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Antifungal strategies for managing invasive aspergillosis: The prospects for a pre-emptive treatment strategy

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Invasive Aspergillosis (IA) is now the leading infectious cause of mortality in patients with haematological malignancies. Studies have identified those patient groups and the time-periods associated with high-risk for IA. The current management strategies for IA include prophylaxis and empiric antifungal therapy (EAFT). The rationale for prophylaxis in high-risk patients exists. However toxicities, drug interactions and breakthrough infections limit the benefits and the optimal duration for prophylaxis remains unknown. In recent EAFT studies in high-risk patients, $<10\%$ of persistent febrile neutropenic episodes were due to IA, suggesting that a disproportionate number of patients are treated with EAFT resulting in unnecessary drug-related costs. An increasing proportion of IA occurs in the absence of fevers and neutropenia, thus use of febrile neutropenia as the trigger to administer antifungal therapy will fail a significant proportion of patients. Recent research efforts have focused on the development of sensitive, specific and rapid diagnostic tests for IA, such as *Aspergillus* polymerase chain reaction (PCR) and antigen testing, which may be of value in guiding pre-emptive treatment strategies. However, studies evaluating the impact of pre-emptive therapy on patient outcomes and health-care costs have yet to be completed. Practical issues such as the combination of tests used and frequency of testing need to be resolved.

Keywords Invasive aspergillosis, prophylaxis, empiric, pre-emptive

Introduction

Invasive Aspergillosis (IA) has emerged as the major infection-related cause of morbidity and mortality in patients who receive intensive combination chemotherapy for acute leukaemia or who undergo allogeneic haematopoietic stem cell transplantation (HSCT) [1–3]. The incidence of IA has increased over the last decade with reported rates of 8–16% [3–7]. A wide spectrum of risk factors for IA has been identified contributing to this increased incidence of IA. and include: increased survival until the late post-transplantation period as a result of successful bacterial, viral and yeast prophylactic strategies; increasing number of patients undergoing HSCT; prolonged neutropenia; prolonged courses of high-dose corticosteroids; graft-versus-host disease (GVHD); receipt of an HSCT from a mismatched or unrelated donor; development of cytomegalovirus (CMV) disease; use of fludarabine- or high-dose cytarabine-based chemotherapy regimens and use of increasingly immunosuppressive therapies for the treatment of leukemia or for the prevention and treatment of GVHD (e.g. alemtuzumab) [2–12]. The identification of risk factors has allowed for accurate risk-stratification and the potential for development of targeted antifungal strategies [4,6,12].

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Historically the high-risk period for IA was early ($<$40 days) after allogeneic HSCT during neutropenia but now most cases of IA occur late ($>$40 days) with the median time to infection being 90–143 days [3–7,10]; related mainly to GVHD and not neutropenia. This late-onset of IA has implications for the duration and cost of all antifungal strategies used for the management of IA.

IA-related mortality rates remain high at 56–90% despite the recent introduction of more potent and less toxic antifungal agents [2–7,13,14]. Often conventional microbiological and histological tests fail to make an early or accurate diagnosis contributing to these high mortality rates [15–18]. Retrospective studies suggest that early diagnosis and prompt institution of therapy will increase survival [19–21].

Strategies such as antifungal prophylaxis and empiric antifungal therapy (EAFT) were developed for patients with haematological malignancies or undergoing HSCT in response to the increasing incidence of IA; the poor survival rates with established disease and the difficulties associated with diagnosis of IA using conventional tests. Whilst these strategies have demonstrated clinical advantages, gained the confidence of clinicians and become the standard of care [22,23], the ongoing high IA-related mortality rates suggest that additional strategies for the management of IA are required.

A pre-emptive antifungal treatment strategy for IA, analogous to the polymerase chain reaction (PCR)-directed ganciclovir treatment strategy used for CMV infection may be an effective additional strategy. However critical to the success of this approach is the availability of rapid, sensitive and specific diagnostic tests. High-resolution computed tomography (HRCT) scanning of thorax and non-culture based assays for the detection of *Aspergillus* antigens and *Aspergillus* DNA respectively have been developed and show promise as methods for the early and accurate diagnosis of IA.

The aims of this review are to briefly discuss the efficacy and limitations of antifungal prophylaxis and EAFT. We will then focus on the utility of HRCT scanning of thorax, serological and nucleic acid amplification assays as diagnostic tests to guide pre-emptive antifungal treatment strategies for IA and the recent progress made in the development and analysis of pre-emptive treatment strategies for IA. Finally the limitations of, unanswered questions about and future directions of pre-emptive treatment strategies will be discussed.

Current strategies

Antifungal prophylaxis

The first antifungal agent to have demonstrated efficacy and safety as prophylaxis was fluconazole. In HSCT recipients, fluconazole at 400 mg per day significantly reduced the incidence of invasive fungal infections (IFI) and IFI-related mortality [9,24]. In addition the administration of fluconazole until day 75 post allogeneic bone marrow transplantation (BMT) was associated with an overall survival advantage [9]. which persisted after eight years of follow-up [25]. A significant limitation of fluconazole prophylaxis is its narrow spectrum of activity as it has no activity against *Candida krusei*, *Aspergillus* and other moulds.

Itraconazole is an azole with a lipophilic four-ring tail that allows additional contact within CYP51, which broadens its spectrum of activity to include moulds and thus makes it an appealing agent for use as prophylaxis in patients at high-risk for IA. Itraconazole capsules have poor bioavailability but this problem has been largely overcome with the oral suspension and the intravenous preparation. Two randomized controlled trials (RCT) compared itraconazole to fluconazole in allogeneic HSCT recipients. In the study by Winston *et al*. [26] patients were randomized to receive intravenous (IV) itraconazole or IV fluconazole for14 days and then oral itraconazole solution or fluconazole tablets respectively until day 100 post transplant. In the itraconazole arm there were significantly fewer IFI caused by yeasts and moulds (9% vs. 25%; $p=0.01$), which resulted in a lower IFI-related mortality rate (9% vs. 18%; $P=0.13$). However the overall mortality rate was no different to that found in patients assigned to fluconazole (45% vs. 42%; $p>0.2$). Marr *et al*. [27] administered fluconazole (oral or IV) or itraconazole at higher doses (oral solution 2.5 mg/kg three times daily or IV 200 mg daily) for a longer period (i.e. until day 180 post transplantation) in very high-risk patients. The results in those able to tolerate itraconazole prophylaxis were similar with significantly fewer invasive mould infections (7% vs. 15%; $P=0.03$) but no difference in overall survival (61% vs. 69%; $P=0.11$). Of note, significantly more patients assigned to the itraconazole arms of both studies had gastrointestinal side-effects with discontinuation due to adverse events (24% vs. 9%; $P=0.02$ (Winston *et al*. [26]) and 23.8% vs. 4.7%; $p<0.001$ (Marr *et al*. [27]) respectively). Itraconazole as prophylaxis has also been studied in a meta-analysis in those undergoing chemotherapy for haematological malignancies and as a suspension significantly reduced the incidence of IA by 48% ($P=0.02$) but in compar-

ison to fluconazole discontinuation rates due to gastrointestinal side-effects were twice as high [28].

The prophylactic effect of itraconazole for IA is seen only at doses of at least 400 mg per day of oral suspension or 200 mg per day of IV solution and only when therapeutic levels (>500 ng/ml) are achieved within two to three days [28–30]. At these doses the rates of adverse events requiring discontinuation of itraconazole are not insignificant at 18–22% [31,32]. and the logistics of performing itraconazole levels at regular intervals prohibits the use of itraconazole in many centres. Drug–drug interactions that occur via cytochrome P450-3A4 and which require the doses of other drugs to be adjusted (e.g. cyclosporine A) add to the complexity of using itraconazole and limit its widespread applicability in the clinical setting.

The echinocandins, a novel class of antifungal agents which inhibit the synthesis of β-1,3-D-glucan in the fungal cell wall, have fungicidal activity against *Candida* and are fungistatic against *Aspergillus* [33]. Serious adverse drug reactions and discontinuations of echinocandins because of drug intolerability are uncommon [34]. Micafungin (50 mg IV daily) has been compared to fluconazole (400 mg orally daily) in 882 adult and paediatric allogeneic or autologous HSCT recipients during the pre-engraftment period as prophylaxis for IFI [35]. In this RCT micafungin resulted in a trend towards the prevention of more proven or probable IFI (80% vs. 73.5%; absolute difference +6.5%; 95% CI +0.9–12%; $P=0.3$); significantly reduced the need to switch to EAFT by 29% ($P=0.024$); decreased the incidence of breakthrough IA (0.2% vs. 1.5%; $P=0.071$) with fewer trial participants discontinuing micafungin due to adverse drug reactions. However, these efficacy results cannot be extrapolated to the post-engraftment period when most IA now occurs. While the echinocandins are suitable alternatives for prophylaxis during the early neutropenic phase post HSCT, the requirement for IV administration and the high costs of these products will limit the widespread use of these agents. Similarly while polyenes are efficacious in preventing IFI it is the requirement for IV administration and the high costs of the lipid formulations that limit the overall applicability of polyenes particularly in the outpatient setting [36,37].

Fluconazole prophylaxis has resulted in a shift to colonization and breakthrough infections with resistant species such as *C. krusei* and *Candida glabrata* [38,39]. Similarly with use of prophylactic antifungal agents that target *Aspergillus* in particular, there is increasing evidence for the emergence of breakthrough infections with resistant moulds [6]. Voriconazole, a triazole which has an extended spectrum of activity against *Aspergillus* and other moulds with the notable exception of Zygomycetes, has superior efficacy in the primary treatment of IA [40]. In patients with neutropenia and persistent fevers EAFT with voriconazole compared to liposomal amphotericin B (LAB) failed to reach the non-inferiority criteria for overall response although it was associated with significantly less breakthrough IFI [41]. Based on these results voriconazole has begun to be used as prophylaxis despite the fact that the trial comparing voriconazole as prophylaxis to fluconazole is not complete [42]. The increasing use of voriconazole as prophylaxis has been associated with an increase in the incidence of breakthrough invasive infections with Zygomycetes [43–46]. This association may limit the widespread applicability of voriconazole prophylaxis.

Posaconazole is a novel triazole that is structurally related to itraconazole and has a broad spectrum of activity against most *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp., *Fusarium* spp., Zygomycetes, *Acremonium* spp., *Paecilomyces lilacinus, Geotrichum* spp., *Trichoderma* spp. and some *Scedosporium* spp. A RCT in allogeneic HSCT recipients with grade 2–4 GVHD compared the efficacy of posaconazole and fluconazole in the prevention of IFI. Posaconazole significantly reduced the incidence of IA (3% vs. 17%; $P=0.001$) and IFI-related mortality (4% vs. 12%; $P=0.041$). Similar to the findings with itraconazole, the all-cause mortality rate was the same in the two treatment groups [47]. The efficacy of posaconazole in the prevention of IFI has been examined in a RCT in patients with acute myeloid leukaemia or myelodysplastic syndrome [48]. The incidence of IA and importantly, overall mortality rates were significantly reduced. Posaconazole was well tolerated in both trials and is the first agent since fluconazole in 1995 to show an overall survival benefit. Whilst the demonstrated overall survival benefit with posaconazole represents a significant advance the experience with voriconazole suggests that posaconazole prophylaxis should be targeted to those who are most at-risk of IFI [12].

Antifungal prophylaxis has decreased the incidence of IFI-related mortality and has in some circumstances improved overall survival (e.g. fluconazole and posaconazole). However factors such as drug toxicities, drug–drug interactions, lack of an oral or IV alternative, high cost, resistance and breakthrough infections create practical challenges that limit the efficacy and the universal applicability of this strategy in the clinical setting. Therefore while antifungal prophylaxis will continue to be a valuable antifungal management strategy for IA in select populations additional strate-

gies for the prevention or treatment of early infections need to be developed.

Empiric antifungal therapy

Empiric antifungal therapy is defined as treatment with systemic antifungal agent(s) for persistent ($\geq$1 temperature $\geq$38°C per 24-h period for a total of three days) or recurrent fevers ($\geq$1 temperature $\geq$38°C after an afebrile period of 48 h) despite broad-spectrum antibiotics in the setting of neutropenia ($<0.5–10^9\,l^{-1}$) and where thorough clinical, radiological and microbiological diagnostic evaluation has excluded other aetiologies but has also failed to document a proven or probable IFI [23,49].

The rationale for empiric antifungal therapy followed from autopsy and clinical studies which demonstrated an increasing incidence of IFI as the cause of death in those with prolonged neutropenia; an improved outcome with early treatment of IFI and retrospective studies which showed a decreased mortality rate from IFI in those treated with amphotericin B for persistent fevers of unknown origin (PFUO) compared with historical controls [50–54]. These observations resulted in the performance of the two sentinel prospective RCT that compared the use of conventional amphotericin B (CAB) for the treatment of PFUO to continuing antibiotics alone and/or discontinuing all antimicrobial therapy [55,56]. In both studies those randomized to CAB had a non-significant reduction in the incidence of IFI (6/32 vs. 1/18, $p>0.1$ and 6/64 vs. 1/68, $P=0.1$ respectively) and IFI-related mortality (6% vs. 0%, $P=0.05$) but importantly did not have an increase in overall survival (21% vs. 16%). Although both studies were non-blinded and underpowered to determine a significant difference in the primary endpoint they were landmark studies of the time and established the use of CAB for PFUO in neutropenic patients as the standard of care.

A new composite endpoint for assessing the efficacy of EAFT, a combination of five factors reflecting success of treatment, survival and tolerability, was developed [55–61]. In an effort to overcome the disadvantages of nephrotoxicity and infusion-related reactions associated with the use of CAB as EAFT, several RCT have been performed to determine if newer, more expensive agents such as LAB, itraconazole, voriconazole, fluconazole and caspofungin are as and in some cases are more efficacious [41,58–62]. Comparison of newer antifungal agents as EAFT using the composite endpoint to determine the overall success rates shows no clear evidence that any newer antifungal agent has superior efficacy to CAB (e.g. LAB (50%) vs. CAB (49%) and LAB (34%) vs. caspofungin (34%)), although there is clear evidence for superior tolerability (see Table 1 for summary of EAFT trials). This lack of superior efficacy when new antifungals are used as EAFT and the failure of EAFT to improve overall survival is related to the fact that the trigger for commencement of EAFT is persistent fever, a weak, insensitive, nonspecific and late sign of IFI.

Although not designed as individual primary or secondary endpoints, the most objective and clinically relevant endpoints for comparison of the efficacy of different antifungal agents when used as EAFT are absence of breakthrough and successful treatment of baseline IFI. LAB and voriconazole significantly decreased the incidence of breakthrough IFI (LAB (3.2%) vs. CAB (7.8%); $P=0.009$ and voriconazole (1.9%) vs. LAB (5.0%); $P=0.02$) and caspofungin was significantly more effective in treating baseline infections than LAB (51.9% vs. 25.9%; $P=0.04$). Based on this data LAB, voriconazole and caspofungin are the most suitable alternatives for use as EAFT. Importantly current rates of use of EAFT in high-risk patients are 40–50% whereas these studies have shown rates of baseline or breakthrough IFI of $<10\%$. Given the high cost of IV voriconazole, caspofungin and in particular LAB significant unnecessary direct and indirect drug-related costs occur with EAFT. Thus alternatives to over-treatment of persistent febrile neutropenia with expensive antifungal drugs are needed.

New non-culture based diagnostic tests for IA

High-resolution computed tomography (HRCT) scan of thorax

The air-crescent sign (i.e. a nodular infiltrate with central necrosis and peripheral crescentic or circumferential cavitation) found on HRCT scan was the first characteristic lesion of IA to be described [63]. However, this sign occurred in 50% of cases after histopathological diagnosis was made and after neutrophil recovery occurred, prohibiting early institution of antifungal therapy and significantly limiting its diagnostic utility [64]. Subsequently, Kuhlman *et al*. [65] defined an earlier radiological sign of IA, a halo sign (i.e. a mass-like pulmonary infiltrate surrounded by a hazy edge of low attenuation) during neutropenia which was diagnostic of IA with a sensitivity of 89%. Two retrospective studies provided indirect evidence that HRCT scan of thorax when performed system-

Table 1 Randomized trials comparing antifungal agents as empiric antifungal therapy (EAFT)

Study/year [references]	Study drugs	Primary endpoint	Sample size	Efficacy outcome	Safety outcome
Walsh *et al*. 1999 [59]	CAB vs. LAB	*Efficacy*: Composite of survival, resolution of fever, successful treatment of any baseline fungal infection, absence of breakthrough fungal infections and absence of premature discontinuation of study drug	702	*Composite*: Equivalence *Individual components*: Significantly less proven breakthrough fungal infections with LAB (3.2% vs. 7.8%)	*Favors LAB*: Significantly less infusion-related fevers (17% vs. 44%) and nephrotoxicity (19% vs. 34%)
Winston *et al*. 2000 [60]	CAB vs. Flu	*Efficacy*: Composite of defervesence, absence of breakthrough fungal infections, survival and tolerability	317	*Composite*: Equivalence	*Favors Flu*: Significantly less adverse events (13% vs. 81%)
Boogaerts *et al*. 2001 [61]	CAB vs. Itra	*Efficacy*: Composite of defervesence, absence of breakthrough fungal infections, survival, tolerability and resolution of neutropenia	384	*Composite*: Equivalence	*Favors Itra*: Significantly less discontinuation due to toxicity (19% vs. 38%)
Walsh *et al*. 2002 [41]	LAB vs. Vori	*Efficacy*: Composite of survival, resolution of fever, successful treatment of any baseline fungal infection, absence of breakthrough fungal infections and absence of premature discontinuation of study drug	849	*Composite*: Vori did not meet the non-inferiority criteria *Individual components*: Significantly less breakthrough fungal infections with Vori (1.9% vs. 5%)	*Variable*: dependent on adverse event examined
Walsh *et al*. 2004 [62]	LAB vs. Caspo-fungin	*Efficacy*: Composite of survival, resolution of fever, successful treatment of any baseline fungal infection, absence of breakthrough fungal infections and absence of premature discontinuation of study drug	1095	*Composite*: Equivalence *Individual components*: Significantly greater successful treatment of baseline fungal infections with caspofungin (51.9% vs. 25.9%)	*Favors caspofungin*: Significantly less discontinuation due to toxicity (10.3% vs. 14.5%) and nephrotoxicity (2.6% vs. 11.5%)

CAB, conventional amphotericin B; LAB, liposomal amphotericin B; Flu, fluconazole; Itra, itraconazole; Vori, voriconazole.

atically (i.e. at weekly intervals while neutropenic or once an infiltrate was detected on chest x-ray in the setting of febrile neutropenia) allowed for an earlier diagnosis of IA by an average of five days and a 66% relative reduction in IA-related mortality [19,20]. Based on these data HRCT scans are now routinely performed in neutropenic patients with persistent or recurrent fevers despite broad-spectrum antibiotics.

High-resolution CT scans of thorax have a number of limitations including the inability to make a microbiological diagnosis. Other infections (e.g. nocardiosis, other invasive moulds infections (e.g. Mucormycosis), tuberculosis etc.) may be misdiagnosed as IA with a resultant delay in appropriate therapy. Changes in HRCT scan may be non-specific requiring further evaluation with invasive procedures such as bronchoscopy. Furthermore HRCT scanning requires patients susceptible to IA to leave their protected environment, urgent HRCT scanning is not always possible at all centres and frequent surveillance scans may prove cost-prohibitive.

Serological assays

Galactomannan

Galactomannan (GM), an antigen component of the *Aspergillus* cell wall, can be detected in serum and other body fluids (e.g. cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL) fluid etc.) of patients with IA. A commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit (Platelia® *Aspergillus* ELISA kit, Bio-Rad, Redmond, Washington, USA) has a lower limit of detection of 0.5–1.0 ng/ml of GM in serum and is the most widely used test [66]. Initial prospective observational studies (see Table 2) demonstrated that when used as a screening test, twice weekly, in neutropenic haematology patients the sensitivities and specificities of the assay were very high at 89.7–94.4% and 94–98.8% respectively [67–70]. Galactomannan was detected in the serum of 63% of patients on average five to 17 days before clinical or radiological signs were present or cultures were positive and in 88.8% of patients a median of six days prior to the

Table 2 Prospective studies evaluating the utility of Platelia *Aspergillus* galactomannan (GM) ELISA

Study/year [references]	Number of patients or (episodes)	GM cut-off	Number of IFI	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Additional analysis
Maertens *et al*. 1999 [67]	(243)	≥1.0	33	92.6*	95.4	93.0	95.0	
Sulahian *et al*. 2001 [68]	797	≥1.5	53	90.5$^{\varphi}$	94.0	–	98.7	
Maertens *et al*. 2001 [69]	(362)	≥1.0	95	89.7$^{\varphi}$	98.1	87.5	98.4	GM positive before radiology signs (e.g. nodules, diffuse infiltrates etc.) in 68% (median of five days)
Maertens *et al*. 2002 [70]	100	≥1.0	27	94.4*	98.8	94.4	98.8	GM positive prior to administration of EAFT in 83.3%
Herbrecht *et al*. 2002 [72]	(797)	≥1.5	153 (31 definite)	29.4‡ 64.5%*	94.8	57.7	84.9	On reanalysis with cut-off of 0.7 sensitivity increased to 73.1% in proven cases
Marr *et al*. 2003 [73]	325	>1.5	54	43.0	99.0	–	–	On re-analysis with cut-off of >0.5 sensitivity increased to 70%
Rovira *et al*. 2004 [74]	74	>1.5	6 (proven and probable IA)	75.0‡	100.0	100.0	97.0	4/6 on EAFT by time GM ELISA became positive
Pinel *et al*. 2003 [75]	807	>1.0	48	50.0†	99.6	85.0	96.8	

EAFT, empiric antifungal therapy; ELISA, enzyme-linked immunosorbent assay; GM, galactomannan; IFI, invasive fungal infections; NPV, negative predictive value; PPV, positive predictive value;*, proven; †, proven and probable; ‡, proven, probable and possible.

initiation of EAFT [69,70]. Galactomannan production is proportional to fungal load in tissue and levels appear to have a prognostic significance, with levels that fail to decrease in the setting of antifungal therapy associated with a poor outcome [69,71].

However subsequent studies have produced less encouraging results regarding the performance of the GM-ELISA (see Table 2) [72–75]. These studies have demonstrated that the sensitivity of the GM-ELISA may be lower at 29.4–64.5%, that the GM-ELISA may not make an earlier diagnosis than conventional diagnostic tests and that only high-risk patients (i.e. neutropenic patients post allogeneic HSCT) should be screened. These findings lead to the recommendation that the optical density index (ODI) cut-off for a positive result of 1.5 be reduced to 0.5 in order to increase the sensitivity of the assay [73]. A number of reasons for the wide variation in sensitivity and the incidence of false-negative results have been proposed including: the presence of anti-*Aspergillus* antibodies [72]; host group studied [70,72]; localization or encapsulation of the infection; transient nature of circulating GM [75]; use of mould active prophylactic agents [76] and inadequate sampling strategies [77].

High rates of positivity detected in the absence of IA have also raised questions with regard to the clinical utility of the GM-ELISA. For many years the specificity of the assay has been recognized to be lower in neonates and children [78]. The neonatal gut is colonized by *Bifidobacterium* spp. and translocation of the bacteria across the immature gut wall allows the lipoteichoic acid component of the *Bifidobacterium* cell wall to cross-react with the Platelia GM assay, contributing to the high positive rates in neonates [79]. Galactomannan has been detected in food (e.g. pasta) and drinks (e.g. milk) and it is likely that the ingestion of exogenous GM and the translocation across an immature or damaged (e.g. mucositis) gut is a cause of this false positivity [80]. More recently false positive GM results have been associated with the administration of antibiotics in particular piperacillin-tazobactam [81,82]. *In vitro* and *in vivo* studies indicate this is likely due to the presence of GM in the antibiotic [83,84]. This has resulted in some centres measuring GM in each new batch of piperacillin-tazobactam and either

recommending that the batch be discarded if GM levels are too high, adjusting the threshold for a positive result or exercising caution in the interpretation of a positive result. Other centres have excluded piperacillin-tazobactam from antibacterial guidelines. In the United States, the Food and Drug Administration (FDA) currently recommends that a positive GM-ELISA result in a patient receiving piperacillin-tazobactam should be confirmed by other diagnostic tests before potentially toxic antifungal therapy for IA is administered [85]. Additionally, the false positivity rate can be decreased by using the criteria that probable IA is diagnosed only when two consecutively positive results (i.e. ODI >0.5) are obtained from two consecutive serum samples [70].

While concerns and controversy regarding the sensitivity, specificity and time to positivity relative to conventional tests exist for GM-ELISA it would be inappropriate to disregard the utility of this assay at this stage. The optimum application of this assay is still being defined but currently it should be used prospectively, a minimum of twice-weekly in high-risk neutropenic patients (i.e. neutropenic patients with acute leukaemia and recipients of allogeneic HSCT when neutropenic), with a cut-off ODI of 0.5 and two consecutively positive results from two consecutive samples required for positivity; for the duration of risk of IA and to use a per-test method of analysis for determination of sensitivity [77]. Only then we will gain valuable information regarding the true utility of GM-ELISA.

1, 3-β-D-glucan

1, 3-β-D-glucan is a component of the cell wall of many fungi including *Candida, Aspergillus* and *Fusarium* but notably not of Zygomycetes or *Cryptococcus neoformans*. Fungitec-G and Fungitell assays produced by Seikagaku Kogyo Corporation (Tokyo, Japan) and Associates of Cape Cod (Falmouth, USA) respectively are the assays that are most widely available and that have been evaluated in the clinical setting. These assays act through 1, 3-β-D-glucan activation of the coagulation cascade within amoebocytes obtained from the haemolymph of horseshoe crabs. 1, 3-β-D-glucan binds to the α subunit of factor G, activating serine protease zymogen β subunit. When this is combined with the proclotting enzyme of the cascade and the chromogenic substrate Boc-Leu-Gly-Arg-p-nitroanilide, 1, 3-β-D-glucan (BD) can be detected and quantified spectrophotometrically [86].

Obayashi *et al*. [87] determined a cut-off for positivity for the Fungitec assay of 20 pg/ml in a study of 202 febrile neutropenic episodes in 179 patients with haematological malignancies. Forty-one proven IFIs were detected in the study population by autopsy or culture and in 37 the assay detected BD at levels >20 pg/ml on one or more samples giving a sensitivity of 90%. A cut-off for positivity of >60 pg/ml for the Fungitell assay was determined using 30 patients with Candidaemia and 30 healthy adults [86]. Serial samples were obtained from 282 patients with acute leukaemia or myelodysplastic syndrome whilst on itraconazole or caspofungin prophylaxis. A minimum of one sample was positive for BD (i.e. >60 pg/ml) a median of ten days prior to diagnosis of proven or probable IFI with a sensitivity of 100% [86]. The Fungitell assay was also recently analysed in six US centres. The overall sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the assay were 69.9%, 87.1%, 83.8% and 75.1% respectively. The sensitivity of the assay did not decrease significantly in the setting of antifungal therapy (i.e. decreased from 83% to 72%; $P=0.69$) [88]. Importantly all these studies included only a few cases of IA (i.e. five cases, four cases and ten cases in the studies by Obayashi *et al*. [87], Odabasi *et al*. [86] and Ostrosky-Zeichner *et al*. [88] respectively). Whilst the reported sensitivity of the 1,3-β-D-glucan assays for IA is high at 80% [88], the assays require further evaluation in much greater number of cases of IA before they can be included in a pre-emptive antifungal treatment strategy.

Nucleic acid assays

The amplification of gene sequences unique to *Aspergillus* spp. offers the potential for rapid, sensitive and early diagnosis of IA. Numerous 'in-house' PCR assays have been developed using different specimens, extraction methods, amplification targets and amplicon detection methods and have reported good performance characteristics [89–91]. However the relative merits of various assays are difficult to assess as the technical differences make it impossible to directly compare sensitivity and specificity results (see Table 3 for details of different PCR assays). Because no commercial standardized PCR-based assay is available for the detection of *Aspergillus* DNA in clinical specimens the widespread use of PCR as a diagnostic modality is currently limited and PCR is not incorporated into the current standardized diagnostic criteria [92].

Several technical factors that affect the performance of the assay have been described. The most sensitive assays are those that have: (i) used whole blood as the

Table 3 Characteristics of 'in-house' polymerase chain reaction (PCR) assays for the diagnosis of invasive aspergillosis (IA)

Study [reference]	Gene target	Detection method	Specimen
Hayette *et al*. [89]	Alkaline protease (ALP)	*Aspergillus fumigatus*- and *A. flavus*-specific nested PCR	BAL
Tang *et al*. [90]	Alkaline protease (ALP)	*A. fumigatus*- and *A. flavus*-specific Southern blot	BAL
Costa *et al*. [91]	*FKS* mitochondrial	*A. fumigatus* ABI PRISM 7700 sequence detection system	Spiked whole blood fractions
Einsele *et al*. [98]	SSU rDNA	Panfungal Southern blot	Whole blood, BAL
Yamakami *et al*. [99]	SSU rDNA	*A. fumigatus*- specific nested PCR assay	Serum
Skladny *et al*. [101]	SSU rDNA	*Aspergillus*-specific nested PCR	Whole blood, BAL
Hebart *et al*. [102]	SSU rDNA	Panfungal (ITS1, ITS4) Southern blot	Whole blood
Williamson *et al*. [104]	LSU rDNA	*Aspergillus*-specific nested PCR sequencing	Serum
Loeffler *et al*. [107]	SSU rDNA	*Candida albicans*- and *A. fumigatus*-specific LightCycler (Roche)	Whole blood
Jones *et al*. [108]	Mitochondrial	*Aspergillus*-specific PCR-ELISA	BAL
Jordanides *et al*. [109]	SSU rDNA	Panfungal LightCycler (Roche)	Whole blood
Halliday *et al*. [110]	SSU rDNA	*Aspergillus*-specific nested PCR	Whole blood
Florent *et al*. [111]	Mitochondrial	*A. fumigatus*- and *A. flavus*-specific PCR-ELISA	Serum
White *et al*. [112]	LSU rDNA	*Aspergillus*-specific LightCycler (Roche)	Whole blood

BAL, Bronchoalveolar lavage; ITS, intergenic transcribed spacer; LSU, large subunit; rDNA, ribosomal DNA; SSU, small subunit.

test-specimen; (ii) used techniques for high-efficiency extraction of *Aspergillus* DNA (e.g. red cell and white lysis buffers, lyticase, heat-alkali treatment, β-mercaptoethanol, EDTA etc.); (iii) targeted multicopy genes such as the rDNA gene complex (i.e. 18S rDNA gene, 5.8S rDNA gene, 28S rDNA gene and ITS region) or mitochondrial DNA and (iv) used a nested PCR or PCR-ELSA format [93–112]. Where the performance of PCR assays was optimized using these techniques sensitivities and specificities of 63.6–100% and 63.5–100% respectively were reported [98,101–105,109–112]. In addition these sensitive PCR assays detected *Aspergillus* DNA in whole blood of patients on average 5–19.5 days before clinical signs or radiological findings were present or cultures were positive and a median of 8–19.5 days prior to the initiation of antifungal therapy in 67% of patients [98,102–104,109–112] (see Table 4).

Importantly all highly sensitive PCR assays have excellent NPV of 97.6–100% [102,103,105,109–112]. Thus repeatedly negative *Aspergillus* PCR results may allow clinicians to withhold *Aspergillus*-directed antifungal therapy and to institute other investigations to determine the cause of persistent or recurrent fevers on broad-spectrum antibiotics. If this approach were taken it has been indirectly estimated that the use of empiric antifungal therapy would decrease by 37–50% [110,112].

All sensitive *Aspergillus* PCR assays are limited by high false positive rates and this is reflected in the reported PPV [102,104]. However, the low PPV may be due to the fact that PCR is a more accurate test than the current reference diagnostic tests of culture and biopsy [109,111]. The PPV increases if two positive PCR results are required to classify a case as probable IA. In a prospective observational study where HSCT recipients were serially screened twice weekly the calculated PPV for a single positive PCR result was 15.2% and increased to 44% if calculations were based on two positive PCR results [102]. Similarly Williamson *et al*. [104] demonstrated that the PPV increased from 80% to 100% when the criterion of a single positive PCR result was changed to the requirement for two positive PCR results for a diagnosis of IA. While consecutively positive PCR results are significantly more likely to occur in cases of proven/probable IA compared with possible IA (60% vs. 14.5% respectively [112]) the requirement of two positive PCR results for a diagnosis of IA decreases the sensitivity of the assay (from 100% to 81%) and also makes the interpretation of the clinical significance of intermittent positive results and single positive results in high-risk patients very difficult (i.e. are these results false positive or early true positive results indicative of sub-clinical IA?) [104].

Halliday *et al*. [110] provided some insight into the controversial issue of interpretation of intermittently positive PCR results. Intermittently positive PCR results (>2 positive results) within a two-week period is more likely to represent probable IA compared with intermittently positive results that are separated by >2 weeks. Additionally patients with intermittently positive results were more likely to be diagnosed with probable or proven IA than possible IA (38.5% vs. 19.1%). Based on these data these investigators recommend starting antifungal therapy and investigating with other diagnostic tests for IA (e.g. HRCT scan of thorax, bronchoscopy etc.) in those who have intermittently positive results within a two-week period. Antifungal therapy should subsequently be modified according to

Table 4 Studies evaluating the utility of polymerase chain reaction (PCR) assays

Study [reference]	Number of patients or (episodes)	Proven/probable IA	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Additional analysis
Einsele *et al*. [98]	121	13	100.0	98.0	–	–	PCR positivity preceded radiological signs by median of four days
Hebart *et al*. [102]	84	7	100.0* 100.0†	65.0* 84.0†	15.2* 44.0†	100.0* 100.0†	PCR positivity preceded radiological signs by median of nine days
Lass-Florl *et al*. [103]	121	3	75.0	96.0	–	–	Antifungal therapy, PCR negativity and clinical responsiveness did not correlate
Williamson *et al*. [104]	37	13	100.0* 81.0†	79.0* 1 00.0†	80.0* 100.0†	–	67% had positive PCR results prior to institution of antifungals
Jordanides *et al*. [109]	(125)	8	75.0‡	70.0‡	15.0‡	98.0‡	PCR positivity occurred a median of 19.5 days prior to commencement of EAFT
Halliday *et al*. [110]	65	13	61.5‡	100.0‡	100.0‡	92.4‡	Inclusion of cases of possible IA decreased sensitivity of assay from 100% to 71%
Florent *et al*. [111]	201	33	63.5‡	89.7‡	63.6‡	89.7‡	Use of PCR and GM-ELISA results in combination increased sensitivity to 83.3%
White *et al*. [112]	203	14	92.3‡	94.6‡	60‡	99.3‡	Good correlation with GM-ELISA results (76.7%)

Calculations based on: *, a single PCR result; †, 2 positive PCR results; ‡, consecutively positive PCR results. BDG, (1→3)-β-D-glucan; EAFT, empiric antifungal therapy; GM-ELISA, galactomannan enzyme-linked immunosorbent assay; IA, invasive aspergillosis; NPV, negative predictive value; PPV, positive predictive value.

clinical progress, the results of ongoing PCR testing and the results of conventional diagnostic tests. In our experience a similar approach should be used for single positive PCR results in those at very high-risk for IA (e.g. those with grade IV GVHD on corticosteroids)

Often, in clinical practice the tests (i.e. biopsy and culture) required for diagnosis of proven/probable IA according to the EORTC-MSG consensus criteria [92]. are either not performed because of inadequate platelets, other clinical factors such as hypoxia etc. or are performed at the wrong time in relation to the timing of infection [113]. In this situation clinicians rely on the detection of the characteristic lesion(s) of IA (i.e. halo-signs, nodules, cavity in area of consolidation) on HRCT scans of thorax for diagnosing IA and for guiding antifungal therapy. However use of characteristic radiological signs without microbiological confirmation allows only for a diagnosis of possible IA [92]. Whether these cases of possible IA are included as true positives, true negatives or are totally excluded from the analysis influences the performance parameters of any PCR assay. Halliday *et al*. [110] showed that if possible cases were included as true positives then the sensitivity of the assay decreased from 100% to 70.6%. Thus some but not all cases of possible IA represent true subclinical or occult IA. This highlights the critical need for better diagnostic tests for IA and for studies with improved methodology to evaluate the performance of any PCR assay and the complexities of distinguishing true positive from false positive PCR results.

There are conflicting data regarding the impact of antifungal therapy directed against *Aspergillus* on the sensitivity of *Aspergillus* PCR assays [103,106,114–116]. Most authors found a decreased sensitivity during antifungal therapy, although Halliday *et al*. [110] noted that in 12 of 13 patients with proven or probable IA receiving antifungal therapy PCR results remained consecutively or intermittently positive. The findings of Halliday *et al*. [110] together with our experience of return of PCR positivity during antifungal therapy despite documented radiological improvement and neutrophil recovery [Morrissey CO, Slavin MA, Szer J *et al*., unpublished data] may provide some insight into the kinetics of *Aspergillus* DNAaemia and the interpretation of *Aspergillus* PCR results during antifungal therapy. PCR positivity during antifungal therapy may be related to improved leucocyte function rather than failure of therapy. Return of normal numbers of functioning leucocytes may lead to destruction of *Aspergillus* at a tissue level and release of and then phagocytosis of *Aspergillus* DNA. Thus it may be circulating *Aspergillus* DNA from dead tissue hyphae that is detected by whole blood PCR while the infection is resolving at a tissue level. *Aspergillus* PCR assay, while of significant value in the initial diagnosis of IA, may not be as useful in monitoring response to therapy

as for example GM-ELISA. For monitoring of antifungal therapy, a combination of diagnostic tests (e.g. PCR, GM-ELISA and HRCT scans), symptoms, signs and degree of risk may be required. Further studies quantifying *Aspergillus* PCR and its kinetics in tissue and correlation with tissue histology and tissue activity of *Aspergillus* by metabolomics are required. The results of tissue studies should also be correlated with whole blood quantitative *Aspergillus* PCR results as well as neutrophil and macrophage counts and function.

In an effort to reduce amplification time, labour and the potential for contamination associated with nested PCR and PCR-ELISA assays; to allow for standardization of PCR and to allow for quantification of the fungal load, real-time PCR assays have been developed [91,107,109,114,117–122]. The limitations of available real-time PCR assays include: (i) decreased sensitivity compared with nested PCR assays [114,117]; (ii) lack of validation in the relevant patient groups [119,122]; (iii) targeting of only *A. fumigatus* and thus inability to detect the 10–15% of cases of IA caused by *Aspergillus* spp. other than *A. fumigatus* [91,107,117,119,122] and (iv) lack of quantification of *Aspergillus* DNA [109,118]. Thus real-time PCR assays need further refinement before their utility in the clinical setting can be fully evaluated.

In summary there are a number of *Aspergillus* PCR platforms currently available that have been optimized which perform reproducibly with excellent sensitivity and NPV and reasonable specificity; that have been adequately evaluated in the correct patient populations against standardized diagnostic criteria and are capable of detecting IA early [109–112]. In addition there is now a growing body of information regarding the optimal use of these assays in the clinical setting such as standardized criteria for interpreting intermittently positive and single positive results; prospectively screening high-risk patients twice weekly for the duration at risk of IA and interpreting a positive PCR result on antifungal therapy in the context of the clinical picture and radiological findings.

Combination of new diagnostic tests for the diagnosis of IA

Invasive aspergillosis has a wide spectrum of pathological and clinical manifestations for example acute invasive pulmonary aspergillosis and *Aspergillus* tracheobronchitis. Thus the available non-culture based diagnostic tests, which detect different components of *Aspergillus,* when used in combination offer the potential for an increased accuracy of diagnosis of IA in its varying manifestations. Several studies have suggested that combinations of GM-ELISA, HRCT scan of thorax and *Aspergillus* PCR assays hold promise for improved sensitivity, specificity, PPV and NPV in the diagnosis of IA.

Becker *et al*. [123] examined the strategy of CT-directed bronchoscopy and testing of the collected BAL fluid with *Aspergillus* GM-ELISA. The sensitivity, specificity, PPV and NPV of this combined-test strategy were all 100%. This compared favourably to the sensitivity, specificity, PPV and NPV of two consecutively positive results on GM-ELISA testing of serial serum samples which were 47%, 93%, 73% and 82% respectively. Florent *et al*. [111] tested 1205 samples from 167 patients who had ≥ 1 episode of febrile neutropenia using a semi-standardized PCR-ELISA assay and *Aspergillus* GM-ELISA. The sensitivity and NPV of the PCR-ELISA for proven and probable cases of IA were improved from 63.6% and 89.7% to 83.3% and 97.6% respectively when used in combination with GM-ELSIA. Yoo *et al*. [124] evaluated nucleic acid sequence-based amplification (NASBA) and *Aspergillus* GM-ELISA for the diagnosis of IA in febrile neutropenic patients. These investigators collected 448 samples from 128 patients during periods of neutropenic fever post chemotherapy or HSCT. Use of the two tests in combination increased the sensitivity of the diagnosis of IA from 86% to 100%. PCR in combination with GM-ELISA and/or HRCT scan should be evaluated as a diagnostic strategy to guide pre-emptive antifungal therapy of IA.

Pre-emptive strategies for IA

We now have available a number of well developed and evaluated non-culture based microbiological and radiological diagnostic tests that are rapid, sensitive, specific and appear to make an earlier diagnosis of IA. The critical question is whether we can incorporate these tests into a highly effective antifungal strategy. An appropriate strategy to examine would be a pre-emptive strategy that uses these new diagnostic tests to prospectively screen high-risk patients (i.e. patients with acute leukaemia and recipients of allogeneic HSCT) in order to (i) detect those patients with early infection and allow for the early and aggressive treatment and (ii) importantly to withhold toxic and expensive antifungal agents in those who have had IA excluded.

The potential impact of such pre-emptive strategies has been explored. Severens *et al*. [125] in a decision analysis using institution based baseline values (e.g institutional EAFT guidelines, local incidence of IA etc.) estimated that the probability of receiving EAFT

could be decreased from 24% to 7.3% (16.7% absolute decrease) as a result of using GM-ELISA, HRCT scan and radionuclide imaging to guide therapy when compared with the conventional diagnostic strategy.

However evaluation of this approach in a clinical setting is limited. In a prospective cohort study in neutropenic paediatric cancer patients Lin *et al*. [126] used a nested PCR assay with the ability to detect *Candida*, *Aspergillus*, *Trichosporon* and *Cryptococcus* spp. in blood to guide the commencement of antifungal therapy. Although this approach was accurate in guiding antifungal therapy to only those with confirmed infection before cultures were positive the predominant infection was invasive candidiasis, not IA.

Maertens *et al*. [127] performed another prospective cohort study in high-risk haematology patients. The triggers for further diagnostic evaluation for IFI included: (i) neutropenic fevers refractory to five days of broad-spectrum antibiotics; (ii) clinical signs and/or symptoms suggestive of IFI; (iii) appearance of a new pulmonary infiltrate whilst receiving corticosteroids or broad-spectrum antibiotics; (iv) isolation of moulds or demonstration of hyphae in respiratory specimens and (v) two consecutive GM-ELISA results. The protocol-prescribed diagnostic evaluation included a HRCT scan and bronchoscopy with BAL fluid collected for microscopy, culture and non-fungal PCR assays (e.g. for CMV). Febrile neutropenia occurred in 117 episodes and 35% of these episodes would have satisfied the current standard criteria for the administration of EAFT. It was estimated that the protocol-prescribed pre-emptive strategy reduced the rate of use of EAFT to 7.7% (a 78% relative reduction). Whilst encouraging, such a cohort study provides only an indirect estimate of the clinical and pharmaco-economic impact of a pre-emptive strategy on patient outcomes.

Hebart *et al*. [128] performed a RCT in a total of 408 patients undergoing allogeneic HSCT comparing PCR-directed therapy with LAB to a fever-driven strategy of EAFT. Patients, randomized to the PCR-based strategy, had PCR testing twice weekly while inpatients and once weekly while outpatients from the time of transplantation until day +100. Antifungal therapy with LAB was administered in those cases with two consecutively positive PCR results. In those assigned to the fever-driven strategy LAB was administered after five days of persistent fevers despite broad-spectrum antibiotics. The primary endpoint was incidence of IFI and an important secondary endpoint was survival. Eleven patients in the PCR-based strategy were diagnosed with IFI compared with 16 in the standard EAFT-based arm ($p > 0.05$). Interestingly, more patients in the PCR-based strategy were treated with LAB (109 vs. 76; $p < 0.05$) and this may be a result of the poor specificity of the PCR assay. Although survival until day 30 post-transplantation for those patients assigned to the PCR-based strategy was significantly greater than in the standard EAFT-based arm (four deaths vs. 13 deaths; $P = 0.03$ respectively) overall mortality rates at day +100 and day +180 were no different between the two arms. The study was underpowered as it was designed to detect a 66% relative reduction in the incidence of IFI. In addition the follow-up to day +100 may not have been long enough to assess the impact of the PCR-based strategy. Thus definitive conclusions regarding the utility of screening with the PCR assay to guide pre-emptive therapy cannot be made and further evaluation is required.

Conclusions

Prophylaxis and EAFT have decreased the incidence of IFI and IFI-related mortality and will remain as useful strategies for the management of IA in select patient groups. Pre-emptive strategies for IA are feasible and studies performed to date suggest that these strategies have the potential to impact on patient outcomes. However, carefully designed RCT which compare a PCR/GM-ELISA/HRCT-directed pre-emptive treatment strategy to the conventional diagnostic strategy for IA (i.e. fever-driven strategy) need to be performed in order to determine which strategy results in better patient outcomes. Such a multicentre RCT is currently underway in eight Australian transplant centres by the Australian Mycoses Interest Group and Australasian Leukaemia Lymphoma Group [129]. In addition European centres have started similar RCTs. These RCTs will provide valuable data regarding the practicality, sustainability and the resource implications (i.e. monetary and labour) for diagnostic laboratories of twice-weekly testing and will address unanswered questions regarding the use of the non-culture based diagnostic tests such as: (i) the true sensitivity of the assays and HRCT scanning as these will be tested in a much larger population than previously; (ii) the ability of the assays and HRCT scanning to detect IA early and the time to positivity relative to conventional diagnostic tests, as all diagnostic tests will be performed at protocol-prescribed time-points; (iii) which of these tests should be used in combination for the diagnosis of IA; (iv) whether different combinations of assays and HRCT scans should be used in different patient groups (e.g. neutropenic patients vs. non-neutropenic patients post allogeneic HSCT with GVHD); (v) whether the recommended duration of antifungal therapy will change with a pre-emptive strategy; (vi) interpretation of single

and intermittently positive PCR results; (vii) the kinetics and mechanisms of *Aspergillus* DNAaemia and (viii) what changes will occur in the natural history of IA and other IFIs as a result of this strategy.

In conclusion rigorous evaluation of the pre-emptive approach is needed in order to determine its impact on important clinical outcomes such as the use of EAFT, IA-related mortality rates, length of hospital stay, antifungal drug toxicity rates, the number of invasive procedures performed to diagnose IA and the changes in the types and antifungal susceptibility profile of IFI that occur with this approach over time. It is crucial to determine if the monetary and labour investments required for this approach translate into better therapeutic strategies for IA and improved allocation of health-care resources. Only if the PCR/GM-ELISA/HRCT-directed pre-emptive treatment strategy results in comparatively better patient outcomes and is more cost-effective can we use this strategy in routine clinical practice to manage IA in high-risk haematology patients. We await the results of these RCTs with interest.

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