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Molecular Approaches to Diagnosis of Invasive Aspergillosis; What we Know and What we do not Know

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Abstract
Invasive aspergillosis (IA) is a major complication in immunocompromized patients where can be serious and rapidly fatal. Early diagnosis and appropriate antifungal treatment is important in reducing mortality and morbidity. Despite many efforts to develop detection methods, the diagnosis of IA still remains challenging and current conventional methods are limited for adequate diagnosis. New rapid methods which can detect IA early in the course of disease with high sensitivity and specificity are needed to treat these infections at an early stage. Using molecular methods for the identification of Aspergillus species can be a cost-effective, rapid, discriminatory, and objective approach for delineating Aspergillus species in a clinical microbiology laboratory. PCR techniques for the diagnosis of IA have been studied for more than a decade and are still considered investigational; however, until now PCR is not included in current EORTC/MSG diagnostic criteria.

Keywords: Invasive aspergillosis; PCR; Molecular approaches; Real-time PCR

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Introduction
Invasive aspergillosis (IA) is a major complication in immunocompromized patients with haematopoietic stem cell or solid organ transplantation, ICU patients, and especially those with prolonged neutropenia which are high-risk populations where IA can be serious and rapidly fatal (1-2). The incidence of IA continues to rise in conjunction with the increasing population of susceptible individual. IA has a high mortality rate of up to 60% or even higher if the diagnosis is delayed (3). Early diagnosis and early appropriate antifungal treatment is important in reducing mortality and morbidity (4). Despite many efforts to develop detection methods, the diagnosis of IA still remains challenging and current conventional methods are limited for adequate diagnosis (5). Several reasons are responsible for these limitations, such as non-specific and variable clinical symptoms that occur late in the course of disease, considerable delay in turn-around time due to slow growth of the fungus in culture, the time needed for its identification, and especially, a lack of diagnostic methods with sufficient sensitivity and specificity (6). Because of the limitations of the aforementioned diagnostic methods, non-culture methods based on the detection of the molecular biomarkers have been developed. Therefore, it is mandatory to develop and evaluate non-culture based methods for the detection of IA. New rapid methods which can detect IA early in the course of disease with high sensitivity and specificity are needed to treat these infections at an early stage (7). The limitations of antibody detection and the problems of sensitivity and false positive associated with antigen detection have prompted the evaluation of the molecular techniques for the diagnosis of IA (8-9). Using molecular methods for the identification of Aspergillus species can be a cost-effective, rapid, discriminatory, and objective approach for delineating Aspergillus species in a

clinical micro- biology laboratory (10). Thus, Polymerase Chain Reaction (PCR) techniques for the diagnosis of IA have been studied for more than a decade and are still considered investigational; however, until now PCR has not been included in current EORTC/MSG diagnostic criteria. This review focuses on utilization of molecular methods, for diagnosis of invasive aspergillosis.

Molecular approaches

Owing to the disappointing performance of serologic assays, research has - since the early 1990s - been focused on molecular methods to detect the fungal pathogen early and with high sensitivity. Early studies to evaluate PCR mediated detection of fungi showed significantly improved sensitivity compared with other diagnostic tools at that time, but were performed with different assays and different objectives partly to optimize culture assays (11, 12), partly for typing in epidemiologic studies, and often in nonclinical settings (13, 14).

The positive benefits of carrying out highly sensitive DNA detection assays should be considered. The first PCR assays detected quantities down to 1-20 pg of genomic DNA from Aspergillus fumigatus in vitro (15). In whole blood between 10-100 fg/ml of Aspergillus DNA have been reported (16). Klingspor L, et al (18) reported that 30 fg/ml of Aspergillus DNA were present in sera in contrast, Challier S et al, (17) between 100 fg/ml to 1 ng/ ml. This indicated that a range of different PCR assays (conventional, nested, and real-time based PCRs) have been developed, targeting different gene regions (cytochrome p450, heat shock proteins, 18S, 5.8S, 28S, and 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 standardized assays are now commercially 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 has still to be reached. Major issues for Aspergillus-PCR based assays to meet these critical points (18).

Polymerase chain reaction (PCR)

PCR detection of Aspergillus nucleic acids is increasingly appreciated as being a method of early detection of IA. In contrast with other non-culture-based methods, PCR has the capability of rapid detection and molecular identification of opportunistic moulds besides Aspergillus at the genus level (19). 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 (iii) as tools to monitor pre-emptive antifungal therapy. Depending 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.

Nested-PCR

Nested PCR formats have been widely used for Aspergillus spp. in an attempt to optimise analytical sensitivity, but the requirement to open reaction tubes means that there is considerable risk of contamination and the subsequent generation of false-positive results (10).

PCR-ELISA

In addition, these technologies those are not easily adapted for use in a clinical microbiology laboratory. The major advantages of the PCR-ELISA identification system, compared to conventional phenotypic identification methods, were included as (A) it was rapid (this test could be completed in a single day); (B) it did not require species of Aspergillus to form specialized identifying structures such as conidia; (C) small amounts of DNA target could be detected (picogram quantities); (D) DNA probes could be easily and reproducibly synthesized and stored ready for use; (E) DNA probes were very specific; (F) interpretation of the results were objective (colorimetric, spectrophotometric readout); (G) the detection system has the potential to be easily automated (20).

Real-Time PCR

Application of real-time PCR technologies allows for rapid and effective post amplification of specific DNA sequences (e.g. ITS1 regions of 18S ribosomal subunits) for genus-and species-specific identification of opportunistic fungi. Furthermore, real-time PCR protocols, which are less prone to contamination, along with automated systems for DNA extraction appear to be a significant step towards standardization and improvement of the reliability of the existin PCR assay. Because real-time PCR allows for quantification of the amount of circulating

Aspergillus DNA, it may also be used as an indirect Parameter of fungal load during monitoring of treatment with antifungal drugs.

Table 1. Major issues for Aspergillus-PCR based assays.

Key issues in PCR-based diagnosis of *Aspergillus*-DNA	Authors' recommendations
Type of specimen	Whole blood for prospective analysis of high risk patients containing intracellular and extracellular fungal elements
Starting sample volume	3-5 ml of EDTA anticoagulated whole blood
Lysis of fungal cells	Bead beating with glass or ceramic beads
Inhibition due to high DNA concentration	Photometric quantification of extracted DNA, dilution if >500 ng/µl
Internal control	Artificial DNA control, phage DNA or bacterial spores to be added to blood samples prior to DNA extraction
Appropriate negative controls	Co-analysis of blood from healthy donors or sterile water, one negative control per 10 clinical samples.
Contamination	Using of detergents and agents for degradation of nucleic acids for cleaning Benches, using of one way gowns and sterile gloves, separated laboratories for DNA extraction and PCR assays, consumables/reagents to be treated with UV light, ethylenoxide etc., and using filter tips aliquot reagents, buffers, primers, and probes.
Sensitivity of the PCR assay	Real- time PCR system with specific probes targeting multi copy genes

The monitoring of accumulating amplicon in real-time has been made possible by the labeling of primers, oligonucleotide probes, or the amplicon itself with molecules capable of fluorescing. These labels produce a change in signal following direct interaction with the amplicon or hybridization to the amplicon. The signal increases as the amount of amplicon increases after each amplification cycle. The fluorescent signal can be obtained by one of several methods. The simplest method employs the SYBR green dye that increases in fluorescene when bound to double stranded DNA allowing quantification. The analysis of the melting curve is indicative of the nature of the amplified fragment. However, for diagnostic purposes, it is of the utmost importance to check the specificity of the amplified products. This can be achieved by using specific hybridization probes.

Table 2. Comparison of different Real Time PCR techniques.

System	Quantity	Measurement	Specificity	Melting curve
SYBER green	++++	Extension	-	product
Taq Man	++++	Any	+++	-
FRET	+++	Annealing	++++	Probe
Beacon	++	Annealing	++++	Probe

There is a range of chemistries currently in use. One relies upon fluorescence resonance energy transfer (FRET) between fluorogenic probes and has been developed for the LightCycler instrument (Roche Diagnostics). The Applied Biosystems apparatus uses the TaqMan technology based on the 50 nuclease activity of the polymerase to release the two dyes of the fluorogenic probe (21). The choice between these two more popular methods depends on specific demands and/or local opportunities. However, other chemistries are in development such as molecular beacons and scorpion probes (22, 23). One of the major disadvantages of SYBR green method is that the SYBR green color nonspecifically binds to all double-stranded DNAs such as primer dimer and other nonspecific bands. Therefore the fluorescent signals derived from the reaction, which is proportional to the amount of double-stranded DNA and the amount of fluorescence increases resulting in decreased specificity and increased false positives. Using this method, Aspergillus species and even other fungal species cannot be distinguished from

each other and only the type of fungi can be reported. For example, it can only be said whether the fungus is Aspergillus or not. However, the SYBR green method has some advantage as well, such as simple design (only 2 primers are required without any probe), low cost, and the ability to analyze melting curve formed after replication in order to verify the specificity of the reaction.

Table 3. Details of the PCR techniques.

Study	Sample type	Sample volume	Target gene	Cell wall disruption	PCR method	Appropriate controls	
						Positive	Negative
Nabili et al (35)	Whole blood	3–5 mL	18S rRNA	Lyticase	Real Time/FRET Probe	Serial dilutions	Yes
Hebart et al (2000) (36)	Whole blood	5 mL	18S rRNA	Zymolyase	PCR-ELISA	Serial dilutions	Yes
Buchheidt et al (2001) (37)	Whole blood and BAL	3–5 mL	18S rRNA	Lyticase	Nested PCR	Serial dilutions	Yes
Ferns et al (2002) (38)	Whole blood	2 mL	mtDNA	Zymolyase	Nested PCR	Internal control (ras oncogene)	Yes
Raad et al (2002) (39)	Whole blood	700 µL	Alkaline proteinase gene mtDNA	SDS, proteinase	Standard PCR	Serial dilutions	Yes
Kawazu et al (2004) (40)	Plasma	200 µL	18S rRNA	QIAamp Blood Mini-Kit	RT-PCR with TaqMan Probe	Blood inhibition control	Yes
Lass-Floerl et al (2004) (41)	Whole blood	10 mL	18S rRNA	Zymolyase	PCR-ELISA	Inhibition control	Yes
Halliday et al (2006) (42)	Whole blood	500 µL	18S rRNA	Lyticase	Nested PCR	Serial dilutions	Yes
Jordanides et al (2005) (43)	Whole blood	10 mL	18S rRNA	Zymolyase	RT-PCR with FRET Probe	Serial dilutions	Yes
El Mahallawi et al (2006) (44)	Serum	-	18S rRNA	Lyticase	Standard PCR	Serial dilutions	Yes
Cesaro et al (2008) (45)	Whole blood	3 mL	18S rRNA	Zymolyase	RT-PCR with FRET Probe	Serial dilutions	Yes

In the TaqMan method, a reporter is placed at the 5' head and Quencher at the 3' head and due to having a probe has higher specificity than the SYBR green method. Each other unless for each species, a specific probe can be designed which requires a high cost. On the other hand, the FRET method is designed with two probes with different fluorescent dyes, the probes are quite specifically bounded to the desiredDNA. The design of these probes is so that in cases of differences in even one nucleotide, it does not bind to the DNA. As in this method the probes are not hydrolyzed, the analysis of the melting curve after replication is possible and considering the melting temperature of DNA, which is a specific parameter of this molecule and depends on the DNA structure and factors such as length and number of nucleotides, the probe concentration, the environment's salt concentration and the percentage of GC, Aspergillus species can be differentiated from each other. Additionally, the ability of the real-time PCR assay to simultaneously quantify the fungal DNA load in a variety of clinical specimens makes its clinical usefulness probable helping establish disease severity, monitor antifungal therapy effectiveness and estimate disease advancement; even though, these advantages has to be finally determined in future prospective clinical studies.

Post amplification analysis

Post-amplification detection techniques provide genus or species specific data but may also increase sensitivity and specificity (24, 25). Real-time detection techniques (eg, TaqMan, LightCycler, molecular beacons) are automated, rapid, and reproducible, thus facilitating comparisons between studies. Southern blotting has had a valuable role in

the evolution of PCR as a diagnostic modality, but is unlikely to have any substantial future role in routine clinical assays. Single-strand conformational polymorphism, (26, 27) restriction fragment length polymorphism digest pattern, (28) Line Probes, (29) fragment size deter- mination (30), DNA sequence analysis (31-33) and PCR-ELISA may have a limited role in specific instances, such as the identification of laboratory isolates (34). Conclusion: Fungal diagnostics has increased dramatically with the introduction of molecular tools, in particular that of PCR. In recent years, many papers related to improved methods and techniques have been published in order to progress or optimize methods and to conquer or decrease methodological drawbacks, confirming the increased interest in the emerging clinical problem of detecting invasive aspergillosis sensitively and specifically. In addition the use of PCR assays for the diagnosis of IA in patients, this extremely sensitive technology can also be performed to monitor patients at risk to develop IA. These new non culture based diagnostic assays are suitable 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 helpful tools for early beginning and monitoring of antifungal therapy. However, lack of standardization of PCR technique is still one of the factors that EORTC/MSG has not been included this technique in an article published in 2008 for the diagnosis of IA (5). In order to solve this problem in 2006, the group consists of 24 centers under the name 'EAPCRI' (European Aspergillus PCR Initiative) ISHAM was created. They began to establish a standard for the diagnosis of Aspergillus-PCR. The ultimate goal of reaching a general consensus for DNA extraction and PCR amplification (46). 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. So the combination of the different diagnostic assays may be an encouraging approach in some cases. In this manner, test sensitivity might be increased and the false-positive results might be decreased. Particularly, the combination of antigenemia and molecular assays is attractive. Alam et al. reported that sensitivities were 88% for Candida semi-nested PCR, 47% for BG, 41% for Candida mannan and 47% for antimannan antibodies in pediatric and adult patients with candidemia (47). When the tests were combined, the sensitivities reached 75% in Candida mannan and antimannan combination, 56% in Candida mannan and BG combination and 88% in combination of mannan, BG and Candida DNA. White et al. have recommended the combination of PCR and antigenemia assays in the management of high-risk patients (48). Nabili and shokohi et al. evaluated the performance of Real Time PCR and GM antigenemia tests from blood in the diagnosis of invasive aspergillosis and they obtained the best performance with a combination of tests (35). Thus, it has been reported that combination could help to increase sensitivity and help eliminate false-positive tests.

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