Medical Mycology September 2006, **44**, S173–S178

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Defining a case of invasive aspergillosis by serum galactomannan

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The timely diagnosis of invasive aspergillosis (IA) remains difficult. In recent years, increased experience has been gained with the Platelia™ *Aspergillus* enzyme immunoassay. However, the excellent sensitivity and high positive predictive value that has been reported in earlier studies cannot consistently be reproduced in some of the more recent studies. As expected, this stems from major methodological and clinical heterogeneities between studies. This article reviews these between-study heterogeneities and concludes that the detection of serum galactomannan can be used to define a case of IA in a well-defined population of at-risk patients.

Keywords Galactomannan, sensitivity, neutropenia

Introduction

In recent years, increased experience has been gained with the Platelia™ *Aspergillus* assay (Bio-Rad Laboratories, Marnes-La-Coquette, France and Bio-Rad laboratories, Hercules, CA) [1]. Available in Europe since the mid 1990s [2,3], this sandwich enzyme immunoassay (EIA) which detects galactomannan (GM) was cleared by the Food and Drug Administration for diagnostic use in the United States in 2003 [4]. The detection of the fungal cell-wall component GM in samples from various body fluids (but especially serum and bronchoalveolar lavage (BAL) fluid) has been incorporated as one of the microbiological criteria in the 2002 European Organization for Research and Treatment of Cancer-Mycosis Study Group (EORTC-MSG) consensus definitions of invasive aspergillosis (IA) [5]. Two or more positive test results from consecutive serum samples (or one from BAL fluid) carry the same weight as the isolation of *Aspergillus* spp. from a lower respiratory tract sample or the microscopical demonstration of hyphae from BAL fluid. The rationale for including the detection of GM in the consensus definitions was supported by the reported excellent diagnostic accuracy of the assay. However, at that time, only a handful of studies had assessed the assay's diagnostic potential. Moreover, virtually all these studies were executed in patients with an underlying haematological disorder [6–12]. However, more recent studies have tapered the initial enthusiasm about the high sensitivity and positive predictive value of the Platelia™ *Aspergillus* assay [1,13–15]. Hence the question arises whether a case of IA can be defined by the Platelia™ EIA.

Recently, Pfeiffer *et al*. [16] presented a meta-analysis of the diagnostic value of GM detection with the Platelia™ *Aspergillus* assay. For proven and probable cases of IA, the pooled sensitivity (derived from 27 studies) was only 61% whereas the overall sensitivity was 93%. However, subgroup analysis showed that the performance of the test differed greatly by study population (adults vs. paediatrics), by underlying immunodeficiency (haematological malignancy vs. allogeneic stem cell transplantation vs. solid organ transplantation), and by set of reference criteria used to define a case of IA (EORTC-MSG criteria vs. other). Such discrepancy usually stems from major methodological and clinical heterogeneities between studies; these undermine the potential for transferability of test results across patient populations.

In reviewing studies on the diagnostic accuracy of serodiagnostic assays, variability can be attributed to flaws in the study design or can be caused by between-study differences in the cut-off point for positivity, in

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DOI: 10.1080/13693780600835732

the type of reference standards, in patient recruitment and clinical setting, or any combination of these factors (Table 3).

Methodological heterogeneity

A study by Lijmer *et al*. [17] empirically examined the impact of study design shortcomings on estimates of diagnostic accuracy and confirmed that the use of a non-representative study population or the application of different reference standards were the deficiencies with the greatest impact on diagnostic accuracy. More recently, this group proposed a minimum checklist of validity criteria to be assessed [18].

Population of recruitment

Studies that evaluate new assays in a population already known to have the disease ('cases') and a separate group of normal individuals ('controls' such as healthy volunteers or blood donors) overestimate the performance of the assay compared with studies that use the relevant clinical population (~threefold overestimation of the diagnostic odds ratio) [17]. The relevant clinical population consists of a group of at-risk patients that is likely to be encountered in the future use of the test. Nevertheless, even to date, case studies with an irrelevant control population are still being reported and must convince us of the usefulness of new serodiagnostic tools [19]. However, controls and cases are not matched for underlying characteristics. In addition, predictive values, which are largely influenced by the prevalence of the disease under study, cannot adequately be derived from such a design [20].

Method of patient selection

Selection bias looms when patients are not entered consecutively into the study. For instance, retrospective studies frequently use frozen serum samples from a non-random selection of patients with established disease status in the recent past.

Method of verification

The stringency of the criteria that are used to verify a positive and/or a negative serological assay is probably the most critical aspect and deserves close attention. With histopathological examination being the reference diagnostic standard for proven invasive mould infections, the ideal study population would be composed of consecutive patients at risk, who, at biopsy or autopsy, are determined to have or not to have fungal disease [21]. This rigid case-definition would circumvent to a high degree the problem of misclassification (although misdiagnosis due to sampling errors cannot be excluded). Hence, the efficacy of serial screening for circulating GM was prospectively evaluated in an autopsy-controlled study in haematological patients at risk for IA [11]. Based on the analysis of 71 patients with confirmed disease status, the sensitivity and specificity were 92.6% and 95.4%, respectively. The positive predictive value was almost 93%; the negative predictive value was 95%. However, most investigators remain reluctant to perform invasive diagnostic procedures such as open lung biopsies or stereotactic brain biopsies in these critically-ill patients. In addition, over the past decades, autopsy rates have dropped dramatically worldwide. Thus, although a significant proportion of the positive test results may be verified by the invasive reference test (usually autopsy), only a small fraction of the negative test results are verified by the same reference test. In most studies, negative test results are 'verified' by a different, often softer, standard (e.g. clinical or radiological follow-up). This introduces a so-called partial verification bias and a differential reference standard bias; the latter results in a more than twofold overestimation of the diagnostic odds ratio compared with studies that use one reference test [19,20]. Unfortunately, relying solely on autopsy or biopsy data excludes a large group of the clinically relevant population and introduces another bias towards selecting the more advanced manifestations of the disease.

As proposed by de Repentigny [21], a stratification of patients according to their probability of disease seems more realistic. Fortunately, uniform stratification has become feasible since the publication of the 2002 EORTC-MSG consensus criteria [5]. Two large prospective studies, a European one and a Japanese one, have applied the same methodology for assessing the value of screening for GM in high-risk haematology patients [12,22]; the pre-mortem EORTC-MSG classification was upgraded via incorporation of all the available relevant histopathological findings (biopsy and autopsy), resulting in a more accurate post-mortem classification. From this latter classification, several estimates of performance were calculated, based upon whether possible cases were regarded as true cases of IA (together with the proven and probable cases) or as true negative cases (no invasive fungal disease). Combining proven and probable IA vs. no fungal disease resulted in an excellent performance of the test (Table 1). However, as shown in Table 1, inclusion of all the possible cases as either truly positive cases or as truly negative cases had a dramatic impact on the sensitivity and positive predictive value [12,15,22].

Table 1 Prospective evaluation of the Platelia™ *Aspergillus* assay in two studies that have used the same methodology [12,22].

	2 5 GM ODI ≥0.5 [12]	2 5 GM ODI ≥0.6 [22]
[Proven + probable IA]* vs. [no IA]*		
Sensitivity	89.7 %	100 %
Specificity	98.1 %	93 %
Positive predictive value	87.5 %	55 %
Negative predictive value	98.4 %	100 %
Efficacy	97 %	93 %
[Proven + probable IA + possible IFI]* vs. [no IA]*		
Sensitivity	41.9 %	58 %
Specificity	98.1 %	NR
Positive predictive value	88.6 %	NR
Negative predictive value	82.7 %	92 %
Efficacy	83.5 %	87 %
[Proven + probable IA]* vs. [possible IFI + no IA]*		
Sensitivity	89.7 %	NR
Specificity	97.1 %	91 %
Positive predictive value	79.5 %	48 %
Negative predictive value	98.7 %	NR
Efficacy	96.3 %	NR

*, Per EORTC/MSG criteria [5]; GM ODI, galactomannan optical density index; IA, invasive aspergillosis; IFI, invasive fungal infection; NR, not reported.

Clearly, possible cases as defined by the 2002 consensus criteria represent a mixed bag of aetiologies, both fungal and non-fungal. Given the uncertain nature of possible cases, these should not be included in the sensitivity analysis of new diagnostic tests for IA.

Method of interpretation of tests

Overestimation of the test's accuracy can potentially result from interpretation of the reference test with knowledge of the results of the test under study. However, in the case of IA, the reference test is not open to subjective interpretation.

Treatment paradox and disease progression bias

A recent update on sources of variation and bias in studies of diagnostic accuracy underscores also the importance of treatment paradox and disease progression bias [23]. In the context of GM detection for the diagnosis of IA, treatment paradox could occur when antifungal therapy is started on the basis of the knowledge of the EIA test results, and the reference standard is applied after treatment has started. Fortunately, in virtually all studies that have assessed the performance of the EIA, test results were not available during the study period but were analyzed and interpreted retrospectively. As such, treatment paradox is unlikely since antifungal therapy was not initiated based on knowledge of the index test but empirically or on the basis of more conventional diagnostic tools (such as culture or radiological findings). Disease progression bias occurs when the index test is performed an unusually long time before the reference standard, so the disease is at a more advanced stage when the reference standard is performed. Given the specific nature of the reference test (usually based on autopsy data), disease progression bias may indeed represent a problem when evaluating the accuracy of the GM EIA. However, data from at least two studies have shown that the assay, when used as a screening tool in high-risk patients, becomes positive within an acceptable period of time (no longer than 10 days) before 'clinical' diagnosis based on radiology and culture/microscopy [24,25].

Clinical heterogeneity

Study population and disease presentation

Disease caused by *Aspergillus* spp. represents a spectrum of manifestations ranging from localized and encapsulated infections with minimal clinical symptoms (such as aspergilloma) to more extensive infections with marked tissue invasion and haematogenous dissemination (such as invasive pulmonary aspergillosis in neutropenics). In addition, not all immunocompromised patients carry the same risk of acquiring IA. Clearly, prolonged use of corticosteroids and prolonged neutropenia have invariably been identified as independent risk factors. However, as recently evidenced in animal models, the pathogenesis of disease differs greatly (Table 2) [26–28]. In corticosteroid-induced immunosuppressed animals, histopathological features are dominated by influx of neutrophils and inflammation; small numbers of conidial elements can be detected and chitin and GM concentrations are low to very low in all organs. However, in chemotherapy-induced neutropenic animals, pathological features consist of necrosis and haemorrhage in the absence of neutrophils and inflammation; large numbers of hyphal elements invade the tissue and blood vessels and chitin and GM concentrations are high in all organs. As such, it has been hypothesized that different cut-offs for GM EIA positivity should be established for neutropenic patients vs. corticosteroid-treated patients. Indeed, a recent analysis by Cordonnier *et al*. [29] demonstrated significant differences in the optical density (OD) index between these two patient populations and is in line with this hypothesis. Of course, within-study differences between these subgroups of patients and severities of disease result in different estimates of diagnostic

Table 2 Differences in the pathogenesis of experimental invasive pulmonary aspergillosis [24].

	Corticosteroid-induced immunosuppression	Chemotherapy-induced neutropenia
Cellular trafficking in BALF	Rapid and extensive increase in PMN	No influx of PMN
TNF-α concentration in BALF	Not detected	High
IL-10 concentration in BALF	Low	High
Histological features	Inflammation	No inflammatory exudate Necrosis with hyphae
Presence of fungal elements	Small numbers of conidia	Large numbers of invading hyphae
Chitin levels in organs	Low in all organs	High in all organs
GM levels in organs	Low to very low	High
Dominant mechanism	Adverse host response	Fungal development

BALF, bronchoalveolar lavage fluid; GA, galactomannan; IL, interleukin; PMN, polymorphonuclear neutrophils; TNF, tumour necrosis factor.

accuracy of a test (both sensitivity and specificity). For example, the absence of neutropenia and the frequent presentation of *Aspergillus* tracheobronchitis can partly explain the low sensitivity of the Platelia™ assay (30%) in lung transplant recipients [30] and liver transplant recipients [31].

Execution and/or interpretation of the test

Very few if any serodiagnostic assays function perfectly well with the use of strictly positive and negative results respectively. The distribution between diseased and non-diseased may not be far apart; so, a 'grey-zone' of indeterminate results may exist, especially when used as a screening assay. If the threshold for positivity is moved towards the (higher) values that are normally seen in the diseased group (e.g. OD index >1.5), the assay will gain specificity but at the expense of lower sensitivity (missed diagnosis). Conversely, biasing the cut-off towards (lower) values that are usually seen in the non-diseased population will increase sensitivity but lower specificity (over-diagnosis) [32]. When first launched in Europe, the manufacturer recommended a threshold OD index of 1.5, whereas an index between 1.0 and 1.5 was considered indeterminate. Meanwhile, many European investigators started to lower the threshold cut-off to 1.0 or less (e.g. 0.7 has been recommended for non-allogeneic transplant recipients [15]), especially in an attempt to make an earlier diagnosis. More recently, the US Food and Drug Administration (FDA) cleared the test as an aid in the diagnosis of IA in cancer patients, accepting an OD index cut-off for positivity of $\geq$ 0.5 [4]. This low cut-off is in line with recent receiver operating characteristic (ROC) analyses that suggest indices of 0.7 and 0.6, respectively [15,22] However, these unfortunate differences further contribute to variations in performance of the test, especially since a low cut-off inevitably increases the risk of misclassification. For instance, in the study by Maertens *et al*. [33], 14.9% of the control episodes tested $\geq$0.5. Although sensitivity remained fairly stable between 0.4 and 0.9, a specificity of 85.1 % for a single positive result was significantly lower compared with previously reported specificities with higher cut-off values.

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 at least two consecutive positive samples ('dynamic' cut-off) for that patient. Recent data corroborate the use of a 'static' cut-off at 0.8 or a 'dynamic' cut-off at 0.5 [33]. A single assay with an OD index $\geq$ 0.8 in the relevant (neutropenic) patient population warrants the initiation of anti-*Aspergillus* therapy, even if the diagnosis cannot be confirmed. Besides, the low rate of false positivity at 0.8 does outweigh the high mortality of established disease, especially since better-tolerated anti-*Aspergillus* agents have become available. A further lowering of the 'static' threshold seems not feasible due to a drop in the positive predictive value. However, given the gradual rise in antigenemia in proven and probable cases of IA – as opposed to the stable or fluctuant results in false positivity – a 'dynamic' threshold at 0.5 is also clinically feasible. The demonstration of at least two sequential sera with an OD index $\geq$ 0.5 increases the specificity and the positive predictive value of the assay to 98.6 % and the clinical efficiency to 98 %. Overall, a rising ratio above 0.5 strongly indicates the presence of IA and should lead to an additional diagnostic work-up.

In conclusion, in a well-defined population of adult patients with chemo-therapy-induced prolonged neutropenia, the presence of one or more consecutive positive GM assays in serum ($\sim$cut-off index for positivity) or the gradual increase in antigenaemia

Table 3 Sources of bias and variation in studies investigating the performance of the Platelia™ enzyme immunoassay (modified from [23])

	Comments
Population	
Demographic features	Sensitivity of the enzyme immunoassay (EIA) is lower in children (including neonates); separate studies should be performed for the paediatric population
Disease severity	Sensitivity of the EIA is lower in non- or minimally invasive manifestations of aspergillosis (e.g. aspergilloma or tracheobronchitis); performance of the assay in these manifestations should be studied separately from invasive presentations
Disease prevalence	Sensitivity and (positive) predictive value of diagnostic assays are highly influenced by disease prevalence. The study population should be restricted to patient populations with an expected high prevalence of invasive aspergillosis (IA) (e.g. =10–15%)
Selection of participants	The spectrum of the study population should be similar to the population in which the EIA will be used in practice (e.g. allogeneic transplants, leukaemia patients, solid organ transplants)
Test protocol: Material and Methods	
Test execution	The execution of the EIA (and reference test) should be described in great detail in the Methods section
Test technology	Variations in the execution of the EIA as a result of technological improvements may affect the performance and should be described in detail in the Methods section (e.g. semi-automation of the original manual assay)
Treatment paradox	Investigators should not be aware of the results of the EIA during the course of the study
Disease progression bias	In diagnostic studies, the reference standard should be performed as soon as feasible (this represents a major problem in diagnostic studies in (IA) given the aggressive nature of the reference standard)
Reference standard and verification procedure	
Inappropriate reference standard	Histopathological demonstration of tissue invasion by hyphae compatible with IA or a positive culture/ microscopy from a normally sterile body site is the only appropriate reference standard
Differential verification bias	All test results (positive and negative) should be verified by the same reference standard. In case of an invasive reference standard (such as histopathology), analysis of the results may be done according to a series of different estimates (regarding all unverified results as either true negative or true positive)
Partial verification bias	Only a selected sample of the index test results (usually the positive samples) is verified by the reference standard. In case of an invasive reference standard (such as histopathology), analysis of the results may be done according to a series of different estimates (regarding all unverified results as either true negative or true positive)
Interpretation	
Incorporation bias	Results of the EIA may not be used to establish the final diagnosis of proven or probable invasive aspergillosis
Observer variability	Intraobserver and interobserver variability should always be assessed in order to define the reproducibility of test results. With respect to the *Aspergillus* EIA, the reproducibility appears to be excellent [35]
Analysis	
Handling of indeterminate results	Indeterminate or uninterpretable EIA results should not be removed from the analysis. As already mentioned, analysis of these data may be done according to different estimates (regarding all unverified results as either true negative or true positive)
Arbitrary choice of threshold value	The performance of different cut-offs should be evaluated through a receiver operating characteristic (ROC)-curve analysis. Optimal EIA cut-offs may vary with different patient populations (e.g. neutropenic versus steroid-treated)

can be used to define a probable case of IA. Besides, recent experience shows that characteristic computed tomography (CT)-scan lesions (halo-sign) are seen in the majority of these cases [34]. However, a number of factors that interfere with the assay's performance result in false positive or false negative tests. These factors (such as the prophylactic or empirical use of mould-active azoles or fungal cell-wall inhibitors and the use of specific antibiotics), patient-related variables, and assay-related variables will be discussed in the article by Verweij *et al.* [this issue].

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