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Issues with galactomannan testing

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Within the past decade detection of the aspergillus antigen galactomannan has become an important and reliable tool for the early diagnosis of invasive aspergillosis. The galactomannan molecule, that is detected by the commercial sandwich ELISA (Platelia Aspergillus, Biorad), was found not to be a single molecule, but a family of molecules that have the epitope that reacts with the monoclonal antibody. Also the cut off level is now world-wide lowered to 0.5 which will help to further standardize and compare this diagnostic tool. Despite the advantages of galactomannan detection, there are several issues that have impact on its use in clinical practice. Both false negative and false positive reactivity is encountered and although the causes of false reactivity are not fully understood, new insights have become available which help us to optimize the use of the assay. This review discusses present issues with galactomannan testing with a view to future research and management.

Keywords Galactomannan, diagnosis, invasive aspergillosis

Introduction

The detection of circulating galactomannan (GM) has become an important tool in the early diagnosis of invasive aspergillosis (IA). GM is part of the outer layer of the aspergillus cell wall, and is released during growth of the fungus at the tips of the hyphae [1,2]. The antigen can be detected using a commercially available sandwich ELISA (Platelia Aspergillus, BioRad, France)(PA-ELISA), which employs a monoclonal antibody (EB-A2) that binds to the galactofuran epitope of the GM antigen [3,4]. The assay has been extensively studied and is now commonly used to monitor patients at high risk for invasive aspergillosis [2,5–8]. There are several issues that hamper the use of the assay, which will be addressed in this review.

One area of controversy has recently been resolved. The PA-ELISA was originally marketed with a cut-off for positive of 1.5. There was no evidence to support this cut off level and in the past years many researchers have proposed lower cut off levels. The assay was released in the USA with a cut off of 0.5 and the producer of the assay recently decided to lower the cut off to 0.5 in all other countries based on ROC analysis of European data sets of GM monitoring in patients with hematological malignancy [9].

The GM antigen

Unlike the name suggests, the so-called 'GM antigen' is not a single molecule but a family of molecules which are better called galactofuranose(galf)-antigens. In addition to GM, fungal glycoproteins also react with the EB-A2 antibody, including phospholipase C and phytase, which were shown to have only one terminal galactofuranose unit that was essential for binding with the EB-A2 antibody [10]. These molecules might not only show different PA-ELISA reactivity's but their expression might also be modulated by the fungal environment. A recent study showed that the release of PA-ELISA reactive antigens *in vitro* is influenced by the growth phase of the fungus [11]. Not only are reactive antigens released during early logarithmic growth, mycelial breakdown during the lytical phase results in a further increase of reactive antigens in the culture supernatant. However, the actual galf antigens that circulate in vivo in the

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body fluids of patients have not been characterized. But it seems likely that the same factors that influence the release *in vitro* might also play a role at the infection site in humans.

The performance of the PA-ELISA depends not only on the mixture of circulating galf-antigens (hereafter referred to as GM for convenience) in patients with IA and their respective reactivity, but also on the PA-ELISA method used. The pretreatment of serum in the PA-ELISA is based on a heat stable polysaccharide like GM. The pretreatment procedure causes denaturation of proteins, which is discarded and the supernatant is used for the ELISA. Protein bound galf-antigens will not be detected and therefore might lead to underestimation of reactivity.

False negative reactivity

The performance of the PA-ELISA is most favorable in patients with hematological malignancy especially those who have undergone a hematopoietic stem cell transplant and are neutropenic, although within this group of patients significant variation in the sensitivity of the assay was observed. The sensitivity varied between 100% and 33% [2]. Causes for the variability are not well understood, although some factors have been identified recently that have significant impact on the performance of the assay. For example, exposure of patients to mould-active antifungal agents reduced the sensitivity to 20% compared with 80% in those not receiving these drugs [12,13].

Cases have been documented in which, despite adequate sampling and proof of invasive aspergillosis, circulating GM was not detected [14–16]. One explanation for lack of reactivity could be that in some patients the levels of circulating GM are below the detection limit of the PA-ELISA. This was recently investigated using a new method in which a larger volume of serum was used, 750 μl compared to the recommended 300 μl, and filtered through a 50-kDa Microcon filter [17]. The thus achieved concentration of the sample resulted in PA-ELISA reactivity in samples that tested negative with the conventional pretreatment method. This resulted in earlier detection of circulating GM as well as detection of GM in patients with invasive aspergillosis who were falsely negative using the standard pretreatment method (Fig. 1) [17]. These preliminary findings might further increase the diagnostic value of the PA-ELISA, but this approach needs to be studied in more detail and reproduced by other investigators.

Besides factors related to the host or the assay, the fungus itself may have impact on the performance of the PA-ELISA. *In vitro* studies show that variable amounts of GM are released by different *Aspergillus* species. In a recent study in which clinical isolates were cultured in liquid culture medium, the most commonly encountered *Aspergillus* species, *A. fumigatus* and *A. flavus*, showed the lowest reactivity in the culture supernatant [18]. Higher GM concentrations were found in the culture supernatants of *A. terreus*, *A. niger* and *A. nidulans*. The clinical significance of this finding, however, remains to be determined. The amount of GM released by *A. fumigatus* and *A. flavus* appeared not to be different for strains from circulating GM-positive and circulating GM-negative patients, again underscoring the multi factorial pathogenesis of detection of biological markers in IA.

False positive reactivity

Most prospective clinical studies show high specificity of the PA-ELISA, with levels of false positive reactivity in adults of approximately 2.5%. Reported false positivity rates in pediatric patients is approximately 10% and the highest rates have been observed in neonates (83%), although the number of studies performed in this patient group is very limited [19–21]. In this respect, false reactivity is best defined as detection of the antigen or the epitope in the serum sample from patients without IA. Although GM is a heat stable antigen, widely distributed through nature and is present in foods and beverages, it remains unclear if consumption of antigen containing substances causes serum reactivity, through translocation of the antigen from the intestine to the circulation. Sporadic cases that have been reported indicate that this might occur in those patients with impaired integrity of the intestinal lining of the gut such as in mucositis [22].

Serum reactivity has been observed in patients that receive certain β-lactam antibiotics [23–29]. Furthermore, piperacillin/tazobactam, amoxicillin/clavulanic acid, ampicillin and phenoxymethylpenicillin have been shown to contain PA-ELISA reactive material [23,25–31]. This is likely to be GM (read one or more galf-antigens) since the fungus *Penicillium* is used in the production process of these antibiotics and this fungus is known to release GM and other galf-antigens. So administration of PA-ELISA reactive batches of the abovementioned antibiotics clearly causes serum reactivity, although there is not always an apparent relationship between the concentration in the batch

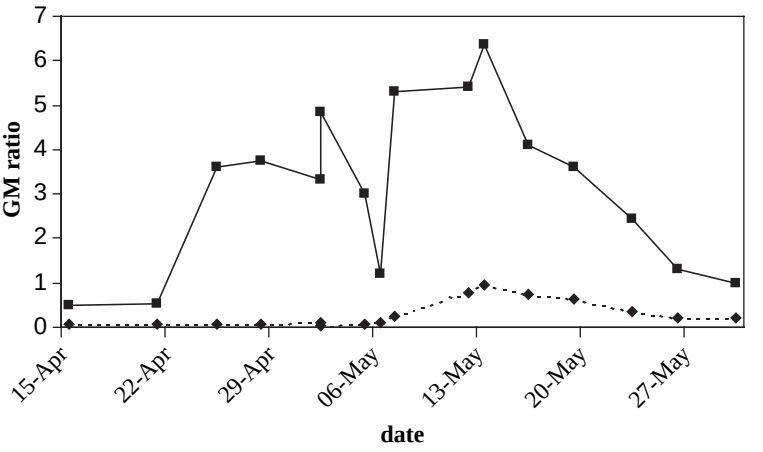

Fig. 1 Levels of circulating GM detected by the conventional pretreatment method (--◆--) compared with the method using filtration by a 50 kDa microcon filter of a larger volume of serum (–■–) [18] in a patient with a probable pulmonary aspergillosis. The filtration of the serum resulted in the detection of higher levels of GM and consequently of earlier detection compared with the conventional pretreatment method.

and the GM indices found in the patients serum [27,29]. Walsh *et al*. showed that repeated administration of the piperacillun tazobactam combination (GM index: 0.75) over 7 days resulted in accumulation of circulating antigen to positive GM index levels [27]. Bart-Delabesse et al. found three patterns of serum reactivity in patients receiving positive batches. Persistent antigenemia, with GM indices above 2, was observed in 65.7% of patients, and GM indices between 0.5 and 1.5 in 25.7%. Variable GM indices were observed in the remaining 14.3% of patients [29]. The majority of patients were classified incorrectly according to the EORTC/MSG consensus definitions, usually in the probable category instead of the possible category, due to the fact that the mycology criterion was met. The mean time needed to clear the antigen after discontinuation of the antibiotic therapy was 5.5 days, with a half life of 2.4 days [29].

The fact that *in vitro* GM index present in the antibiotic may not predict *in vivo* GM titers seems logical if one realizes that this reactivity might be due to several galf-antigens with different PA-ELISA reactivity's and different rates of clearance from the blood. Furthermore, it is possible that the high salt concentrations present in the antibiotic samples cause underestimated PA-ELISA results (BioRad, personal communication). Although the observation of the presence of PA-ELISA reactivity in beta-lactam antibiotics was first published over 2 years ago, at present batches still show reactivity.

The epitope detected by the EB-A2 monoclonal antibody is not exclusively present in *Aspergillus* species. Other moulds including *Penicillium* and *Paecilomyces* also release antigens containing that epitope, but invasive infections caused by these moulds are very rare. Unexpectedly, reactivity was also found with antigen released by *Cryptococcus neoformans* [32]. PA-ELISA reactivity was noted in serum from a patient with cryptococcemia, and reactivity with *C. neoformans* was confirmed *in vitro* [32]. The authors suggested that the *C. neoformans* galactoxylomannan contains one or multiple epitopes that cross-react with aspergillus GM [32]. Another study showed that the β-1,5-galactofuranose chain is present in certain bacteria, most notably bifidobacteria [33,34]. These bacteria have lipoglycans in their cell wall with the galf-epitope detectable as a surface antigen. When these lipoglycans are secreted, they might produce micelles with multiple galf-epitopes exposed to the outside [34]. In vitro, these bacteria as well as their culture supernatants showed reactivity with the PA-ELISA [34]. Since the intestinal microflora of neonates predominantly contains bifidobacteria, it was suggested that translocation of these bacteria or the cross-reacting antigen might contribute to the high false serum reactivity observed in this patient group [33,34].

The course of the antigen titer generally corresponds with the clinical response to antifungal therapy. This may not be the case in patients treated with the echinocandin caspofungin. Although, in patients receiving salvage therapy with the drug the GM titer corresponded with clinical response [35], a case was recently described in which a paradoxal rise of the GM titer was observed [36]. This patient received primary therapy with the drug and the GM index reached a peak of 33 shortly after therapy was commenced. Exposure of the *A. fumigatus* strain to caspofungin *in vitro* caused increased release of GM in the culture supernatant compared with strains exposed to voriconazole [36]. This observation would suggest that the course of the antigen titer in patients receiving caspofungin might not be in accordance with the clinical response.

Strategy and value of GM monitoring

Regular monitoring of patients with hematological malignancy, who are neutropenic and do not receive mould-active antifungal drugs is the setting in which the PA-ELISA appears to be of most value in the management of high risk patients [7,8,15]. In other patient groups the benefit of this assay and the optimal strategy which incorporates GM detection is less well established [37]. In hematology patients, the most appropriate strategy appears to be a combination of GM monitoring and high resolution CT scan, although some investigators find no additional value of GM-monitoring [38]. This pre-emptive approach was recently found to be feasible in hematology patients [39]. Patients with IA were better identified compared with a historic control group which received empiric antifungal therapy [39]. The number of patients requiring antifungal drugs was also lower in those undergoing the pre-emptive strategy than those receiving empiric antifungal therapy; 17% vs 35%. Only one patient, who died of invasive zygomycosis, was not diagnosed using this approach [39]. Although these results are encouraging, there is at present no study that shows that GM monitoring or the pre-emptive management strategy leads to a survival benefit.

Conclusions

GM detection remains a useful tool in the diagnosis of IA despite the current drawbacks as discussed above. Future research and improved GM detection techniques, such as described with filtration as part of the pre-treatment procedure, will certainly further contribute to early diagnosis of IA. At present monitoring of circulating GM is the only noninvasive tool with proven usefulness. However, new biological markers like (1→3)-β-D-glucan and fungal DNA might show an additional value. Management strategies should include all possible options for early diagnosis in order to increase the chance of successful treatment of patients with this disease.

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