Medical Mycology September 2006, **44**, S191–S198

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Aspergillus PCR – Platforms, strengths and weaknesses

P. LEWIS WHITE* & ROSEMARY A. BARNES†
**NPHS Cardiff, and †Department of Medical Microbiology, University Hospital of Wales, Cardiff, UK*

PCR is a useful tool to aid in the diagnosis of invasive aspergillosis. However, it is essential that an optimal method be agreed to allow inclusion in future consensus diagnosis criteria. It should be used in conjunction with other methods (e.g., galatomannan (GM) ELISA and high resolution computed tomography (HRCT)) to enhance the opportunity for detection of this devastating infection. This manuscript will try to highlight the benefits but mainly the limitations occurring throughout the process of molecular testing. It will focus on real-time methods although many of the points will be relevant to block-based amplification.

Keywords Sample type, DNA extraction, PCR amplification, result interpretation

Introduction

Current methods for early diagnosis of invasive aspergillosis (IA) are limited. With an increasing 'at risk' population the incidence of IA will continue to rise, but will *ante-mortem* diagnostic procedures be able to detect the infection or will definitive diagnosis still remain poor? The European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) criteria for diagnosing invasive fungal infections [1] (currently being updated) have gone someway to improve confidence but can only use current methods that have had extensive approval.

The publication of molecular methods to aid in the diagnosis of IA is widespread although the performance of these methods varies greatly [2]. The main reason behind this diversity is the lack of an agreed consensus method, with many groups developing individual assays. Whilst this creates variety and improves understanding it is time for multi-centre evaluation of molecular procedures to standardize methodology [3,4].

The lack of an agreed method has resulted in the use of a range of specimens [2,5–7] extraction methods [8–11] and PCR assays [2,7,12–14], all of which can impact on the performance of the test [2,4,15]. The limitations of molecular methods are often overlooked in publications as most focus on the positive features of an assay. However, every assay will have many limiting factors, some of which can be improved upon but all should be brought to the user's attention.

Correspondence: P. Lewis White, Department of Medical Microbiology and NPHS Cardiff, University Hospital of Wales, Heath Park, Cardiff, CF14 4XN, UK. Tel: +44 [0]29 2074 6581; Fax: 029 2074 2161; E-mail: lewis.white@nphs.wales.nhs.uk

The benefits of real-time PCR

The limitations of conventional techniques have led to the development of PCR methods with high sensitivities and prompt results. Earlier techniques utilized block-based amplification with assay specificity and enhanced sensitivity derived by post-amplification techniques such as sequencing [16], single strand confirmation polymorphisms (SSCP) [17], or Southern hybridization [18]. Other techniques avoided time-consuming post-amplification techniques and obtained specificity by using *Aspergillus* specific primers, although to increase sensitivity a nested PCR was necessary [19,20]. With the development of real-time PCR the need for these techniques in clinical diagnostics has diminished and with it the opportunity for post-amplification cross-contamination and laboratory contamination with amplicons.

Compared to classical microbiological culture techniques early PCR methods represented a rapid result. In the current real-time PCR climate these methods are now considered slow with real-time results generated within 2 h, although when combined with an extraction procedure, same-day results are the norm. Real-time results can be generated in a variety of ways, the simplest being the use of the fluorescent dye Sybr

DOI: 10.1080/13693780600898003

Green that binds to the minor groove in double stranded DNA. Used with specific primers and melt curve analysis the specificity of the amplicon can be identified but non-specific amplification and primer dimers may clutter melt-curve analysis and quantification. As specificity is essential in a diagnostic setting TaqMan (hydrolysis) or hybridization probes are used to provide amplicon identification. Both allow accurate copy number quantification but only hybridization probes can be used for melt curve analysis that will be necessary if genus/species level identification is a requirement [13]. In addition hybridization probes have the potential to detect single nucleotide polymorphisms that may represent antifungal resistance within an organism and will have implications in the treatment of patients [21].

PCR methods also have a role in histological investigations where the discovery of hyphal elements only proves a case of invasive filamentous fungal infection, as the morphological characteristics typical for *Aspergillus* sp. (slender, septate hyphal elements, with a branching angle of 45°), are also characteristic of other filamentous fungi (e.g., *Fusarium* spp.). Identification of the organism can be deduced by molecular methods [22–24].

The development of real-time PCR platforms has enhanced the quality of diagnostic PCR assays. Reduced contamination rates and the ability to rapidly quantify microbial loads and identify organisms improves their value in clinical settings. With the new generation of real-time platforms permitting the use of larger volumes and multiplexed assays the benefits are evident, although the design of assays may become complex and the use of multiple oligonucleotides may affect the kinetics of the reaction altering its performance.

Use of Nucleic Acid Sequence-Based Amplification (NASBA) has been applied to the detection and monitoring of viral infections. Of the few publications used to detect fungal infections most have focused on *Candida*. The technique utilizes avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H and T7 RNA polymerase for the isothermal amplification of RNA. Amplification can be determined by enhanced chemiluminescence (ECL, or 'endpoint') or in real-time by the use of molecular beacons. Like all molecular techniques NASBA has a high sensitivity and specificity. Performance was comparable to an established real-time PCR assay for the detection of *Aspergillus* [25]. This coupled with an amplification process that is more efficient than PCR and the requirement for less starting material makes NASBA a viable alternative [25]. However, the use of a RNA protection buffer and/or RNase inhibitor is essential to minimise RNA degradation.

Alternative non-culture methods

Alternative non-culture laboratory-based approaches to aid in the diagnosis of IA have focused on the detection of fungal antigens, in particular galactomannan (GM) and β-D-Glucan (BDG). The basis of both these methods has been described previously and this report will comment on their comparisons with PCR where assays have been directly compared using the same study population. A summary of performances of these methods is shown in Table 1. A range of specimen types have been used but while the GM and BDG methodologies are consistent, a variety of PCR protocols are utilised, highlighting the necessity for a consensus PCR method.

When testing bronchoalveolar lavage (BAL) both PCR and GM ELISA perform to similar standards (Table 1). Using serum/plasma the GM ELISA generated better sensitivities than PCR in four out of five publications (Improvement range: 25–45%, Mean: 31.3%) with little or no effect on specificity [26–29]. The one report where PCR was optimal utilised a nested PCR and the GM ELISA performance may have been improved by using a lower positivity index (0.7) [30]. The use of a nested PCR was also reported to be superior to GM ELISA when testing serum by Williamson *et al*. [20]. The use of serum allows easy sample processing and a single specimen to be used for GM ELISA and PCR. It has been extensively assessed for the GM ELISA and can be considered as an optimal specimen type for this method. However, this may not be the case for PCR (see Limiting factors – specimen type).

When whole blood is used for PCR and serum for GM ELISA, four out of five publications reported better sensitivities for PCR than GM ELISA (Improvement range: 21–40%, Mean: 29.8%) with only one suffering a lose in specificity [31–35]. Furthermore, using 4–5 ml of whole blood, Jordanides *et al*. reported that in 12 cases of probable/possible IA, PCR detected 10 cases whereas the GM ELISA was consistently negative [13]. The one report where GM ELISA was superior in an animal model only used 0.5 ml of whole blood [34].

Four of the five PCR methods with improved sensitivity over GM ELISA involved post-amplification handling (2nd round of PCR or ELISA detection), indicating that a single round amplification process may not provide sufficient sensitivity even when detection is determined by probe hybridization. In addition,

Table 1 Comparison of laboratory-based non-culture methods for the detection of invasive aspergillosis.

Patients (no.)	Sample type	Sample volume[a]	Assays performed	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Ref.
44	BAL	1.5 ml	Real-time PCR	90	100	100	92	58
			Nested PCR	90	100	100	92	
			GM ELISA I = 1.5	100	100	100	100	
100	BAL	1.5 ml	Real-time PCR	67	100	100	76	7
			GM ELISA I = 1.0	61	98	97	72	
			GM ELISA I = 0.5	76	94	93	80	
83	Serum	0.1 ml	Nested PCR	89	100	100	89	30
			GM ELISA I = 1.5	57	100	100	67	
			GM LA	30	100	100	56	
96	Serum[b]	0.2 ml	Real-time PCR	55	93	40	96	26
	Plasma[c]		GM ELISA I = 0.6	100	93	55	100	
			BDG	55	93	40	93	
122	Serum[b]	0.2 ml	Real-time PCR	79	92	79	92	31
	WB/Plasma[d]		GM ELISA I = 1.5	58	97	86	86	
			BDG	67	84	61	87	
42[e]	WB[f]	1.0 ml	Real-time PCR	15	100	100	16	32
	Serum[b]		PCR ELISA	62	100	100	28	
			GM ELISA I = 1.5	35	100	100	19	
25	WB[f]	1.0 ml	PCR ELISA	100	85	63	100	33
	Serum[b]		GM ELISA I = 1.5	60	95	75	80	
41	Serum	0.2 ml	PCR/Probe	55	100	100	66	27
			GM ELISA[g]	82	95	95	82	
50	Serum	0.5 ml	Real-time PCR	55[h]	100[h]	100[h]	77[h]	28
			GM ELISA I = 1.5	80[h]	100[h]	100[h]	88[h]	
29	Serum	0.2 ml	Real-time PCR	50	64	69	44	29
			GM ELISA I = 1.5	78	100	100	73	
60[e]	WB[f]	0.5 ml	PCR/Probe	41[i]	97[i]	92[i]	64[i]	34
	Serum[b]		GM ELISA[g]	100[i]	100[i]	100[i]	100[i]	
165	WB[f]	5–7 ml	Nested PCR	64	64	16	94	35
	Serum[b]		GM ELISA I = 0.7	33	99	75	94	
24[e]	Serum	0.1 ml	Nested PCR	68	100	100	22	59
			GM LA	64	100	100	20	
			BDG	62	100	100	20	

Key: BAL, Bronchoalveolar lavage; GM, Galactomannan; I, Positivity index; LA, Latex Agglutination; BDG, β-D Glucan; WB, EDTA whole blood.
[a]Refers to volume used for DNA extraction.
[b]Serum used for GM ELISA.
[c]Plasma used for PCR and β-D-glucan assay.
[d]Plasma or whole blood used for DNA extraction. Plasma used for β-D-glucan assay.
[e]Animal Model used.
[f]EDTA whole blood used for DNA extraction.
[g]Positives samples had a GM concentration $\geq$1 ng/ml (Corresponding I = 1.0).
[h]Performance statistics calculated using multiple positive results.
[i]Performance statistics calculated using day 7 results.

comparison of real-time PCR, PCR-ELISA and GM ELISA in an animal model showed PCR-ELISA to be optimal and real-time PCR to be the worst [32].

Direct comparison of BDG with PCR and GM ELISA has shown it to perform adequately in the clinical scenario [26,31]. In testing 122 patients (33 definite IPA) PCR was the optimal assay although BDG was more sensitive but less specific than GM ELISA [31]. However, a follow-up study on 96 patients (11 proven/probable IA) using the same methods reported that the GM ELISA was the optimal assay [26]. The improvement in GM ELISA performance can be explained by a reduction in the GM ELISA positivity index from 1.5 to 0.6 for the second study. Whereas the reduction in sensitivity for PCR and BDG is possibly a result of the cut-offs used to maintain comparable high specificities between the assays. Using lower cut-offs for PCR and BDG increased sensitivity to 91% and 82%, respectively, albeit at the expense of specificity and PPV. PPV was particularly poor due to the low number of proven/probable cases.

In setting cut-offs it is important to decide what is required from a diagnostic assay. Is it essential to rule-in or exclude an infection and in screening at-risk patients it may be necessary to perform more than one test to achieve both.

The limitations of molecular methods

As highlighted previously the performance of any PCR assay is limited by each step of the molecular method [4,15,16]. This manuscript will try to expand on previous publications by commenting on the factors that are limiting to each individual step of the process but also on how they impact steps downstream.

Limiting factors – specimen type

When choosing a sample various factors come in to consideration and the correct choice is essential as an inappropriate sample may cause false results. Primarily the patient's condition controls sample choice. A clinician is unlikely to perform invasive procedures on critically ill patients so obtaining BAL or CSF specimens, particularly on more than one occasion, is usually difficult even if it is 'your preferred specimen'. In clinical practice obtaining blood specimens is easy and allows routine screening to be performed. This is important, as intensive testing is paramount for optimal detection with some PCR methods detecting *Aspergillus* DNA in only 11–12% of patients with proven/probable IA [35,36]. Low positivity rates may represent the transient nature of the circulating target. Frequent large volume ($\geq$4 ml) sampling may alleviate this as relationships between sample size and positive PCR result have been noted [37].

Frequent testing of patients may also limit the impact of contamination and inhibition. *Aspergillus* contamination and false positive PCR results with BAL and sputum samples are apparent due to inhalation of airborne spores or colonization of the lung [38–40] whereas contamination of blood is less evident. However, *Aspergillus* contamination of vacutainers used to take blood specimens has been seen [41]. A small study by Williamson showed that a high proportion of citrate vacutainers and to a lesser extent EDTA vacutainers were contaminated with *Aspergillus*, whereas plain vacutainers were not affected [41]. The size of study limits its impact and highlights the need for an extensive study before the recommendation of a sample type. These contaminations represent clinical false positive results, as the contamination has not entered during the molecular process, nevertheless in a diagnostic service any false positive result is significant.

Inhibition of PCR due to the presence of haemoglobin as led to thorough extraction methods to remove the inhibitory blood component. Other research has shown that anticoagulants may be inhibitory sources that can effect *Aspergillus* PCR methods [42]. Using DNA spiked in plasma (0.15 mg/ml) and a previously published nested PCR [43] (by design highly sensitive and hampered by false positive results). Garcia *et al*. reported that use of heparin and citrate inhibited PCR whereas EDTA did not. In our experience PCR inhibition has been limited to patients receiving heparin after heart by-pass surgery. The use of an internal control PCR avoids the reporting of false negative results and is essential in a diagnostic assay [15].

The final factor determining specimen selection is the fungal element targeted. The source of DNA is unlikely to be viable circulating fungal fragments. These should be detected by lytic blood culture methods [44,45] and 1–10 cfu of *Aspergillus* conidia inoculated into blood culture bottles can be detected within 24 h [46]. In the clinical scenario this rarely occurs. An alternative source of DNA is non-viable fungal fragments. Whether these are phagocytosed is not clear but is an important consideration when testing neutopenic patients. As part of the innate immunity macrophages are thought to be mainly responsible for ingestion of conidia, whereas neutrophils act predominantly but not exclusively on hyphae [39]. As such, it could be argued that amount of phagocytosed fungal fragments within a neutropenic patient during infection would be low. Alternatively circulating fungal fragments may suffer cell wall damage due to platelet attachment [39], possibly introducing a non-phagocytosed, non-viable fungal source. Targeting circulating fungal fragments involves the processing of EDTA whole blood specimens and labour intensive procedures [9,18]. If circulating DNA is targeted then serum and simpler extraction methods can be utilised [43,47].

Publications reporting the successful use of serum or EDTA whole blood are widely available.

We believe that non-viable and cell associated fungal fragments may be a major contributor to the DNA source. As previously reported automated extractions using whole blood inoculated with known quantities of free *A. fumigatus* DNA were unsuccessful [14], although results for this method correlated well with proven/probable cases of IA, indicating an alternative DNA source [14]. However, excluding free DNA as a source would be unwise as others have reported detectable DNA levels in both serum and plasma [28]. The choice of EDTA whole blood specimens targets both circulating free DNA and non-viable fungal fragments (phagocytosed or not) minimizing variation

[3] and is recommended. The development of automated extraction procedures has reduced demanding labour time and the human error involved [11].

Limiting factors – extraction method

As mentioned above the specimen type directly influences the efficiency and technicality of the extraction method. Sample volume and type will limit the available target and may complicate the procedure. The efficiency may also be directly affected by the complexity of the method. Technically demanding systems with more steps introduce greater opportunity for DNA loss and will imply greater demands on the experience of the worker. In addition methods targeting conidia or hyphae will need to lyse the cell wall. The use of zymolase has been abandoned due to its contamination with fungal DNA [48,49] and replaced, at great expense, with recombinant lyticase [50,51]. Mechanical disruption by bead-beating has provided a cheaper non-enzymatic alternative without effecting detection limits [8,10,11,14].

Recently the use of automated procedures has been described [11,14,28]. These reduce work burden, minimize crossover contamination and allow improved interlaboratory comparison when compared to manual methods [15]. However, they require the purchase of relatively expensive equipment.

The importance of an efficient extraction procedure is exemplified by the perceived DNA losses that occur during extraction. In a clinical scenario the fungal load in blood may be equivalent to <1conidia/ml therefore in a typical positive sample (4 ml) there may be <4 conidia. By utilizing the multicopy (10^2 copies/organism) rRNA operon for PCR amplification the available target is greatly improved. However, DNA extracted and quantified as described previously [14] revealed losses of at least 10^2 copies during extraction (L. White, unpublished data, Table 2). This would have implications if a single or low copy number gene had been used for PCR. Additional research using alternative automated extractors demonstrated the efficiency of extraction could be improved 10–100 fold (L. White, unpublished data) and that development of new and/or improved technologies is essential and beneficial.

Limiting factors – PCR amplification

Optimal PCR amplification relies on high quality purified DNA that is dependent on the extraction procedure (see above, 8–10). The extraction, although not effected by inhibitors, is responsible for their removal and failure to do this will impact on PCR amplification.

Oligonucleotide design is also paramount. The targeting of the rRNA operon, particularly the 18S rRNA gene is common practice and designing primers to be pan-fungal allows the assay to be modified for additional fungi by using specific probes. The completion of the Human Genome Project has made the human 18S rRNA gene available for sequence comparison and large sections of the gene are highly similar to regions, particularly conserved regions within the homologous fungal gene. This has implication for block-based/Sybr Green-based assays where inappropriate panfungal primers will amplify human DNA generating false positive results unless further specific analysis is performed.

For assays utilizing inappropriate primers with *Aspergillus* sp. probes false negative results may occur due the amplification of abundant human DNA rather than the small quantities of any fungal DNA present in the sample, although no probe binding would occur. In designing an *Aspergillus* PCR it is preferential that both the primers and probe should exclude the possibility of binding to human DNA.

Another consideration is that assay performance may vary between different amplification platforms. Part of the work of the UK fungal PCR consensus group was to evaluate *Aspergillus* PCR methods. Two optimal methods were identified. One, published by Kami *et al*., used panfungal primers and an *Aspergillus*

Table 2 Quantification of *Aspergillus fumigatus* 28S rRNA gene copy numbers after extraction with the Roche MagNa Pure.

Sample (conidia)	PCR result	Crossing Point	Copy number available[a]	Detected[b]
1000	Detected	34.9	10^5	144
500	Detected	36.4	5×10^4	56
100	Detected	37.6	1×10^4	25
75	Detected	37.8	7.5×10^3	23
50	Detected	38.1	5×10^3	19
10	Detected	38.0	1×10^3	20
0	Not detected	0	0	0

[a]Calculated by multiplying the number of conidia by a copy number of 10^2.
[b]Calculated from Crossing Point and regression line $Y = -1.5705\ln[X]+42.71$ [14]

specific hydrolysis probe [31] and it was designed for use on the TaqMan. However, most centres used Light Cycler systems. As it is possible to run hydrolysis probes on the LC it was decided to evaluate the method on other platforms [52].

Using the Rotorgene the detection limit was 25–50 conidia, and this fell to 500 conidia using the Light Cycler, compared to 10–20 conidia on the TaqMan [52]. Electrophoresis of the Light Cycler products revealed amplification had occurred but was independent of the fungal load. Sequencing of the Light Cycler products showed that the human 18S rRNA gene had been amplified [52]. A definitive explanation for this difference has not been given.

The second optimal assay [14] showed significant but much less dramatic differences between platforms [52]. Designed for use on the Light Cycler and utilising *Aspergillus* spp. primers and probes the results on the Light Cycler were much improved. However, 100% detection limits were set at 100 conidia/ml whereas the 100% thresholds on the Rotorgene and TaqMan were 25 conidia/ml and 10 conidia/ml, respectively [52].

Limiting factors – result interpretation

One of the most challenging aspects of *Aspergillus* PCR testing is the interpretation of results in a clinical context. There have been a number of publications with promising performances but confidence in PCR detection remains varied [14,18–20,50,53–55]. These studies reported high sensitivities ($>92\%$) and negative predictive values ($>99\%$). As such, confidence in negative results should be high. Most assays also showed good specificity but positive predictive values will be affected by the small number of proven/probable cases of IA and a single false positive result will exert a large impact on the positive predictive value. Large-scale studies are needed to improve the number of proven/probable cases.

It is essential that all the available clinical information be used when interpreting PCR results. Antifungal therapy will lower the fungal burden. It will not alter the extraction efficiency but may reduce the load below PCR thresholds and combined with accepted DNA extraction losses lead to negative PCR results [35,56]. More efficient extraction procedures may minimize this effect and possibly explains why others have not seen an antifungal – PCR response [37]. Larger sample volumes may help combat an antifungal effect but this must be balanced against the possible introduction of more inhibitors.

The nature of the infection can also influence the result. Again it will not alter extraction or amplification performance but fungal burden may be lowered with varying disease status giving negative PCR results. Yamakami *et al*. reported that the degree of tissue invasion was important and less invasive infections such as aspergilloma had a lower frequency of positive results compared to proven/presumptive cases of invasive pulmonary aspergillosis [57].

When interpreting PCR results the EORTC-MSG consensus criteria should be used to classify the disease and weight the power of the result [1]. Others have reported the difficulty in comparing studies due to the use of different case definitions [37] and the use of the EORTC-MSG criteria would be a common starting point that is not prejudiced by an 'in house' PCR result.

Currently, two consecutive positive PCR samples are required to exclude contamination but in a clinical setting a variety of other factors may cause loss of positivity. Halliday *et al*. [37] have proposed guidelines with the potential to reduce costly empirical therapy in febrile neutropenic allogeneic HSCT and acute leukaemia patients. In addition to consistently negative and positive PCR results they have commented on intermittently positive PCR results recommending that intermittent results within two weeks should be considered significant.

Another controversial interpretation is in the result reporting where one replicate is positive and the other negative. Generally the positive result would be considered as contamination and the result given as PCR negative. However, a recent study proposed that the amount of *Aspergillus* DNA quantified in PCR positive samples could be very low and below the 100% reproducibility thresholds of PCR systems [29].

It is possible that patients with non-reproducible or intermittent positive PCR results could be representative of the initial stages of infection, or a lowered fungal burden due to antifungal therapy or type of disease. Alternatively contamination or patient colonization may be responsible. This highlights why frequent testing, radiological, GM ELISA and collation of all available clinical information should be used to interpret the result, remembering that a positive PCR result could also represent the initial stages of infection that have gone undetected by current diagnostic techniques [13].

Conclusion

PCR methods have shown their usefulness in a number of independent studies but their major limitation is the lack of a definitive inter-laboratory study to define a standardized method. They can be used in a clinical setting as adjunct assay in a carefully constructed

neutropenic care pathway, also involving the concomitant frequent use of galactomannan ELISA and carefully timed HRCT.

The lack of a standardized method must be resolved and address all stages of the molecular process from sample type and processing to interpretation of results. Screening methods to find an optimal protocol will allow major widespread evaluation that will address the issue of limited cases of proven/probable IA, allowing representative clinical evaluation. The substantial cost may be offset against savings in anti-fungal usage [3,37]. Only this strategy will permit the introduction of molecular methods in to future consensus criteria for diagnosing IA.

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