Aspergillus PCR formidable challenges and progress

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Invasive aspergillosis (IA) is an important cause of morbidity and mortality in immuno-compromised patients, with an incidence of 4–10%, and with a mortality rate of 80–90%, such as in allogeneic stem cell transplant recipients. Conventional diagnostic tests, like blood culture, are not useful in the diagnosis of *Aspergillus* spp. fungemia. Furthermore, non-culture based techniques that have been used in the past have lacked sensitivity and specificity in immunocompromised patients. New rapid methods which can detect IA early in the course of disease with high sensitivity and specificity are needed since treating these infections at an early stage is often essential for favourable outcomes. In this regard, the polymerase chain reaction (PCR) offers great promise for the rapid diagnosis of fungal infections, including detection of fungi that do not grow in blood cultures such as *Aspergillus* spp. At Karolinska University Hospital we have established a diagnostic assay, using a combination of a manual extraction and a robot for automated extraction of *Candida* and *Aspergillus* DNA, together with real-time PCR. To assess its clinical applicability, a large number of samples from patients with suspected invasive fungal infection have been analyzed with real time PCR. Data will be presented with focus on *Aspergillus* R-T PCR results in immunocompromised patients. A range of different PCR assays have been developed, targeting different gene regions and including a variety of amplicon detection methods. These molecular assays provide high potential in terms of sensitivity and specificity, but vary widely in their feasibility and have not, until now, been standardized. Despite this progress, there are certain questions to be addressed using these assays, such as the frequency of prospective sampling, as well as the number of positive results of a PCR assay required to initiate antifungal therapy. Furthermore, there are only a few standardized assays that are commercially available. This particular challenge will be addressed by the Working Group 'EAPCRI' (European Aspergillus PCR Initiative) under the auspices of ISHAM. Twenty-four centres have started to establish a European standard for *Aspergillus*-PCR. The principal goal of this initiative is to achieve a standard for PCR that can be incorporated into the next revision of the EORTC/MSG definitions for IA. Future prospective studies evaluating the potential benefits of early therapy based on R-T PCR in patients at high risk for IA infections are needed.

**Keywords**  *Aspergillus*, PCR, diagnostic PCR

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Introduction

Opportunistic invasive fungal infections are a major cause of morbidity and mortality in immunocompromised individuals. Patients at highest risk are those with prolonged periods of neutropenia, for example during treatment for acute leukaemia or after bone marrow hematopoietic stem cell (HSCT) and solid organ transplantation [1–4].

*Aspergillus fumigatus* is a ubiquitous saprophytic mould that forms airborne conidia. Humans inhale, on average, hundreds of these conidia daily. Fungal conidia range in sizes from 1–3 μm and are easily spread into the environment through many routes including air. In immune competent hosts, conidia are killed and cleared by cells of the pulmonary immune system. However, immunocompromised patients may develop invasive pulmonary aspergillosis, a life-threatening infection, mainly caused by *Aspergillus fumigatus*.

Early detection of invasive aspergillosis (IA) is crucial for the outcome of the patient. The increasing incidence of IA, in immunocompromised patients, emphasizes the need to improve the currently limited diagnostic tools.

Conventional diagnostic tests, such as blood culture, are not useful for the detection of *Aspergillus* spp. [5–7].

There has been some progress in the diagnosis of invasive aspergillosis (IA) in recent years, mainly due to use of high-resolution CT-scanning and other imaging procedures. Still, established IA is difficult to treat with a death rate of 80–90% [8–10].

Therefore, it is mandatory to develop and evaluate non-culture-based methods for the detection of systemic fungal infections. The galactomannan assay (Platelia®, BioRad) is commercially available and approved by the FDA, as well as in Europe and has been included in the criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC-MSG) for classification of IA [11].

A further antigenemia ELISA assay detects the polysaccharide β-1,3-glucan [12]. However, this assay is not able to distinguish among fungal etiologic agents [13]. The assay is approved by the FDA and has been included in the EORTC/MSG criteria for the diagnosis of possible IA.

More recently, PCR protocols for diagnosing fungal infections have been described [14–20]. A range of different PCR assays (conventional-, nested-, real-time-based) have been developed, targeting different gene regions (cytochrome p450, heat shock proteins, 18S, 5.8S, 28S, ITS). Furthermore they include a variety of amplicon detection methods, such as gel electrophoresis, hybridization with specific probes, ELISA and restriction fragment length polymorphism (RFLP).

These molecular assays provide high potential in terms of sensitivity and specificity, but vary widely in their feasibility and are up to now not standardized. Furthermore, only few commercially available, standardized assays are available.

This highlights the problems when evaluating publications designed to compare PCR methodologies. Yet, a consensus concerning the type of specimen, the extraction method and the PCR format and platform still has to be reached.

Types of sample

As for the best blood fraction to test, a consensus has yet to be reached on the method for nucleic acid extraction and purification, and of course, the choice of specimen has a great influence on the extraction methodology. Different types of specimens have been analyzed, most often blood or serum samples since they are easy to obtain and offer the possibility for prospective analysis.

Testing serum specimens by PCR relies on the detection of free circulating *Aspergillus* DNA, while the use of EDTA-whole-blood samples allows the detection of conidia, hyphal fragments and/or free circulating DNA.

The pathogenesis of IA is poorly understood and there is a lack of knowledge regarding the source of the fungal nucleic acid in the blood. No consensus has been achieved on the source for fungal DNA in plasma/serum or whole white blood cell pellets. Some scientists recognize that as blood culture samples rarely yield actively growing *Aspergillus* spp. [21], it is unlikely that this source would have viable conidia or fungal cells. On the other hand the spread of viable *Aspergillus* cells through the bloodstream to internal organs must occur since *Aspergillus* can be cultured from tissue biopsies such as heart and brain.

Loeffler et al. found that whole blood was a better specimen for fungal DNA extraction than plasma [22]. White and Barnes hypothesized that, depending on the patient population being studied, the fungal DNA associated with the white blood cell fraction could be that in conidia previously ingested and partly digested by macrophages, hyphae attacked by neutrophils, or free fungal fragments damaged through platelet attachment [23].

We would therefore recommend the collection of whole-blood in EDTA tubes, in order to have access to both the free- and the cell-associated DNA. Moreover, EDTA is known to inhibit DNases that are naturally present in the blood without interfering with the PCR
assay which often occurs in the presence of citrate [24] (and heparin has to be avoided because of Taq-inhibition (Table 1).

**Extraction methods**

A consensus has yet to be reached on the method of choice for nucleic acid extraction and purification. DNA is generally considered the target of choice due to its relative stability, and ease of extraction (compared to RNA) [24]. Specimens such as plasma and serum, in which DNA would be free in solution, allow for more rapid and less complex protocols (red cell and white cell lysis buffers are not needed) for nucleic acid extraction as compared to whole blood [16]. If hyphal elements or conida are present in the specimens, the protocols have to rely on longer and more complex processes. This is due to the fact that the fungal cell wall is difficult to break in order to release DNA. For whole blood (hypotonic red cell and white cell lysis buffers are often used), enrichment of fungal DNA by decanting human DNA with the supernatant increases the sensitivity.

Protocols currently used to break the fungal cell wall usually rely on enzymatic digestion, such as recombinant lyticase [25], or mechanical disruption by bead-beating [26]. If enzymatic lysis is performed, recombinant lyticase has to be used instead of zymolyase which might be contaminated by fungal DNA [27].

For DNA extraction, either spin columns or an automated method can be performed. Sources of contamination during fungal DNA extraction may also include buffers and spin columns (the silica membranes) [28].

**Fungal nucleic acid target for the assay**

The advantage of selecting a multi-copy gene is obviously that it increases the chances to successfully amplify it to detectable levels. During the last few years, the ribosomal DNA gene region composed of the 18S (1800 bp) gene, the 5.8S (159 bp) gene and the 28S (3396 bp) gene has been shown to be a promising target. These are respectively separated by internal transcribed spacer 1 (ITS 1) (361 bp) and ITS 2 (231 bp). Up until now, numerous studies made use of its different components, 18S [18,29], 28S [30,31], ITS1 [32] and ITS 2 [20].

**Polymerase chain reaction (PCR)**

Hundreds of manuscripts have been published dealing with the detection of fungal DNA. However, there has been little standardization in procedures and very few inter-laboratory validation studies.

Which PCR assay that is most suitable is dependent on the demands of the method. PCR assays can be used as (i) diagnostic tools only, (ii) as a means for the early diagnosis of IA, ideally prior to the onset of clinical symptoms and finally (iii) as tools to monitor preemptive antifungal therapy. Dependent on the purpose of the assay, different technical aspects have to be considered, including the types of samples, the extraction of the nucleic acids, the fungal DNA targets and the frequency of sampling.

**Table 1** Major issues for Aspergillus-PCR based assays and recommendations of the authors to meet these critical points.

<table>
<thead>
<tr>
<th>Key issues in PCR-based diagnosis of Aspergillus-DNA</th>
<th>Authors’ recommendations</th>
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<tr>
<td><strong>Type of specimen</strong></td>
<td>Whole blood for prospective analysis of high risk patients containing intracellular and extracellular fungal elements</td>
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<tr>
<td><strong>Starting sample volume</strong></td>
<td>3–5 ml of EDTA anticoagulated whole blood</td>
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<td><strong>Lysis of fungal cells</strong></td>
<td>Bead-beating with glass or ceramic beads</td>
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<td><strong>Inhibition due to high DNA concentration (human, bacterial)</strong></td>
<td>Photometric quantification of extracted DNA, dilution if &gt;500 ng/ml</td>
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<td><strong>Internal control</strong></td>
<td>Artificial DNA control, phage DNA or bacterial spores to be added to blood samples prior to DNA extraction</td>
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<tr>
<td><strong>Appropriate negative controls</strong></td>
<td>Co-analysis of blood from healthy donors or sterile water, one negative control per 10 clinical samples</td>
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<td><strong>Contamination</strong></td>
<td>Use of detergents and agents for degradation of nucleic acids for cleaning benches</td>
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<td><strong>Sensitivity of the PCR assay</strong></td>
<td>Use of one-way gowns and sterile gloves</td>
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<td>Separated laboratories for DNA extraction and PCR assays</td>
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<td>Consumables/reagents to be treated with UV-light, ethyleneoxide etc., use filter tips</td>
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<td></td>
<td>Aliquot reagents, buffers, primers and probes</td>
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<td>Real-time PCR system with specific probes targeting multi-copy genes</td>
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White and colleagues recently published a multicentre study, comparing the use of two primer sets (28S and 18S) and three machines (LightCycler (Roche), Applied Biosystems 7500 Real-time PCR system with hydrolysis probes and Rotor-Gene (Corbett Research) with a panel consisting of 8 negative and 8 positive samples (10–5000 conidia per ml) [23]. The study revealed that the 28S assay was more specific than the 18S assay, particularly since the 18S was found to amplify a portion of the human rDNA gene in the absence of DNA from \textit{A. fumigatus}. This interference was particularly strong with the LightCycler and was responsible for a decrease in sensitivity. Overall, the sensitivity, specificity, negative (NPV) and positive (PPV) predictive value were higher with the 28S than the 18S primer set, regardless of the platform used. The platforms themselves were found to have a major influence on the assay, as sensitivity and NPV were 100% on the Applied Biosystems 7500 Real-time PCR system, whereas specificity and PPV were at 100% using the Rotor-Gene system [23].

However, the sensitivity of PCR assays with blood samples from healthy donors spiked with \textit{Aspergillus} conidia might not reflect the sensitivity of the assay when clinical blood samples are analyzed because we do not know if conidia, hyphal fragments or free circulating DNA are detected.

**Practical considerations in the clinical laboratory**

If a test has to be used routinely in a clinical laboratory, there are additional factors of great importance. The volume of blood to be processed has to be within the range that is usually taken from a patient. Again, a big discrepancy exists among studies, with blood sample volumes ranging from 200 \mu l [16] to 10 ml [17]. As the fungal load in the circulation of a patient may be less than 1 genome per ml, we recommend using between 3 and 5 ml, which corresponds to the volume of blood accommodated by most EDTA collection tubes in hospitals. Larger blood volumes (5 ml vs. 1 ml vs. 200 \mu l) increase the sensitivity of the assay due to the higher number of fungal cells (unpublished data).

Another crucial consideration in the development of a diagnostic laboratory test for invasive aspergillosis would be the potential for automation of the method. In our laboratory, we have developed a real-time LightCycler assay for the detection of \textit{Aspergillus} spp., which employs a combination of manual extraction (we use this for whole blood hypotonic red cell lysis buffers and mechanical disruption by bead-beating) and a robot, the MagNa Pure LC Instrument (Roche Diagnostics, Basel, Switzerland) for automated extraction of fungal DNA [18]. The assay takes 5–6 h to perform. The oligonucleotide primers and probes used for species identification were derived from the DNA sequences of the 18S rRNA genes of various fungal pathogens [18].

Samples are screened for \textit{Aspergillus} to the genus level in the real-time PCR assay which detects most of the clinically relevant \textit{Aspergillus} spp. The lower detection limit of \textit{A. fumigatus} conidia is 5–10 genome equivalents.

To assess clinical applicability, 1,650 consecutive samples (1,330 blood samples, 295 samples from other body fluids and 25 biopsy specimens) from patients with suspected invasive fungal infections were analyzed and the results compared to those obtained with cultures, direct microscopy, serology and CT scans. Of the total number of samples, 28 (1.7%) were PCR-positive for \textit{Aspergillus} spp. In patients with positive PCR results for \textit{Aspergillus}, verification with conventional methods was possible in only 50% of cases.

Since conventional methods, such as blood cultures, are not as useful diagnostic tools for the detection of \textit{Aspergillus} fungemia, we could only verify the \textit{Aspergillus} PCR positive results in 50% of the patients. The feasibility of PCR testing to detect \textit{Aspergillus} spp. was further demonstrated not only in blood samples, but also in bronchoalveolar lavages (BAL) and other body fluids [18].

We have found that the DNA fungal extraction method are crucial since DNA extracted from different body fluids contains not only human DNA but may also contain fungal, bacterial, viral and parasite DNA. The total amount of DNA may significantly vary from sample to sample. Using a probe system in the LightCycler, we have noted that it is essential to know the total amount of DNA. If this exceeds 500 ng per sample, inhibition may occur in our assay and give a false negative result. Thus, we routinely measure the DNA concentration in all samples using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE, USA). If the concentration is too high, we dilute the sample before running the PCR.

We started to use this assay as part of our daily routine in June 2002. Samples for PCR have been analyzed from patients at risk for fungal infection, those in which there is clinical suspicion of fungal infection, and for confirmation of fungal infection. Samples have been taken before, during and after treatment. Between the period June 2002–September 2006 (52 months), 5,408 samples have been analyzed for \textit{Aspergillus} spp., which 227 (4.2%) were PCR positive.
Aspergillus of hyphae were found. The total number of Aspergillus positive PCR blood samples was 127/4,565 (2.8%). In 78 patients (108 Aspergillus PCR positive blood samples) the underlying disease was known, i.e., 49 had undergone allogeneic HSCT (with 81 PCR positive samples), two had received solid organ transplantation, 19 were leukaemic patients and eight patients were in ECMO (Extracorporeal Membrane Oxygenation) treatment.

In the immunocompromised patient, fungal conidia and/or hyphae, as well as naked DNA may be released into the bloodstream, with or without clinical signs of disease. The lack of symptoms does not necessarily indicate the absence of disease. Some authors claim that at least two consecutive positive PCR results are required to initiate antifungal therapy [33–36].

How to interpret a single positive PCR result is unclear. A transient presence of fungal DNA in clinical specimens might be possible but an infection cannot be excluded.

The interpretation of a single Aspergillus PCR positive test has to be seen in relation to host factors, individual risk factors, the clinical signs of the patient [11] and the specificity of the PCR assay. As an example, in a critically ill, neutropenic high-risk patient, a single Aspergillus PCR positive would indicate the immediate initiation of antifungal therapy.

During the same period, 120 BAL samples were analyzed by PCR. Thirty-one (26%) from 23 patients were Aspergillus-PCR positive, of which 6 from 16 patients (38%), could be confirmed by culture. In seven patients the result of the cultures were unknown.

At present, only positive results from conventional tissue cultures or histological examination in combination with culture or other indirect procedures such as immunohistochemistry [37] provide definitive proof of invasive aspergillosis [38]. However, there are data supporting the considerable clinical value of this PCR assay for confirming and improving diagnosis of pulmonary aspergillosis in high-risk patients [39].

Recently, the greater sensitivity of the galactomann EIA or PCR compared to culture in the detection of Aspergillus spp. in BAL fluid has been confirmed [40,41]. However, BAL is difficult to perform or to repeat, but testing BAL samples may help in diagnosing invasive pulmonary aspergillosis.

During the 52 months period, 31 biopsies were analyzed by PCR in our lab and 12 were positive for Aspergillus. One hundred percent could be confirmed by culture, as well as by direct microscopy in which hyphae were found.

The high sensitivity of a PCR assay in the detection of Aspergillus spp. has been demonstrated in patients with cerebral aspergillosis [42]. In addition, tissue biopsy [43] has been employed. However, obtaining such samples requires invasive procedures which might not be performed in critically ill patients.

Despite this progress, there are certain questions to be addressed when using these assays, such as the risk of contamination with conidia and amplicons (which can be minimized by the use of cabinets and real-time PCR assays) (Table 1). The frequency of prospective sampling, as well as the number of positive results obtained with a PCR assay required to initiate antifungal therapy is not known and needs to be addressed. Is frequent PCR testing (2–3 times per week) in high risk patients necessary to detect IA early enough to achieve reduction of mortality by the immediate initiation of treatment?

Besides the use of PCR assays for the diagnosis of IFI in symptomatic patients, this highly sensitive technology can also be performed to preemptively monitor patients at risk to develop IFI, a strategy successfully used to monitor opportunistic viral infections. The objective is to clearly define negative (no therapy) and positive (initiation or continuation of therapy, additional screening tests) samples. In these patients, maximum sensitivities and high negative predictive values are mandatory to minimize the risk of false negative results.

Taken together, those new non-culture-based diagnostic assays are appropriate as simple and rapid screening tests with high sensitivities and quick turnaround times. Thus, they might help to reduce empirical antifungal therapy and might be valuable tools for early initiation and monitoring of preemptive antifungal therapy.

However, the lack of standardization of the techniques is a main reason why the EORTC/MSG has not yet included PCR in the recently published list of criteria for the diagnosis of IA.

This particular challenge will be addressed by the Working Group ‘EAPCRI’ (European Aspergillus PCR Initiative) under the auspices of ISHAM. Twenty-four centres have started to establish a European standard for Aspergillus-PCR. The principal goal of this initiative is to achieve a standardized consensus for Aspergillus-DNA extraction and PCR amplification.

Future prospective studies evaluating the potential benefits of early therapy based on real-time PCR in patients at high risk for IA infections are needed. To be able to validate Aspergillus PCR, we need to perform multi-center studies in order to include enough patients with proven and probable infection according to the EORTC/MSG criteria.
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**References**


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