LEADING ARTICLE

PCR-Based Methods with Aspergillosis as a Model

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According to the definitions of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC-MSG), invasive fungal diseases (IFD) are classified as proven, probable, and possible IFD. A recent revision of the EORTC-MSG definitions has incorporated (1→3)-β-D-glucan diagnostic tests for mycological evidence of IFD; however, polymerase chain reaction (PCR)-based diagnostic techniques are presently absent from the guidelines owing to a lack of standardized methodology. This is in spite of the huge potential of PCR and the fact that such tests have been in use for >15 years. In this review, the current issues associated with PCR-based diagnostic tests – specifically for *Aspergillus* – including the choice of samples, probes, and platforms, are discussed. Furthermore, the "European *Aspergillus* PCR Initiative" (EAPCRI), which was set up by the present authors, is described. This initiative aims to establish a standard for *Aspergillus* PCR that is validated as a screening tool. The Laboratory Working Party of the EAPCRI hopes to complete its work and to propose a standard for *Aspergillus* DNA extraction and PCR assays by the end of 2008. *J Invasive Fungal Infect* 2008;**2**(2):46–51.

State of the art

With a mortality rate >90%, invasive aspergillosis (IA) remains a significant cause of death of immunocompromised patients, particularly in those who have received a hematopoietic stem-cell or solid organ transplantation, and in patients who have been treated for hematological malignancies [1]. The successful treatment of IA in these individuals depends not only upon reversing the compromised immunity, for example by reducing the dose of or stopping immunosuppressive drugs, but also on the early diagnosis and initiation of effective antifungal therapy. Invasive fungal diseases (IFD) are classified as proven, probable, and possible IFD, according to the definitions of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) [2]. Briefly, proven IFD requires demonstration of fungi in tissue or sterile material obtained from an affected organ, whereas probable and possible IFD are defined according to the presence of three elements, namely host factors, clinical features, and mycological evidence. The original definitions have undergone revision to allow groups of patients who were initially omitted in the first set of definitions to be included (solid organ transplant patients and those with chronic granulomatous disease [CGD]). Other changes include refining the clinical features and the addition of (1→3)-β-D-glucan tests for mycological evidence. However, polymerase chain reaction (PCR)-based diagnostic techniques were excluded as no standard or validated method was available; this is despite the huge potential of PCR and the fact that the methodology has been in use for >15 years. The lack of standardization is partly attributable to the multitude of molecular targets, variety of specimens, variations in extraction protocols, and the different PCR platforms used. This lack of standardized methodology was discussed in a recent systematic review by Tuon [3].

However, EORTC/MSG consensus definitions are intended to provide diagnostic stringency for clinical trials and the exclusion of PCR from the consensus does not imply that PCR is not being employed as a diagnostic tool for IA. Many PCR-based techniques have been (and continue to be) used to diagnose IA, but no single method has provided clinicians with the rapid, precise, and inexpensive diagnostic

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information they require. Efficiency and reliability may have been provided by PCR-enzyme-linked oligosorbent assay (PCR-ELISA) [4,5], but the protracted time required to obtain results (approximately 2 days), as well as the prohibitive costs associated with the procedure, have discouraged its use. The development of quantitative PCR and intercalating agents, such as SYBR green and more recently LC Green, may offer a cheaper and faster approach, although it is generally admitted that intercalating agents do not provide the sensitivity nor the specificity achieved with molecular probes [6]. The present authors would like to argue, however, that this may be due to methodological problems or the poor choice of primers as SYBR green was found to perform equally well as TaqMan technology when appropriately used [7]. Several groups are working on new protocols that make use of more recent technologies such as Molecular Beacon (developed by the Public Health Research Institute, Newark, NJ, USA) and Scorpion (developed by DxS Ltd, Manchester, UK), but the authors are currently not aware of any publication reporting the use of these techniques to diagnose IA. It would also appear, from the literature, that the scientists involved in the development of a PCR-based diagnostic for IA prefer a combination of primers and probes.

What are the current issues of *Aspergillus*-PCR-based detection?

A limited number of preliminary standardization trials have been performed (see section Standardization of *Aspergillus* PCR detection – the "European *Aspergillus* PCR Initiative" below), although several questions remain unanswered. The first factor is the specimen. The UK–Irish fungal PCR consensus group recommended the use of 5 mL of whole blood collected in ethylene diamine tetraacetic acid (EDTA), whereas others use different specimens such as bronchoalveolar lavage (BAL) [8], cerebrospinal fluid (CSF) [9], or tissue biopsy [10]. However, obtaining such samples requires invasive procedures that are seldom suited to critically ill patients. Although BAL has the advantage of allowing investigation of the primary site of infection, the procedure is prone to contamination as spores of *Aspergillus* may be present in the upper airways making it difficult, if not impossible, to discriminate between the presence of extraneous conidia and true tissue invasion [3]. In contrast, it is relatively simple and straightforward to obtain a blood sample, leading to this becoming the sample of choice [11].

Nevertheless, no consensus has been reached regarding whether a cellular component, or plasma or serum, should be used for PCR [12]. The method for extracting DNA is also a matter for debate. Indeed, extracting nucleic acids from the serum or plasma (where they are free in solution) involves rapid and less complex protocols, whereas extracting nucleic acids from the cellular phase generally requires long and complex protocols with either a mechanical disruption step such as bead-beating [13], or an enzymatic step such as the use of recombinant lyticase [14].

The third key element to be agreed upon is the molecular target. DNA rather than RNA is generally considered to be the target of choice owing to its relative stability and ease of extraction [12]. Several genes of the *Aspergillus fumigatus* genome, such as the ribosomal operon, are present in multiple copies. Targeting such genes affords the advantage of increasing the chances that amplification will yield DNA at detectable levels. Other genes such as those of the mitochondria are not true multi-copy genes, but may in fact be considered as such since it has been estimated that there are 12 copies of the mitochondrial genome to every one copy of the *Aspergillus* genome [15].

It is also important to consider the environmental nature of *Aspergillus* as its hyphae and spores are ubiquitous in the environment posing a challenge to scientists at every level [16]. Indeed, the risk of encountering the fungus in the atmosphere justifies the use of class 2 laminar flow cabinets equipped with high-efficiency particulate air filters, and filter sterilization of all solutions used in these assays.

Which PCR methods have been tested and how do they perform?

Most recently published studies have made use of quantitative PCR – in particular the LightCycler (Roche Applied Science, Indianapolis, IN, USA) – platforms. One of these studies was published by White et al. who used a nested PCR approach with primers targeting the 28S region of the ribosomal DNA operon to investigate a group of 203 at-risk patients over a 1-year period [17,18]. The authors reported a good correlation between the PCR and galactomannan (GM) test results, as well as good sensitivity (92.3%) and specificity (94.6%), with a limit of detection of one input copy (five were required for complete reproducibility). The nested PCR approach is susceptible to cross-contamination between samples, but White and colleagues, by focusing on the negative predictive value (NPV) instead of the positive predictive value (PPV) and by reprocessing any positive sample, managed to overcome this problem. However, this approach only allows the diagnosis to be excluded and the nested step renders any precise quantification of the infective agent impossible. Nevertheless, this criticism would be valid for most of the recent studies that make use of real-time PCR for the diagnosis of IA. In fact, few studies report the amount of DNA found in the clinical specimen processed, rather they simply use real-time PCR as an endpoint, with the samples being described as either positive (crossing the fluorescence threshold before a certain number of cycles) or negative (not different from the negative

controls). Indeed, most of these techniques are based on the NPV rather than PPV [19–21], semi-quantitative (nested) analysis [22,23], or a combination of both [17].

Using the NPV is justified when the prevalence is low and the infective agent is found in the environment, leading to a high risk of false-positive test results [24]. The present authors would like to suggest that real-time PCR could be used quantitatively to enable closer monitoring of the outcome of the IFD to therapy, provided that the PPV of the assay is the focus and the assay affords a very high specificity and sensitivity. Appropriate controls would need to be added to the assay to achieve this level of confidence. Controls for DNA extraction – both positive and negative – are essential each time a group of specimens is processed [25]. The positive extraction control ensures extraction efficiency is maintained and would consist of the same type of specimen "spiked" with a known amount of positive material (conidia, fungal cells, or fungal DNA, depending on the target of the assay). The negative control ensures the absence of contamination. The introduction of additional PCR controls provides evidence of consistency of inter-assay PCR performance. Controls should be included to detect the presence of PCR inhibitors. This is achieved through spiking clinical specimens with an alternative nucleic acid target (plasmid) that is amplified under the same conditions but does not interfere with assay specificity. To date, many studies undertaken have not included all of these stringent controls. For example, spiking samples with an internal control plasmid post-extraction will only monitor for PCR inhibition and any losses downstream of this stage of specimen processing [12]. This may fail to provide a reliable estimate of the total efficiency of the nucleic acid extraction, and could be inadequate if assays were used in a quantitative rather than qualitative fashion.

Aspergillus-PCR – for primary screening or to confirm aspergillosis?

Different management strategies of preventing and treating high-risk patients for IA exist [23]:

- Prophylaxis.
- Empirical antifungal treatment.
- Pre-emptive or diagnostic-driven antifungal treatment.
- Therapy of existing IA.

Besides the use of PCR to diagnose IA as the cause of disease, this highly sensitive technology can be also employed to monitor patients who are at risk of developing IA – a strategy successfully used to monitor opportunistic viral infections in allogeneic hematopoietic stem cell transplant (HSCT) recipients. The aim of this approach is to clearly define a negative test so that therapy can be withheld (or if positive, to allow the initiation of early therapy). The highest possible sensitivity and NPVs are a prerequisite to minimize false-negative results.

Quantitative real-time PCR assays might be useful to monitor antifungal therapy by quantifying the fungal load in blood or tissue specimens. Analogous to the monitoring of antiviral therapies, a reduction of the fungal load or negative PCR results over time should indicate a favorable response, whereas persistence or increase of fungal DNA would be correlated with an unfavorable outcome [26]. Furthermore, these PCR tests may precede other signs of IFD (such as radiological abnormalities) by days or even weeks.

However, there are reports of false-negative results during antifungal therapy, even when there is clear progression of disease [27]. This might be due to the rapid disappearance of fungal DNA from the blood, albeit intermittently. Loeffler and colleagues found that the fungal load in whole blood specimens is very low, usually around 10 colony-forming units (CFU)/mL, with a maximum of several hundred CFU/mL, even in patients with proven IA with a fatal outcome [28]. This underlines the need for PCR assays with extremely high sensitivities.

Other issues to be addressed include the simple fact that the frequency of sampling required for screening has yet to be established, as does the number of positive PCR assay results required for initiation of antifungal therapy. Most authors suggest obtaining at least two blood samples per week during the risk period. However, this might prove inadequate, as the circulation of fungal DNA in blood is not continuous and the amount is often close to the lower detection limit of the assays [29]. This could explain the phenomenon of inconsistent positive results. Moreover, the significance of a single positive PCR result is unclear, particularly as contamination or transient presence of fungal DNA in the clinical specimen cannot be excluded. Consequently, there is a consensus that two consecutive positive PCR results are required to initiate antifungal therapy [19,30–32].

In early intervention strategies for treating IFD, the trigger for starting therapy may be a known host factor such as profound neutropenia or prolonged exposure to corticosteroids (in those for whom radiological signs of IFD are already apparent). Others rely on the detection of laboratory markers and some rely on both for instigating such strategies. Irrespective of the approach, the major goal of pre-emptive therapy is to provide early targeted therapy to improve the outcome while reducing toxicities, attenuating the risk of drug interactions, and lowering costs. With this in mind, it is clear that sensitive, reliable, and standardized tests are essential. Many centers still opt for

empirical therapy for patients who are ill and for whom IFD has not yet been excluded and the source of infection is unknown [33]. The primary aim of empirical therapy is to achieve the best outcome by preventing IFD altogether, or treating incipient disease early enough to increase the chance of resolution of the IFD. However, there are concerns that too many patients are being exposed unnecessarily to antifungal drugs leading to higher-than-desirable costs, toxicities, and drug interactions. Furthermore, this approach makes little use of the laboratory markers that are now available.

In a recent study, Maertens et al. explored an approach that relied on GM testing and high-resolution thoracic computed tomography scanning for assessing the feasibility of pre-emptive antifungal therapy, initially analyzing 4170 serum samples by GM assay [34]. In total, 41 of 136 (35%) treatment episodes qualified for empirical antifungal therapy, whereas nine of 136 (6.6%) would have been classed as pre-emptive. Pre-emptive therapy was also started for 10 episodes, not because of fever, but rather because of a positive GM enzyme-linked immunosorbent assay result. This approach led to less than half of the patients needing therapy with no increase in mortality rate; thus, Maertens et al. concluded that a strategy based on the detection of GM, pulmonary complications, or persistent unexplained fever could form the basis of a pre-emptive treatment strategy. If successful, this would reduce exposure to expensive and potentially toxic drugs while allowing cases of IA to be identified. However, it should be noted that the approach failed to detect IFDs due to other moulds [34].

In a separate study, empirical and pre-emptive therapy (based on a variety of clinical signs and symptoms and detection of GM antigen) were compared in 293 patients with hematological malignancies (mainly acute myeloid leukemia) [35]. Patients were screened twice weekly for the presence of GM antigen. The authors concluded that although significantly more IFD were observed in group managed by the pre-emptive strategy ($p<0.02$) the fungal infection-related mortality rate was similar with the two treatment approaches [35].

The group from the University of Würzburg (Würzburg, Germany) has compared the efficacy and safety of PCR-based versus empirical liposomal amphotericin B treatment after allogeneic SCT [36,37]. Recipients of allogeneic bone marrow or peripheral blood SCT were randomized to pre-emptive therapy that would be started after a single positive PCR result or after 5 days of febrile neutropenia refractory to broad-spectrum antibacterial therapy (group A; n=198), or to receive antifungal therapy empirically for fever persisting for >5 days (group B; n=211). Therapy consisted of 3 mg/kg per day liposomal amphotericin B, which was lowered to 1 mg/kg for patients who were stable on day 4 of therapy. Rigorous PCR monitoring until day 30 after allogeneic SCT led to a reduction in early mortality and a trend towards a lower rate of early IFD, but the survival rate between groups was not different at day 100. Hence, the impact of close PCR monitoring and PCR-based therapy beyond day 30 should be studied in future trials [37]. Finally, to further confirm pulmonary aspergillosis, bronchoalveolar lavages can be useful; Tuon recently published an extended review on this topic [3].

Standardization of *Aspergillus* PCR detection – the "European *Aspergillus* PCR Initiative"

The amplification of *Aspergillus* DNA by PCR has been described since the early 1990s, and many studies on the topic have been published. However, as mentioned earlier, protocols vary widely in terms of the selection of appropriate clinical specimens (whole blood samples, serum, or BAL), the DNA extraction procedure (mechanical or enzymatic lysis procedures), and the selection of the adequate target sequences, including primers, probes, and PCR systems. Furthermore, there are only a few standardized assays that are commercially available, such as SeptiFast (Roche Diagnostics, Basel, Switzerland), MycXtra (Myconostica, Manchester, UK), and the Affigene *Aspergillus* tracer (Cepheid, Sunnyvale, CA, USA).

The variety of different DNA extraction and PCR protocols, their variable inter-laboratory reproduction, and the lack of standardization of the techniques led to attempts to define a consensus for PCR-based detection of *Aspergillus*. White et al. described the first multicenter study of PCR methods for the detection of *Aspergillus* species, used in the UK and Ireland by distribution and analysis of multiple specimen control panels [38]. The group consisted of 10 different laboratories. They reported the comparison between two primer sets (28S and 18S) and three platforms (LightCycler [Roche], Rotor-Gene [Corbett Research, Mortlake, NSW, Australia], and TaqMan [Applied Biosystems, Foster City, CA, USA]), on a panel consisting of eight positive samples (10–5000 conidia per mL) and eight negative samples [34]. In this study, the 28S assay was found to be more specific than the 18S assay. This was mainly due to the fact that the set of primer/probe used to amplify/detect the 18S amplicon was found to amplify and detect a portion of the human rDNA gene in the absence of DNA from *A fumigatus*. This phenomenon was particularly noticeable with the LightCycler and was responsible for a decrease in sensitivity. Overall, the sensitivity, specificity, NPV, and PPV were higher with the 28S primer set than with the 18S primer set, regardless of the platform used. The type of platform used was also found to have a major

influence on the assay; sensitivity and NPV were 100% on the TaqMan machine, whereas specificity and PPV were at 100% using the Rotor-Gene system [38].

An Austrian–German consensus collaboration involving six laboratories has also been founded to evaluate DNA extraction and PCR methods currently in use and under research in laboratories in Austria (Vienna, Graz, and Innsbruck) and Germany (Tübingen and Würzburg) for the detection of *Aspergillus* spp. It would appear that real-time PCR-based assays in combination with effective lysis of the fungal cell wall show high inter-laboratory reproducibility (personal communication). Nonetheless, the consensus group for defining IFD had to conclude reluctantly that there had been too little progress in establishing a validated, standardized PCR assay to allow inclusion of the technique in the revised definitions of IFD [39].

Recently, a Working Group of the International Society for Human and Animal Mycology (ISHAM) has been formed with the title "Towards a standard for *Aspergillus* PCR". Besides the chairman (JPD), there is a Laboratory Working Party (chaired by JL) and a Clinical Working Party (chaired by RB) and collaboratively they have set up a foundation called the "European *Aspergillus* PCR Initiative" (EAPCRI) to help raise funds for its work. Its goal is to establish a standard for *Aspergillus* PCR that is validated as a screening tool.

Since the foundation of the Working Party, four panels of extracted DNA and spiked blood samples have been distributed to the 24 participating laboratories. Preliminary results show that the extraction of DNA appears the major obstacle rather than the various PCR techniques, which performed consistently. The group could demonstrate that mechanical lysis of *A fumigatus* conidia is superior to enzymatic lysis, and that a blood volume of ≥3 mL offers a better yield than 0.2–1 mL blood; furthermore, real-time PCR assays, independent of the type of assay, are superior to conventional PCR assays. The Laboratory Working Party of the EAPCRI hopes to have completed its work and propose a standard for *Aspergillus* DNA extraction and PCR assays by the end of 2008. Work is also in progress to design and undertake a clinical validation study.

Conclusion

Establishing the utility of *Aspergillus* PCR, at least for screening purposes, has arguably never been as close as at the present time. Failure to achieve standardization of *Aspergillus* PCR may well consign the technique to the history's archives, whereas success may prove the tipping point whereby PCR assays will establish its place not just for *Aspergillus* assessment but also for other etiological agents of invasive fungal diseases.

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Disclosures

The authors have no relevant financial interests to disclose.

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