**: 745–52.
 - 111 Odabasi Z, Mattiuzzi G, Estey E *et al.* Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff, development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. *Clin Infect Dis* 2004; **39**: 199–205.
 - 112 Ostrosky-Zeichner L, Alexander B, Kett D *et al.* Multi-center clinical evaluation of the (1→3) beta-D-glucan assay as an aid to diagnosis of fungal infections in humans. *Clin Infect Dis* 2005; **41**: 654–9.
 - 113 Upton A, Leisenring W, Marr K. (1→3) beta-D-Glucan assay in the diagnosis of invasive fungal infections. *Clin Infect Dis* 2006; **42**: 1054–6.
 - 114 Mennink-Kersten M, Warris A, Verweij P. 1,3-beta-D-Glucan in patients receiving intravenous amoxicillin-clavulanic acid. *N Engl J Med* 2006; **354**: 2834–5.
 - 115 Pickering J, Sant H, Bowles C *et al.* Evaluation of a (1→3)-beta-D-glucan assay for diagnosis of invasive fungal infections. *J Clin Microbiol* 2005; **43**: 5957–62.
 - 116 Klont RR, Mennink-Kersten MASH, Verweij PE. Utility of *Aspergillus* antigen detection in specimens other than serum specimens. *Clin Infect Dis* 2004; **39**: 1467–74.
 - 117 Kawazu M, Kanda Y, Nannya Y *et al.* Prospective comparison of the diagnostic potential of real-time PCR, double-sandwich enzyme-linked immunosorbent assay for galactomannan, and a (1,3)-β-D-glucan test in

- weekly screening for invasive aspergillosis in patients with hematological disorders. *J Clin Microbiol* 2004; **42**: 2733–41.
- 118 Chamilos G, Luna M, Lewis RE *et al*. Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989–2003). *Haematologica* 2006; **91**: 986–9.
- 119 Pazos C, Ponton J, del Palacio A. Contribution of (1 → 3)-β-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. *J Clin Microbiol* 2005; **43**: 299–305.