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MOLECULAR DIAGNOSTICS IN MICROBIOLOGY

Molecular diagnostic methods for invasive fungal disease: the horizon draws nearer?

C. L. HALLIDAY[1,2], S. E. KIDD[3], T. C. SORRELL[2,4] AND S. C-A. CHEN[1,2]

[1]Clinical Mycology Reference Laboratory, Centre for Infectious Diseases and Microbiology Laboratory Services, ICMPR – Pathology West, Westmead Hospital, NSW, [2]Centre for Infectious Diseases and Microbiology, Westmead Millennium Institute, NSW, [3]National Mycology Reference Centre, SA Pathology, SA, and [4]Marie-Bashir Institute for Infectious Diseases and Biosecurity, University of Sydney, NSW, Australia

Summary

Rapid, accurate diagnostic laboratory tests are needed to improve clinical outcomes of invasive fungal disease (IFD). Traditional direct microscopy, culture and histological techniques constitute the 'gold standard' against which newer tests are judged. Molecular diagnostic methods, whether broad-range or fungal-specific, have great potential to enhance sensitivity and speed of IFD diagnosis, but have varying specificities. The use of PCR-based assays, DNA sequencing, and other molecular methods including those incorporating proteomic approaches such as matrix-assisted laser desorption ionisation-time of flight mass spectroscopy (MALDI-TOF MS) have shown promising results. These are used mainly to complement conventional methods since they require standardisation before widespread implementation can be recommended. None are incorporated into diagnostic criteria for defining IFD. Commercial assays may assist standardisation. This review provides an update of molecular-based diagnostic approaches applicable to biological specimens and fungal cultures in microbiology laboratories. We focus on the most common pathogens, *Candida* and *Aspergillus,* and the mucormycetes. The position of molecular-based approaches in the detection of azole and echinocandin antifungal resistance is also discussed.

Key words: DNA sequencing, fungal infections, molecular diagnosis, PCR.

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INTRODUCTION

Invasive fungal disease (IFD) causes significant morbidity and mortality in hospitalised patients despite advances in antifungal therapies. Early diagnosis, which necessarily includes species identification, is essential for improving patient outcomes, but standard histological and culture methods are slow and insensitive.[1,2] To overcome these limitations, rapid culture-independent molecular (and serological) tests, which are also non-invasive, are increasingly used.

This article summarises recent advances in molecular methods, in the context of the clinical mycology laboratory, for the detection and identification of fungal pathogens in (i) clinical specimens, and (ii) fungal cultures with emphasis on invasive candidiasis (IC), invasive aspergillosis (IA) and mucormycosis. Diagnosis of *Pneumocystis jirovecii* is not discussed. We also review the molecular approaches in detection of resistance to antifungal agents since these, and their applicability as potential alternatives to phenotypic methods of resistance detection, have become increasingly important. More recent applications of matrix-associated laser desorption ionisation-time of flight mass spectroscopy (MALDI-TOF MS) that complement molecular-based diagnostics are also briefly discussed.

GENERAL CONSIDERATIONS

Parameters influencing selection of a molecular test and its utility include: (i) the local epidemiology of fungal pathogens; (ii) body site(s) of infection; (iii) appropriate specimen selection; (iv) method used to isolate and concentrate fungal DNA from different clinical specimens; (v) selection of the fungal target gene; and (vi) the amplification and detection method.[2,3] Regardless of the efficiency of a polymerase chain reaction (PCR) assay and the platform employed, its overall performance will be limited by the effectiveness of DNA extraction, which in turn is influenced by the specimen tested.[4] Here we discuss only the laboratory-related issues of importance.

PCR and other molecular tests are used to either (i) screen for a particular IFD, i.e. to pre-emptively diagnose this IFD in high-risk patient groups, or (ii) enable a definite diagnosis where an IFD is clinically evident. The selection of specimen to be tested, frequency of testing and result interpretation necessarily depends on the indication, as above, for testing. In both settings, PCR is often used to complement culture methods but may also represent the primary diagnostic approach.

Specimens

Determining the most appropriate specimen for PCR testing is usually straightforward but will depend on whether the assay is to be utilised for screening high-risk patients for IFD, or as a diagnostic test *per se*. Blood specimens (whole blood, plasma, serum) are easy to obtain and are the most widely used for screening for infection. The choice of blood fraction determines whether free circulating DNA is targeted during extraction, as for serum/plasma, or if cell-associated DNA is targeted as for whole blood.[4] Where DNA is predominantly cell-associated, large volumes (>3 mL) of whole blood should be centrifuged to obtain sufficiently concentrated DNA.[5] In the setting of pulmonary pathology, testing respiratory tract specimens other than lung tissue is often performed. Since these specimens are non-sterile, both pathogens and commensal fungi will be detected resulting in higher 'clinical' false positives.[5] Bronchoalveolar lavage (BAL)

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fluid is an example of a common specimen that is tested but it is not suitable for high-frequency screening.[4]

Conversely, the detection of fungal DNA from normally sterile tissue and other samples is diagnostic of IFD. This is particularly helpful in the diagnosis of mucormycosis where the aetiological agent may not be cultured.[6] Fresh tissue is preferred to paraffin embedded specimens.[3]

Gene target

Ideally the fungal gene target to be amplified should be: (i) present in multiple copies to maximise PCR sensitivity, and (ii) sufficiently conserved to allow amplification of target fungi, but with adequate sequence variation to define a particular genus or species. The majority of fungal PCR-based assays target one or more regions of the multi-copy (50–100 copies in the haploid genome) ribosomal DNA (rDNA) gene cluster comprising the 18*S*, 5.8*S* and 28*S* genes and the intervening internal transcribed spacer (ITS) regions, ITS1 and ITS2 (Fig. 1).[7] These regions contain both highly conserved and variable regions, allowing the design of universal primers within the conserved regions to amplify DNA from a large number of fungal species. At the same time, genus or species specific primers/probes can be designed based on the variable gene regions.[3] Since the ITS region is the most variable, it is the most likely to enable species identification. As such, it has been proposed as the primary fungal barcode marker by the Consortium for the Barcode of Life[8] (see later). Other useful multi-copy targets include mitochondrial cytochrome (*mtCytB*), alkaline proteinase, and cytochrome P450 lanosterol C-14α-demethylase genes.[3] In some instances, single copy genes, e.g., house-keeping candidates such as RNA polymerase I (*RBP1*), RNA polymerase II (*RBP2*),[8] β-tubulin (*BT2*), or translation elongation factor (*EF-1α)* may also be suitable targets.[9]

DETECTION OF FUNGI IN CLINICAL SPECIMENS

There are two main PCR-based approaches to detect fungi directly in clinical specimens: broad-range or panfungal assays, and more directed genus- or species-specific tests.

Panfungal approach

The use of panfungal PCR assays mirrors the growing need to detect a broad range of 'unknown' fungi in clinical specimens, as well as the more common pathogens, *Aspergillus* and *Candida* species. Ideally, the PCR target will be: (i) of a suitable product size (≤500 bp) to allow ease of sequencing; (ii) long enough to provide sufficient species-specific discriminatory information; and (iii) available in a public sequence database.[2] Identification of the PCR product is usually achieved by DNA sequencing or by the inclusion of genus- or species-specific probes and melting curve analysis.[10–15] Sequencing is time consuming but comprehensive, whilst assays based on probe design are targeted at specific pathogens and therefore fail to identify other fungi.

Lau *et al.*[10] developed a panfungal PCR assay targeting the ITS1 region of the rDNA gene cluster followed by sequencing to detect and identify fungal DNA in fresh and paraffin embedded tissue specimens from patients with culture- or histologically-proven IFDs. The assay identified a diverse range of fungi and was successfully applied to other specimen types including vitreous fluid and cerebrospinal fluid (CSF). In our hands, the panfungal PCR approach is complementary to culture and, as reported by others, most valuable for identifying fungi in culture negative, histologically-proven infection, where species identification helps guide antifungal therapy.[10,11,13,14] However, in our experience, the clinical utility of applying broad-range PCR to non-sterile samples, including BAL fluid, is poor due to amplification of commensal fungi, e.g., *Candida* species. We recently reviewed panfungal PCR results from 136 BAL fluid/washing specimens; 48% ($n=65$) tested positive by PCR, however all but two of the organisms identified were not considered to be clinically significant [*Candida* species ($n=35$), non-*Candida* yeast ($n=7$), saprophytic moulds ($n=6$), mixed fungi ($n=15$)] (Halliday *et al.,* unpublished).

Panfungal PCR assays have also been used to detect fungi in the blood of high-risk patients,[12,15–17] although the majority of the studies targeted patients with suspected IA and IC. Sugawara *et al.*[15] used a 18*S* rDNA-targeted panfungal PCR assay to prospectively screen blood for IFD ($n=64$ at risk episodes). They reported 44.4% of fungi detected were neither *Aspergillus* or *Candida* species, and in those cases PCR provided valuable information for selecting suitable therapies.

The greatest drawback of panfungal PCR assays is exogenous contamination of specimens and/or PCR master mixes by environmental fungal spores. To minimise contamination, it is essential that laboratory staff follow strict precautions

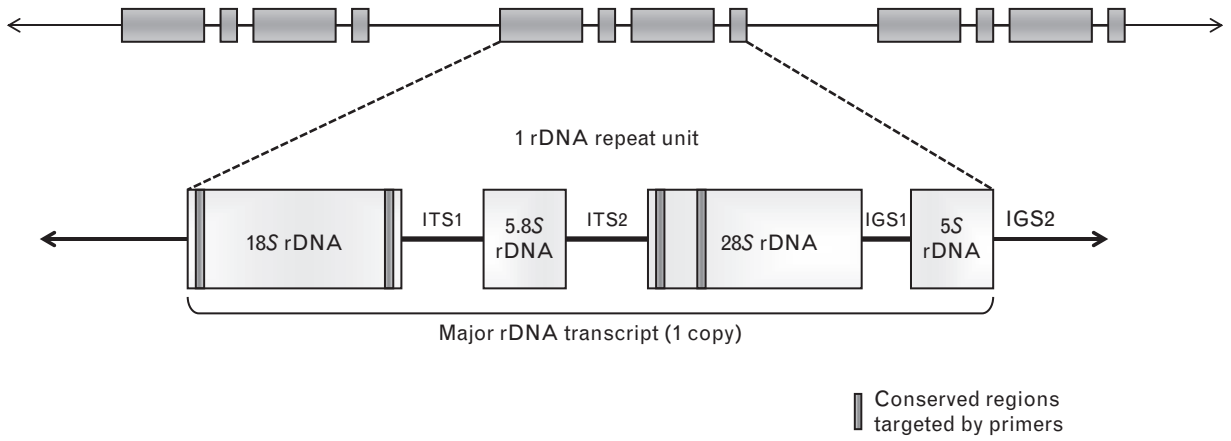

Fig. 1 Schematic diagram of the fungal rDNA gene cluster (adapted from CLSI[7]). The 18*S*, 5.8*S* and 28*S* rDNA genes are separated by the internal transcribed spacers 1 (ITS1) and 2 (ITS2). The 28*S* and 5*S* rDNA genes are separated by the intergenic spacer 1 (IGS1). The intergenic spacer 2 (IGS2) separates the repeat units from each other.