Advancing the Field: Evidence for New Management Strategies in Invasive Fungal Infections

C. Orla Morrissey

Abstract Invasive fungal infections (IFI) are a significant cause of morbidity and mortality in the immunocompromised. The traditional diagnostic methods of culture and histological examination lack sensitivity and often only make a diagnosis late when the fungal burden is high, reducing the chances of cure even with the availability of new more potent and less toxic antifungal agents. New non-culture-based serological and PCR assays have been developed. These appear more sensitive and are able to make an earlier diagnosis as compared with traditional diagnostic methods. Early diagnosis is central to reducing IFI-related morbidity and mortality. This review describes the diagnostic potential of the new serological and PCR assays and outlines how these assays have been incorporated into algorithms to improve the management of IFI.

Keywords Invasive fungal infections · Candidiasis · Aspergillosis · Cryptococcal meningitis · BDG · Mannan · Galactomannan · PCR · Early treatment strategies

Introduction

Invasive fungal infections (IFI) remain as a major cause of mortality in those undergoing hematopoietic stem cell or solid organ transplantation, chemotherapy for hematological malignancies, patients in intensive care units (ICU) and those who are HIV infected. *Candida* is now the fourth most common cause of bloodstream infections and invasive candidiasis (IC)

has been associated with an attributable mortality of 49 % in some ICUs in the United States of America (USA)[1, 2]. More recently, mortality rates of up to 60 % have been reported with invasive *Aspergillus* infections in high-risk hematology patients [3, 4•]. The numbers that die from cryptococcal meningitis (CM) is estimated at 625,000 with 80 % (500,000) of these deaths occurring in sub-Saharan Africa [5•].

The economic costs of IFI are also substantial. In the USA the mean total hospital costs of invasive aspergillosis (IA) have been estimated at US$96,310 [6]. A study performed in one Australian center reported that treatment of IFI in allogeneic hematopoietic stem cell transplantation (HSCT) recipients and patients with acute myeloid leukemia was estimated at AU$30,957/case, increasing to AU$80,291/case if admitted to ICU, as compared to matched controls [7].

These high mortality rates and economic costs occur despite the availability of antifungal agents with improved potency and less toxicity. Early treatment has been related to improved outcomes but this is dependent on diagnostic tests that are both rapid and highly sensitive [8]. As traditional culture- and histology-based diagnostic methods have poor sensitivity and often only make a diagnosis late when the fungal burden is high, research has turned its focus in the last two decades to the development of serological and molecular assays with improved accuracy and rapidity of diagnosis [9].

This review will focus on the diagnostic capabilities of new serological and molecular assays and how these assays are incorporated into screening and pre-emptive (or as more accurately renamed diagnostic-driven (DD)) strategies for improved management of IFI.

Serological Markers for the Diagnosis of IFI

(1→3)-β-D-Glucan (BDG)

The BDG, a polysaccharide cell wall component of many fungi including *Candida*, *Aspergillus* and *Fusarium*, is

C. O. Morrissey (✉)
Infectious Diseases Unit, Alfred Health, Level 2, Burnet Building,
85 Commercial Road,
Melbourne, Victoria 3004, Australia
e-mail: o.morrissey@alfred.org.au

C. O. Morrissey
Department of Infectious Diseases, Central Clinical School,
Monash University, Melbourne, Australia

released during invasive infection. Four commercial tests have been developed for the detection of BDG including Fungitell (Associates of Cape Cod, East Falmouth, MA, USA), Fungitec-G (Seikagaku Cooperation, Tokyo, Japan), Wako-WB003 assay (Wako Pure Chemical Industries, Osaka, Japan) and Maruha (Maruha-Nichizo Foods Inc, Tokyo, Japan). These kits vary according to cut-off value used for a positive result and the detection method (colorimetric or turbimetric). The BDG is detected in a patient's serum by incubating serum with lyophilized horseshoe crab coagulation factor. If BDG is present, then the coagulation cascade is initiated.

The BDG assay has been extensively examined for its diagnostic performance in patients undergoing chemotherapy for hematological malignancies. A recent meta-analysis reported variable sensitivity and specificity (50 %-8 5 % and 80 %-99 %, respectively) [10••]. This variability is due to the heterogeneity of study designs and the populations included. The BDG has also been shown to make an earlier diagnosis of IFI as compared with culture by a median of 5–10 days [11, 12]. As a result, BDG assay has been included as an indirect microbiological criterion in the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) definitions of IFI and has been recommended for use in clinical trials and routine clinical practice by the European Conference on Infections in Leukemia (ECIL) guidelines [13, 14•].

The BDG has been less extensively examined in ICU patients. Posteraro et al., examined the diagnostic performance of the assay for IC in critically-ill ICU patients and compared it to the *Candida* score and colonization index of Leon and Pittet, respectively [15••, 16, 17]. The diagnostic accuracy was found to be greater for a single positive BDG value of >80 pg/ml (cut-off of Fungitell assay) as compared with the *Candida* score and colonization index (receiver operating characteristic (ROC) area under the curve (AUC) 0.98, 0.80 and 0.63, respectively). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 93.7 %, 83.6 %, 75 % and 98.6 %, respectively. In addition, BDG was detected 1–3 days prior to blood culture detection.

The evaluation of the BDG assay in bronchoalveolar lavage (BAL) fluid has been very limited, especially in comparison to galactomannan and PCR. The PPV was low (20.0 %) for 109 immunocompromised cases of proven, probable or possible IFI detected using the EORTC/MSG definitions [18]. In comparison to *Candida* PCR, the Fungitell BDG assay has been reported as having lower sensitivity for IC (80% vs. 56%; *p*=0.03), with similar specificity (70 % vs. 73 %; *p*=0.31) [19••].

Glucan is ubiquitous in the environment so false positivity can occur in patients undergoing hemodialysis or given blood products using cellulose materials, surgical patients in contact with cotton gauze, receiving β-lactam antibiotics and with bacteremia. Levels decline with antifungal therapy and a reduction in BDG levels with antifungal therapy has been associated with improved survival. However, the assay is not available in many institutions and is costly. Based on studies to date, the current role of the BDG assay is as an adjunct microbiological test for the diagnosis of IC.

More recently, BDG has been used to diagnose *Pneumocystis jirovecii* pneumonia (PJP). BDG is a component of the cyst wall of *P. jirovecii*. In a bivariate meta-analysis of 14 studies that included 357 cases of PJP and 1723 controls without PJP, the average sensitivity and specificity were determined as 94.8% and 86.3%, respectively, with positive and negative likelihood ratios (LR) of 6.9 and 0.06, respectively [20]. The area under the hierarchical summary ROC curve was 0.97. This study indicates that BDG may have potential for the diagnosis of PJP but further clinical evaluation is required.

Candida Mannan Assays

Mannan is a polysaccharide cell wall component of *Candida* and is released into blood during IC. *Candida* mannan antigen and antimannan antibody assays (Platelia *Candida* Antigen and Antibody assays) are commercially available from Bio-Rad (Marnes-la-Coquette, France) in Europe only. The cut-offs for test positivity are defined as 0.5 ng/mL for mannan and 10 arbitrary units/mL for antimannan antibody. Values of 0.25-0.5 ng/mL and 5–10 arbitrary units/mL are considered as indeterminate. Use of both tests in parallel improves sensitivity. A review published by ECIL showed that the sensitivity of the mannan antigen and antimannan antibody assays when used individually were 58 % and 59 %, respectively, but when used together, the sensitivity increased to 83 % with no significant reduction in specificity [21]. Based on these data it is now recommended that the tests are used in combination to diagnose IC. In addition, the assays performance varies according to *Candida* species. It performs best for *C. albicans* (sensitivity of 80–100 %), followed by *C. glabrata* and *C. tropicalis*, and it performs least well for *C. parapsilosis* and *C. krusei* (sensitivity of 40–50 %).

Bio-Rad has developed new detection assays, namely, Platelia *Candida* Ag Plus (Ag-Plus) and the Platelia *Candida* Ab Plus (Ab-Plus) which have been evaluated using a collection of stored serum samples obtained from 21 patients with microbiologically proven IC and 30 controls [22•]. All patients included in this study were undergoing myeloablative chemotherapy for hematological malignancies. The sensitivity and time to detection of IC were not significantly better than for the conventional *Platelia* Candida assays and specificity was reduced by 50 %. The reduction in specificity was determined by logistic regression analysis to be due to the detection of cases of superficial candidiasis by the Ag-Plus assay.

In a retrospective study of 45 patients either the Platelia *Candida* Antigen and Antibody assays were positive a minimum of two days prior to blood cultures. In addition, the performance depends on the patient population and the phase of treatment. Non-neutropenic surgical patients are more likely to have positive antibodies firstly but neutropenic populations undergoing chemotherapy are more likely to present with antigenemia [23]. Overall, further evaluation of the precise role of *Candida* mannan antigen and antimannan antibody in different populations is required before these assays are adopted into clinical practice for use in diagnosing IC.

Galactomannan Assay

Galactomannan (GM) is a heat-stable polysaccharide present in the cell wall of most *Aspergillus* species. It is also present in the cell wall of some *Penicillium* species. During invasive infection it is released from growing hyphae and can be detected in bodily fluids including serum, BAL and cerebrospinal fluid (CSF). Bio-Rad has developed a commercially available assay, the Platelia *Aspergillus* GM enzyme-immunosorbent assay (EIA). The original cut-off was an optical density index (ODI) of 1.5 but subsequently the Food and Drug Administration (FDA) has approved a lower ODI cut-off of 0.5 [24, 25]. Bio-Rad has very recently released a new assay which has been validated for testing on BAL as well as serum. There are no published data comparing both GM assays, unlike the comparison of the various Platelia *Candida* mannan assays.

The GM-EIA has been extensively examined for the earlier and more accurate diagnosis of IA in hematology patients. The sensitivity has varied between studies from 33 % to 100 % [24]. Like BDG analysis, this variation is related to differences in study design. In a meta-analysis the overall sensitivity was determined as 71 % with a specificity of 89 % [26]. The PPV is low (26–53 %) but the assay has an excellent NPV (95–98 %), indicating it may have more value as a screening tool to exclude IA. Some studies have also reported that GM can be detected a median of 5–8 days before culture positivity or clinical or radiological signs are present [27]. As a result it has been included as a microbiological criterion in the revised EORTC/MSG definitions of IA [13].

Whilst an ODI cut-off for positivity of 0.5 for serum is generally recommended it is less clear as to how many positive serum samples are needed to classify a case as IA. Maertens et al., found using ROC analysis that a cut-off of 0.5 in two sequential samples was associated with an optimal sensitivity of 92.1 % [25]. As a result, it is recommended that a positive result (ODI>0.5 in serum) indicating IA should be verified by retesting another aliquot of the same sample or obtaining a new sample and demonstrating reproducibility. Furthermore, studies have also indicated that the Platelia *Aspergillus* EIA may be useful for determining

fungal burden and responses to antifungal therapy [28, 29]. The ODI values over time are strongly correlated with treatment outcomes. Miceli et al., reported that the κ correlation coefficient for the ODI and survival was significant at 0.87 (95 % confidence interval 0.814-0.93; $p<0.001$) [29].

The sensitivity of the GM-EIA is lower in solid organ transplant recipients as compared with neutropenic and HSCT recipients. The assay had a reported sensitivity of 56 % in liver transplant recipients and only 30 % in lung transplant recipients indicating that this assay when used in serum may not have a diagnostic role in these populations [30, 31]. The assay has also been examined in serum samples from HIV infected patients diagnosed with penicilliosis (15), cryptococcosis (22) and 11 HIV infected patients with no IFI (controls) [32]. The ODI was significantly elevated in those with penicilliosis indicating that the test may have a role for the early diagnosis of this infection in endemic areas.

The GM-EIA has also been evaluated in BAL. A meta-analysis of 13 studies demonstrated that at a cut-off ODI of 0.5 the pooled diagnostic odds ratio (DOR), sensitivity, specificity, positive and negative LR for proven or probable IA of 52.7, 87 %, 89 %, 8.0, 0.15, respectively [33••]. When a cut-off ODI of 1.0 was used the DOR (112.7), specificity (95 %) and positive LR (17.0) increased with similar sensitivity (86 %) and negative LR (0.15), indicating that for BAL the optimal cut-off is 1.0. In addition, the sensitivity of GM-EIA was higher in BAL as compared with its use in serum (65 %) [33••].

Mold-active antifungal agents may decrease the performance of GM-EIA causing false negative results [34]. False positive results may occur in those on antibiotics (especially piperacillin-tazobactam or amoxicillin-clavulanate), dialysis, Plasmalyte solution, other fungal infections, myeloma and neonates.

Cryptococcal Antigen-Based Assays

Cryptococcal polysaccharide antigens can be detected in serum and CSF using latex agglutination (LA) or EIA methods which are more sensitive than microscopy and culture (93 % for the LA compared with 50–80 % for microscopy) [35–38]. The LA required minimal laboratory infrastructure and is easy to perform. Its distinct disadvantage is its subjectivity related to reading the agglutination reaction. The EIA is as sensitive as the LA. The EIA has advantages over the LA. The EIA does not need pretreatment of serum samples and has greater reproducibility as results are generated objectively using a spectrophotometer [35–37]. However, EIA requires technical expertise to perform and more advanced laboratory infrastructure. It is best suited to larger reference and diagnostic laboratories.

More recently, a new cryptococcal antigen assay has been developed, namely, the lateral flow assay (LFA; Immuno-

Mycologics, Norman, OK, USA). The LFA detects crypto-coccal antigen by dipping an immunochromatographic test strip into a drop of diluted serum or CSF and incubating for 10 minutes. A positive result is when two bands develop in the test zone. The LFA has comparable sensitivity and specificity to the LA and EIA and has received approval from regulatory authorities both in Europe and USA for use in serum [39••]. The LFA may play a substantial role in the management of CM in developing countries. It requires minimal training, can be performed by non-laboratory staff and the assay requires no refrigeration. In addition, Immuno-Mycologics have adopted a global access pricing strategy such that it will be affordable in developing countries.

Molecular-Based Diagnosis of IFI

The PCR assays have the potential for the rapid, sensitive and early diagnosis of IFI. Many *Candida*- and *Aspergillus*-specific as well as panfungal assays have been developed for use in blood and other clinical specimens. Detailed discussions of different PCR methods can be found in in another recent review [40].

A meta-analysis of the use of PCR to diagnose IC has reported a sensitivity ranging from 73–100 % and a speci-ficity of 92–100 %, with pooled sensitivity and specificity exceeding 90 % [41]. In this meta-analysis it was noted that higher sensitivities were achieved by using whole blood rather than serum samples, a commercial DNA extraction kit, panfungal ribosomal RNA or multi-copy gene targets and *Candida*- or fungal-specific PCR rather than multiplex assays. Specificity was increased by testing serial samples. Serial sampling and using consecutive results may assist in differentiating between colonization and true invasive infec-tion. Similar to other non-culture-based methods it appears that PCR for IC diagnosis can make the diagnosis earlier than blood cultures (median of three days) [42].

Aspergillus PCR assays have been used for screening high-risk hematology patients with varying sensitivities (63-100%). A meta-analysis of *Aspergillus* PCR assays for use in blood has reported that high-risk hematology patients should be screened twice-weekly and that a single PCR-negative result rules out IA but to confirm IA two consec-utively positive PCR results are recommended [43]. The variation in sensitivity is not only related to study design but also to the design of the PCR assay. A number of technical factors have been determined as affecting sensi-tivity and include DNA extraction methods (e.g., use of bead-beating), and volume of blood tested [44, 45•, 46]. The diagnosis of IA by *Aspergillus* PCR assays has preced-ed the diagnosis of IA by other methods (e.g., high resolu-tion computed tomography (HRCT) scan and culture/histological diagnosis by up to 3 weeks) [13, 47, 48].

Aspergillus PCR assays have also been examined and compared to GM in BAL specimens [49••]. The ROC indi-cated that a GM-ODI of 0.5 was optimal. Using this ODI, sensitivity and specificity was higher for GM-EIA than for *Aspergillus* PCR (79 % vs. 59 % and 96 % vs. 87 %, respec-tively). Use of positive results from both assays to define IA was associated with a sensitivity of 55 % and a specificity of 100 %. If either assay could be used to define IA, then the sensitivity and specificity were calculated at 83 % and 83 %, respectively. This indicates that if both tests are positive, then IA is effectively confirmed and that the combined use of both tests may improve the diagnosis of IFI.

There are conflicting data in the literature as to the effect of antifungal therapy on the sensitivity of PCR assays in both blood and BAL samples. Most report that *Aspergillus*-active antifungal therapy reduces the sensitivity but others have reported no effect [50–56]. This may be due, in part, to the form that *Aspergillus* DNA circulates in the blood and to the DNA extraction methodology. The PCR assays are particularly prone to contamination by air-borne spores which may result in false-positivity. Thus, a number of procedures need to be implemented in the laboratory to minimize contamination and the results need to be inter-preted in the context of clinical and radiological findings.

A commercial *Aspergillus* PCR assay is available [57]. In comparison to a validated in-house assay it has a sensitivity of 60–70 % and a specificity of 90.5-100 %. The current limitations of fungal PCR assays are that they are not stan-dardized nor have they undergone extensive multi-center clinical evaluation. As a result they are currently not includ-ed as a mycological criterion in the EORTC/MSG defini-tions of IFI. However, the European *Aspergillus* PCR Initiative has made significant advances in the area of stan-dardization providing recommendations for extraction of *Aspergillus* DNA from whole blood [46, 47].

Early Treatment Strategies for IFI

A randomized controlled trial (RCT) comparing a DD-strategy that guided the administration of anidulafungin therapy based on a positive BDG result (>80 pg/ml; Fungi-tell assay) to the administration of anidulafungin empirically based on physician's preference reported that BDG levels were significantly higher in those with an IFI versus those who had no IFI (117 vs. 28 pg/ml; $p<0.001$) [58]. Analysis of test performance indicated that the use of two sequential BDG results >80 pg/ml had a sensitivity, specificity, PPV and NPV of 100 %, 75 %, 30 % and 100 %, respectively. Twenty-one DD-strategy arm patients received anidulafun-gin as compared to five empiric arm patients. Anidulafungin was well tolerated and safe. However, a comparison of mor-tality outcomes was not reported for this trial. Two additional

RCT have been completed but the results are not published to date (MSG-01 and INTENSE, www.clinicaltrials.gov). These trials may provide data to help determine the best management strategies for IC in the ICU.

Several DD-strategies for IA have been examined. A number of prospective non-comparative studies have been performed to determine the feasibility of a more targeted antifungal approach based on positive non-culture based assays or characteristic imaging findings rather than the traditional empiric approach of administering antifungal therapy to all with persistent or recurrent fevers despite broad-spectrum antibiotics [59–62]. These studies indicate that DD-driven strategies can reduce empiric antifungal therapy (EAFT) by up to 78 % (relative reduction), have an excellent NPV (100 %), don't miss cases of IA, can reduce antifungal drug costs (by £52, 839) and identify cases of IFI even in the absence of persistent fevers. However, these data only provide an indirect estimate of the impact of DD-strategies.

A number of RCT have been performed comparing a DD-strategy to the traditional EAFT approach. Cordonnier et al., enrolled 293 patients including low-risk patients undergoing autologous HSCT [63]. Significantly more IFI were diagnosed in the DD-strategy arm (13 vs. 4; $p < 0.02$) but overall survival was no different between the arms (95 % vs. 97 %; $p = 0.12$). On sub-group analysis, in those receiving induction chemotherapy, non-inferiority in terms of overall survival was not demonstrated in the DD-strategy arm. Overall, there was a significant reduction in antifungal drug costs in the DD-strategy (€1470 vs. €2252; $p < 0.001$). In addition, a significant delay was detected between fever onset and initiation of antifungal therapy in the DD-strategy arm (median 13 vs. 7 days; $p = 0.01$). However, this may be related to the delay in instituting the DD-strategy until 96-hours of febrile neutropenia despite broad-spectrum antibiotics. Hebart et al., enrolled 403 allogeneic HSCT recipients and randomized them to a PCR-based DD-strategy versus the traditional EAFT approach [64]. In the PCR-based arm, antifungal therapy was administered if one PCR assay was positive or if PCR negative they had persistent febrile neutropenia despite broad-spectrum antibiotics for five days. Significantly more antifungal therapy was administered in the DD-strategy arm (112 vs. 76; $p < 0.001$) due, in the main, to the lack of specificity of the DD-strategy used in this trial. No difference in the number of cases of proven and probable IFI was detected between the arms. Whilst a significant mortality benefit was seen for the DD-strategy at day 30 post allogeneic HSCT, this was not evident at day 100 post transplantation. This finding may be explained by the lack of intensive monitoring (twice weekly) with PCR beyond day 30. Two further RCT have been published; one reported that a GM-based DD-strategy reduced EAFT use without decreasing survival and the second reported that PCR was a poor indictor of IA early after non-myeloablative SCT. However, both were small and inadequately powered [65, 66].

Overall, a DD-strategy will replace the EAFT approach and provides a suitable alternative to broad-spectrum mould-active prophylaxis but questions regarding types of non-culture-based assays that should be used, the patient groups that a DD-strategy should be used in, timing of implementation of, duration of screening with a DD-strategy and cost-effectiveness still need to be answered.

Early diagnosis of CM allowing for early treatment appears to be the most practical approach in resource-limited settings. Such a strategy involves screening HIV-infected patients with CD4 counts <100 cells/μL using one of the cryptococcal antigen assays (i.e., LA, EIA or LFA). If one of these assays is positive in an asymptomatic patient, then therapy using fluconazole should be instituted. Such a strategy has been studied in Uganda and has been shown to be associated a survival benefit (71 % at 30 months vs. 0 % if untreated) [67•]. Further, it has been estimated that such a screening strategy would be cost-effective in the setting of a prevalence of asymptomatic cryptococcal antigenemia of greater than 3 %. Currently, the prevalence of cryptococcal antigenemia in sub-Saharan Africa and South-East Asia is between 6 and 13 % [68, 69]. Micol et al., compared primary prophylaxis to a screening approach and found that primary prophylaxis was more effective when the CD4 count was less than 50 cells/ μL, but screening was a more cost-effective strategy when the CD4 count was less than 100 cells/μL [69]. The availability of the new LFA, especially when used as a point-of-care test, may increase the scope of such a screening strategy by increasing the availability of the assay and decreasing losses-to-follow-up. However, before the widespread roll-out of such a screening strategy occurs a number of barriers need to be overcome including the training of health-care workers in the use of the LFA and the administration of fluconazole.

Conclusions

Numerous non-culture-based assays are now widely available for the diagnosis of IFI. Now, the challenge is how we integrate these assays into algorithms so that we can best target antifungal therapy to those who have an IFI and prevent unnecessary antifungal therapy in those without an IFI on one hand and on the other hand reduce mortality through earlier diagnosis.

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