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Molecular diagnosis of endemic and invasive mycoses: Advances and challenges

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ABSTRACT

The diagnosis of endemic and invasive fungal disease remains challenging. Molecular techniques for identification of fungi now play a significant and growing role in clinical mycology and offer distinct advantages as they are faster, more sensitive and more specific. The aim of this mini-review is to provide an overview of the state of the art of molecular diagnosis of endemic and invasive fungal diseases, and to emphasize the challenges and current need for standardization of the different methods. The European *Aspergillus* PCR Initiative (EAPCRI) has made significant progress in developing a standard for *Aspergillus* polymerase chain reaction (PCR), but recognizes that the process will not be finished until clinical utility has been established in formal and extensive clinical trials. Similar efforts should be implemented for the diagnosis of the other mycoses in order to fully validate the current methods or reinforce the need to design new ones.

This manuscript is part of the series of works presented at the "V International Workshop: Molecular genetic approaches to the study of human pathogenic fungi" (Oaxaca, Mexico, 2012).

Diagnóstico molecular de las micosis endémicas e invasivas: avances y retos

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RESUMEN

El diagnóstico de las micosis endémicas e invasivas continúa siendo difícil. Hoy día, las técnicas moleculares para la identificación de los hongos desempeñan un papel importante y cada vez mayor en la micología clínica ya que ofrecen diversas ventajas, como su mayor rapidez, sensibilidad y especificidad. El objetivo de esta revisión es ofrecer una visión de conjunto de las técnicas moleculares más recientes para el diagnóstico de las micosis endémicas e invasivas, y destacar los retos y la necesidad actual de estandarizar los diferentes métodos. El grupo EAPCRI ha contribuido con avances muy significativos al desarrollo y estandarización de las técnicas de reacción en cadena de la polimerasa para el diagnóstico de aspergilosis, y reconoce que este proceso no estará terminado hasta que se establezca la utilidad clínica mediante ensayos clínicos, efectuados a gran escala y prolongados. Se precisan esfuerzos similares para implementar las técnicas moleculares de diagnóstico de otras micosis con el objetivo de validar por completo los métodos actuales o reforzar la necesidad de formular nuevos métodos.

Este artículo forma parte de una serie de estudios presentados en el «V International Workshop: Molecular genetic approaches to the study of human pathogenic fungi» (Oaxaca, México, 2012).

Endemic mycoses, candidiasis, aspergillosis, and other invasive fungal diseases (IFDs) continue to be significant sources of morbidity and mortality in immunocompromised and critically ill patients. Many studies indicate that delayed or inaccurate diagnosis and treatment are major causes of poor outcomes in these patients.[15,17,48,50] The diagnosis of endemic and IFDs remains challenging. Current fungal diagnostic techniques include traditional techniques, namely direct examination, culture, histology, antigen and antibody detection, but they have some limited sensitivity/specificity and incur significant delays in diagnosis. Current culture-based diagnostic tools continue to be the "gold standard"

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for diagnosis, but in many cases the results come too late to guide appropriate initial treatment of critically ill patients, and in many cases they remain negative.[49,53]

Recent efforts to improve the sensitivity and specificity of diagnostic tests have focused on culture-independent methods, in particular, nucleic acid-based methods such as polymerase chain reaction (PCR) assays.[33] Molecular techniques for identification of fungi now play a significant and growing role in clinical mycology as they offer distinct advantages in their ability to: (a) offer higher sensitivity and specificity than standard diagnostic approaches, aiding earlier diagnosis and initiation of antifungal therapy; (b) identify organisms that are seen on direct microscopic examination but do not grow on culture; (c) identify molds that do not produce microscopic reproductive structures (conidia or spores), produce only unrecognized structures, or identify yeasts that are not represented in the laboratory' commercial phenotypic identification systems database; and (d) definitively identify organisms whose characteristics closely resemble those of other fungi.[16] However, the present downside is that there is a lack of standardization of methods, results vary across laboratories and there is also often a lack of a collection of reliable, specific reference sequences. Availability of standard or commercial tests is also limited and for this reason many laboratories have developed their own "in house" methods.[33,48,53] There are currently no FDA-approved nucleic acid-based assays for fungal diagnosis.[48]

A consensus on the standardization of molecular techniques, along with validation from large prospective studies, is necessary to allow widespread adoption of these assays[33,66] and very importantly, to ensure that they become included as mycological evidence for the diagnosis of endemic and invasive fungal diseases by the guidelines of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG).[17] The value of PCR in aiding diagnosis is often overlooked. Exclusion from the EORTC/MSG criteria is related less to unsatisfactory performance than to the fact that little standardization is being done, so that reproducibility among laboratories is often not achieved.[68]

Challenges for molecular fungal diagnostic assays

To understand the challenges associated with any molecular diagnostic assay, one needs to understand the incidence and the pathology of the mycoses and the impact on the sample selection for the procedure (blood, serum, plasma, corporal fluids, or biopsy material, either fresh or paraffin embedded), and to carefully evaluate the basic steps of the molecular assay: first, nucleic acid extraction, second, selection of the fungal target(s) and third, amplification method.[33,68] While it is important to evaluate the combined performance of these processes it is also important to determine the performance of the individual processes; this overcomes the potential effect of combining, for example, a poor extraction technique with an optimal PCR test, leading to poor PCR performance.[68] DNA extraction and purification techniques will remove molecules other than fungal DNA that would interfere with the PCR reaction (e.g., hemoglobin and some anticoagulants inhibit the Taq DNA polymerase).

The success of fungal molecular identification depends on the choice of a reliable target sequence or gene. Optimal gene targets possess several characteristics which may also depend on the approach chosen. The targets are: (a) often present in multicopies, in order to provide good PCR sensitivity; (b) sufficiently conserved to ensure amplification from the desired range of fungi (for example, following amplification using panfungal primers, variable regions within the amplicon are desirable for species and genus specific identification); (c) of optimal size (approximately 500 bp), e.g., short enough to be easily sequenced but long enough to provide adequate information for identification; (d) present in sequence databases, for reliable comparison and accurate interpretation of results. The majority of fungal assays target multicopy loci, in particular, the ribosomal DNA (rDNA) genes (18S, 28S, and 5.8S) and the intervening internal transcribed spacer or ITS regions (ITS 1 and ITS 2), in order to maximize the yield of amplified DNA and allow high sensitivity.[16,46] The ITS 1 and ITS 2 regions are relatively variable among species, making them good target for species identification. Other targets commonly used for fungal identification include β-tubulin, calmodulin, the D1/D2 region of the 28S rDNA, and elongation factor (EF-1α).[14,16]

Simulated samples are often chosen for standardization and for quality control (QC) and they should contain quantities and targets representative of the clinical scenario as well as both negative and positive samples. The sample matrix should be also screened for contamination before use and, very importantly, it is essential to use strict aseptic conditions and clean room facilities (e.g., laminar air flow) in order to prevent preparation-borne contamination.[16,65,68]

The DNA amplification and detection procedure could be manual or involve commercially available real time PCR platforms or Luminex technology.[16,18,24,27,33,37,46,53,56,68] Among the current molecular methodologies, diagnostic laboratories and researchers have a broad spectrum of techniques from which to choose, from identification methods using amplification but not sequencing, such as PCR, PCR ELISA, nested PCR, real time PCR, fluorescence resonance energy transfer (FRET), microarrays, and repetitive-element PCR, to sequencing-based identification methods such as Sanger sequencing, pyrosequencing or next generation sequencing (NGS), and DNA barcoding.[16,19,58] As the need for sequencing grows, new high-throughput sequencing techniques are being developed at a rapid rate. More recently, commercial platforms have been also developed: PNA FISH® (peptide nucleic acid-fluorescence in situ hybridization), LUMINEX xMAP® (a platform that utilizes PCR amplification of the sample, followed by probe hybridization on an array of beads) and MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight), which is unique in that it does not depend on analysis of genetic information for identification of an isolate, but is instead based on analyses of the spectrum of organic biomolecules present in the isolate.[16,63]

It is important for researchers to be aware that currently there is little consensus on how best to perform and interpret quantitative real time PCR experiments. To solve this problem, MIQE guidelines have been proposed minimum information for publication of quantitative real time PCR experiments[13,29] their aims are to ensure the reliability of published results, enable integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. These guidelines should be incorporated in all new assays to be published and all efforts to standardize methods by any consensus group.

PCR-based assays for the detection of invasive aspergillosis (IA), invasive candidiasis (IC), endemic mycoses and other invasive fungal diseases are rapidly developing as sensitive tools for early diagnosis, although many challenges remain, including the need for proper molecular laboratory infrastructure. Much work still needs to be done to reach a consensus for extraction protocols, amplification targets and best approaches and platforms for measurement. Extensive clinical validation of the techniques is also much needed.

Nucleic acid based diagnostics for invasive aspergillosis

The amplification of *Aspergillus* DNA by PCR has been described since the early 1990s, and many studies on the topic have been