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Difficulties with molecular diagnostic tests for mould and yeast infections: where do we stand?

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Abstract

PCR assays have not reached the same level of acceptance for the detection of human fungal pathogens as for other micro-organisms, mainly because the low number of micro-organisms challenges the detection limits of PCR. Therefore, whereas meta-analyses focusing on clinical validation suggest interest in adding PCR results to the diagnostic workup for invasive fungal disease (IFD) along with clinical evaluation, CT scans, classical mycology and antigen detection, no consensual PCR method has emerged. Compared with the end-point format of the 1990s, real-time quantitative PCR is a major breakthrough. This format prevents contamination with previously amplified products, provides the yield of amplification, allows for developing consensus procedures and should therefore be the only format used. An internal control is now mandatory to avoid false-negative results. Primer design strongly impacts on the objectives: pan-fungal primers can provide false-positive results due to environmental fungal DNA contamination; conversely, species-specific primers miss infections caused by untargeted fungi. Unresolved issues include the best specimens to be used; serum is currently preferred to blood because of the ease of the DNA extraction step. Work is in progress to establish standards at least for *Aspergillus* PCR, and the implementation of quality controls should help centres to improve assays. Eventually, the classical analysis of biomarker performance does not consider the evolving risk factors and changing treatments during IFD, which can lead to variable conclusions. New statistical methods such as event history analysis should be considered.

Keywords: Candidiasis, clinical validation, diagnosis, invasive aspergillosis, real-time quantitative PCR
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Introduction

Molecular diagnostic tests for invasive fungal diseases (IFD) have a huge spectrum of applications, such as species identification and genotyping, which are not discussed here. The main field of investigation of clinical importance is the detection of *Candida* and *Aspergillus* in human specimens. The aim is to include molecular tests, mainly PCR assays, in the diagnostic workup of diagnosing IFD, which remains a multidisciplinary analysis of clinical and CT scan data, and microbiological findings, including classical mycology and antigen detection [1]. In addition, PCR assays can be designed for the detection of antifungal drug resistance, which is a growing concern [2].

The number of publications dealing with PCR assays aiming at improving diagnosis and treatment management of IFD has been increasingly expending in the past 20 years to overcome the limits of other diagnostic methods in mycology [3,4]. Thus, it may seem strange that PCR results are still not included in the consensus criteria for definitions of IFD [1], despite numerous meta-analyses for *Aspergillus* [5–8] and

Candida [9], showing that the sensitivity and specificity of these PCR tests are at least as good as antigen detection. Unfortunately, the PCR format is usually not considered as a critical point in published meta-analyses. As a consequence, no consensus had emerged regarding the implementation of PCR tests in a routine laboratory. For instance, a PubMed interrogation (January 1 2013) with "Aspergillus," "PCR" and "diagnosis" over the last five years provided 252 publications and 24 reviews in English with numerous differences regarding (i) DNA targets, (ii) probes (hydrolysis probes, hybridization probes or molecular beacons) and (iii) the PCR equipment used. To reach a consensus on a given assay is unrealistic in contrast to a consensus on PCR procedures.

The aim of this review was to describe some technical limitations specific to invasive aspergillosis (IA) and candidiasis. The discussion of these limitations may also lead clinicians to pay more attention to PCR tests and to be more critical when assessing the conclusions of some studies.

PCR Format for Diagnosing IFD

The generally very low amount of fungal DNA in clinical specimens challenges the limits of PCR assays. In focusing on increasing sensitivity, the risk of false-positive results due to contamination by either PCR amplicons or environmental DNA increases as well. To prevent false-positives due to amplicon contamination, uracil-N-glycosylase treatment has been proposed for more than 20 years [10,11], but enzymatic methods have been slowly implemented in PCR assays. Currently, the best means to prevent amplicon contamination are the closed format provided by real-time quantitative PCR (qPCR) [12,13].

The qPCR format dramatically reduces the risk of carry-over contamination by environmental amplicons and the potential for false-positive results. By opening tubes between two runs of amplification, nested PCR negates the main advantage of the closed-tube format. The technical performance of a qPCR test should also be optimized following the "Minimum Information for the publication of real-time Quantitative PCR Experiments" (MIQE) guidelines [14,15]. An analysis of the literature shows that the analytical features of qPCR tests should be improved, particularly in highly ranked journals [16].

Monitoring the Yield of Amplification

In addition to the risk of false-positive results, PCR presents a risk of false-negative results often linked to PCR inhibitors. To prevent false-negatives, the use of an internal control (IC) of the amplification has been recommended in recent years [10,17]. The aim of the IC is to be sure that the yield of the reaction in a given clinical specimen is as established after experimental validation of the PCR assay. Any DNA fragment exogenous to the considered PCR assay can be used, which excludes human DNA genes when dealing with human specimens. Indeed, the quantity of human DNA is often huge compared with the target and is also highly variable, giving no clue regarding the yield of the PCR. With an appropriate IC, a slight decrease in PCR yield can be detected and adequate measures can be implemented to improve DNA extraction steps and to reject samples with incorrect IC amplification to avoid false-negative results.

Primer Selection

The risk of false-positives in diagnostic PCR has led to the delineation of physical measures of prevention, well-known for years although not always easy to maintain in a routine laboratory (a unidirectional workflow environment with physically separated laboratories for pre-, peri- and post-PCR analysis, wearing a gown and gloves, the use of aerosol-resistant tips and specific pipettes). However, these measures are inefficient against another source of environmental DNA, that is, DNA from micro-organisms present in the environment. For instance, false-positive results have been reported due to fungal DNA in sampling tubes [18] and in IV drugs [19]. Regular tests of reagents or any suspected products using negative extraction controls should be as numerous as possible. Nevertheless, these contaminations generally correspond to low levels of fungal DNA (<10 DNA copies), and alternatively positive and negative results may lead by chance, according to Poisson's law, to falsely validate a negative control.

To decrease the risk of detecting environmental fungal DNA, one can design primers very specific for the main target, for example A*spergillus fumigatus* for IA. Fig. 1 shows the results of using qPCR to detect other mould DNAs when different primer sets are used. *Penicillium* DNA, for instance, can be amplified as efficiently as or more efficiently than *A. fumigatus* DNA. If for any reason *Penicillium* DNA is present in the PCR tube, it can be preferentially amplified due to the competitive nature of PCR, leading to false-negative results, even in presence of *A. fumigatus* DNA. On the other hand, when using very specific primers, one must accept not being able to detect other fungi, which may be clinically relevant, such as *Fusarium* spp. or mucormycetes [20]. Therefore, more specific assays lower the risk of false-positives with environmental DNA but, unfortunately, are more likely to miss non-*fumigatus* infection.