



## Mycologic Forum

# Molecular diagnosis of endemic and invasive mycoses: Advances and challenges



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## ARTICLE INFO

### Article history:

Received 22 August 2013

Accepted 27 September 2013

Available online 16 November 2013

### Keywords:

Endemic mycosis

Invasive mycoses

Polymerase chain reaction

Molecular diagnosis

Standardization

## 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).

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

## 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).

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### Palabras clave:

Micosis endémicas

Micosis invasivas

Reacción en cadena de la polimerasa

Diagnóstico molecular

Estandarización

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.<sup>15,17,48,50</sup> 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.<sup>49,53</sup>

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.<sup>33</sup> 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's commercial phenotypic identification systems database; and (d) definitively identify organisms whose characteristics closely resemble those of other fungi.<sup>16</sup> 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.<sup>33,48,53</sup> There are currently no FDA-approved nucleic acid-based assays for fungal diagnosis.<sup>48</sup>

A consensus on the standardization of molecular techniques, along with validation from large prospective studies, is necessary to allow widespread adoption of these assays<sup>33,66</sup> 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).<sup>17</sup> 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.<sup>68</sup>

### 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.<sup>33,68</sup> 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.<sup>68</sup> 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.<sup>16,46</sup> 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  $\beta$ -tubulin, calmodulin, the D1/D2 region of the 28S rDNA, and elongation factor (EF-1 $\alpha$ ).<sup>14,16</sup>

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.<sup>16,65,68</sup>

The DNA amplification and detection procedure could be manual or involve commercially available real time PCR platforms or Luminex technology.<sup>16,18,24,27,33,37,46,53,56,68</sup> 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.<sup>16,19,58</sup> 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<sup>®</sup> (peptide nucleic acid-fluorescence in situ hybridization), LUMINEX xMAP<sup>®</sup> (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.<sup>16,63</sup>

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<sup>13,29</sup> 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

published. However, as mentioned earlier, protocols vary widely with respect to the PCR assays used, sample target and DNA extraction methods, and this variability makes it very difficult to compare the different “in house” methods developed through the years.<sup>20,22,33,68</sup> There are only a few standardized assays that are commercially available. They include LightCycler SeptiFast (Roche Diagnostics, Basel, Switzerland), MycXtra (Myconostica, Manchester, UK), and the Affigene® *Aspergillus* tracer (Cepheid, Sunnyvale, CA, USA), and their preliminary clinical validations look promising, although patient numbers are limited when compared to the more frequent evaluations of “in-house” protocols.<sup>71</sup>

The variety of different DNA PCR-based assays of blood for detecting *Aspergillus* species have shown a wide range of sensitivities, ranging from 36% to 100%.<sup>33,65</sup> Of importance, the positivity of two serial samples helps to define positivity but when a simple sample is measured the specificity is lowered.<sup>41</sup> Independent meta-analysis studies show that performance of PCR assays can be comparable to that of other biomarkers, e.g., galactomannan and  $\beta$ -D-glucan that use a standard commercial approach;<sup>31,35,41</sup> this suggests and encourages that standardization of an optimal PCR protocol could lead to superior performance. Some other independent studies have already suggested that PCR based assays appear to be more sensitive and more specific for the diagnosis of IA than other assays and that when they are combined, they can improve sensitivity and the negative predictive value (NPV).<sup>23,59</sup> In addition to blood, PCR can also be used to detect *Aspergillus* in BAL, respiratory tract biopsy and even sputum. A 100% sensitivity and 86% specificity were reported on CT-guided lung biopsy specimens, compared with 88% and 94% for the galactomannan assays.<sup>34</sup> However, obtaining BAL specimens and biopsies may not be possible for a variety of reasons including thrombocytopenia in critically ill patients.

The UK and Ireland fungal PCR consensus group offered a first approach to compare PCR assays developed by different groups as “in house methods” for *Candida* and *Aspergillus*, and found that *Candida* PCR methods provide comparable and satisfactory performance, achieving a reproducible detection limit of 10 organisms.<sup>66</sup> The results for *Aspergillus* PCR methods were more variable: two optimal protocols were assessed by multicenter evaluation (10 centers), and the performance of the assays varied with both PCR platform (LightCycler [Roche], Rotor-Gene [Corbett Research, Mortlake, NSW, Australia], and TaqMan [Applied Biosystems, Foster City, CA, USA]) and sample type.<sup>66</sup> The type of platform used was found to have a major influence on the assay; sensitivity and NPV were all 100% on the TaqMan machine, whereas specificity and positive predictive value (PPV) were all 100% using the Rotor-Gene system. An Austrian–German consensus collaboration involving six laboratories evaluated DNA extraction and PCR methods currently in use and under research for the detection of *Aspergillus* spp. It appears that real time PCR-based assays in combination with effective lyses of the fungal cell wall show high inter-laboratory reproducibility.<sup>54</sup> The two studies mentioned highlighted the need for standardization, and soon after the UK and Ireland consensus group’s publication in 2006,<sup>66</sup> a European *Aspergillus* PCR Initiative (EAPCRI, [www.eapcri.eu](http://www.eapcri.eu)) was created, partly supported by the International Society of Human and Animal Mycoses (ISHAM). The aim of the initiative was to establish a standard for PCR methodology for the diagnosis of IA and to validate this in clinical trials, so that PCR could be incorporated into future consensus definitions for diagnosing invasive fungal disease. EAPCRI has over 62 participating centers from 25 countries in four different continents (Europe, North and South America and Australia) and is now a global initiative with several publications and important contributions to the field.<sup>67,69,70</sup> Since the foundation of the working party, four panels of extracted DNA and spiked blood samples have been distributed to the participating laboratories.

Results showed that the extraction of DNA appears to be the major obstacle rather than the various PCR techniques, which performed consistently.<sup>67</sup> The group could demonstrate that mechanical lyses of *Aspergillus fumigatus* conidia are superior to enzymatic lyses, and that a blood volume of at least 3 ml offers a better yield than 0.2–1 ml blood; furthermore, real time PCR assays, independent of the type of assay, are superior to conventional PCR assays.<sup>67,70</sup> The centers compliant with the EAPCRI recommendations showed further improvements in sensitivity and specificity reaching 88.7% and 91.6%, respectively when testing the distributed panel. On completing whole blood PCR standardization the group also evaluated serum samples. It was hypothesized that simple nucleic acid extraction protocols could be used with serum specimens. For this purpose, commercial kits provide greater standardization and QC. These are necessary prerequisites for widespread use of PCR outside specialist molecular mycology laboratories.<sup>69</sup> Initially, when evaluating whole blood, less than half of methods were able to attain the designated threshold. For serum testing, this figure rose to 82.8%, confirming the hypothesis that methods testing serum required less standardization. Overall sensitivity was 86.1% and specificity 93.6% and these values were comparable to those for whole blood PCR, compliant with EAPCRI recommendations.<sup>69</sup> Significant positive associations were noted between sensitivity and the use of larger sample volumes ( $\geq 0.5$  ml), an internal control PCR, and PCR assays targeting the ITS region. Significant negative associations were noted between sensitivity and eluting nucleic acid in volumes  $>100 \mu\text{l}$  and using PCR assays targeting mitochondrial regions.<sup>69</sup>

The EAPCRI group is currently collaborating with the Invasive Aspergillosis Animal Model (IAAM) group, which is developing standardized in vivo models of IA to provide representative pathological samples, and with the *Aspergillus* Technology Consortium (AsTeC), which is developing a bank of samples from well documented patients with IA to provide source material for evaluating future diagnostic tests.<sup>69</sup>

In addition, samples for PCR testing are currently being collected in two prospective trials, one comparing pre-emptive and empirical strategies and the other focusing on the prevention of invasive fungal infections in subjects receiving chemotherapy for acute lymphoblastic leukemia.<sup>68</sup> These studies will be crucial to accurately determine the clinical utility of PCR as a diagnostic tool for IA.

### Nucleic acid based diagnostics for invasive candidiasis

To date, a variety of nucleic based approaches have been studied for the diagnosis of IC, using a wide range of DNA extraction methods, primers and amplification and measurement approaches, and varied results have been reported for sensitivity (33.3% up to 100%) and specificity (greater than 75% with the exception of some nested PCR assays); see review by Kourkoumpetis et al.,<sup>33</sup> PPVs are also extremely variable, with most of the studies reporting PPVs of approximately 50%. Of importance, NPVs are more consistent and range from 88 to 100%.<sup>33,48,49,53</sup> As for the molecular diagnosis of IA, comparing the varied methodologies that have been used by the different groups reporting “in house methods” is a difficult task. This is well illustrated in a recent review by Kourkoumpetis et al.<sup>33</sup> and also in a systematic review of the literature available by Avni et al.<sup>1</sup> Some groups have dedicated efforts to the comparison of DNA extraction protocols and also compared serum and whole blood as specimens for the detection of *Candida* DNA in critically ill patients,<sup>42,43</sup> but no further validation of these methodologies with other centers have been done.

It is important to mention that best results for diagnosing IC (high sensitivity, specificity, and positive and NPVs) have been reported in studies using real time PCR<sup>40</sup> and when using in situ hybridization with commercial probes<sup>72</sup> for *Candida* spp. in blood.

McMullan et al.<sup>40</sup> conducted a rigorous study with 157 critically ill patients but honestly stated that only 23 of the patients had proven *Candida* infection following the guidelines of the consensus group EORTC and MSG. Two TaqMan-based real time PCRs were developed to detect the main medically important *Candida* species, with target gene 18S for *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida dubliniensis* and a target region spanning ITS 1, 5.8S, and ITS 2 for *Candida glabrata* and *Candida krusei*. Overall sensitivity was reported to be 87%, specificity 100%, PPV 100% and NPV 99.6%. The very high sensitivity afforded by the real time PCR approach is particularly appealing, given a report that >50% of initial positive blood cultures for *Candida* species had <1 CFU/ml.<sup>51</sup> A recent study reported real time PCR was even more sensitive than the  $\beta$ -D-glucan assay in diagnosing candidemia, and deep-seated candidiasis with comparable specificity.<sup>47</sup>

A very good illustration of how diverse techniques, samples, fungal target(s), and amplification methods have been used is described in a recent review and meta-analysis that included 54 studies with a total of 4694 patients, 963 of whom had proven/probable or possible IC.<sup>1</sup> Serum samples were used in 13 studies and blood in 41. Amplification methods included PCR in 23 studies, nested PCR in 16, and real time PCR in 16. Fungal targets were 18S in 32 studies, 5.8S and 28S in 8, other rRNA in 2, cytochrome P450 *LAL1* genes in 6, and other genes: *SAP*, *EO3*, *HSP*, *ERG11*, *CHS1*, *ACT1* ( $n = 1$  each). The meta-analyses concluded that sensitivity and specificity were very good when the sample included only patients with proven candidemia, but sensitivities tended to decrease when the patients were with probable or possible invasive candidemia. Sensitivity for proven or probable candidiasis was 85% (78–91%) when compared with blood cultures 38% (29–46%). Reported specificity for the same patients was above 90%. However, PCR performance was observed to correlate with the degree of certainty of the diagnosis. The use of whole-blood samples, rRNA, or P450 gene targets and a PCR detection limit of <10 CFU/ml were associated with improved test performance.<sup>1</sup>

It is necessary to reinforce the need for standardization of methods and also that authors use standard definitions of IC such as those well described by the consensus group of the EORTC and MSG.<sup>17</sup>

Drawbacks of PCR and other nucleic acid based diagnostics for IC are that they are prone to false-positive results (for example due to an inability to distinguish colonization or contamination from real infection or disease) that they are often not standardized, and that until very recently they were not commercially available. New commercial assays are now becoming available, e.g., the LightCycler SeptiFast assay (Roche, Molecular Diagnostics), a multiplex real time PCR assay that is able to detect a wide range of bacterial and fungal organisms commonly involved in systemic infections, and among the yeasts it identifies are *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis*.<sup>7</sup> Further studies are needed to validate these assays and to determine whether or not the information provided would translate into tangible clinical benefits.

### Nucleic acid based diagnostics for endemic mycoses

Several molecular methods have been described for the diagnosis of endemic and systemic mycoses including histoplasmosis, paracoccidioidomycosis, blastomycosis, and coccidioidomycosis. However, all PCR assays have been developed as “in-house” methods and have not been yet established as regular diagnostic tools, there is so far no nucleic acid assay commercially available for use in clinical samples, and the existing assays have not yet been fully standardized and validated by multi-centric studies.

Comparing with the number of publications available for the molecular diagnosis of IA and IC respectively, less attention has

been given to the molecular diagnosis of endemic mycoses but current efforts are underway to advance the field. Genes coding for specific proteins or the ITS regions of the rDNA have been used as targets for designing and developing the “in house” PCR-based molecular assays described in the literature. Molecular tests include conventional PCR, nested PCR, and real time PCR.

Culture identification tests based on chemiluminescent DNA probes targeting multicopy ribosomal genes are commercially available (The AccuProbe tests, Gen-Probe, San Diego, CA) for the detection of *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Coccidioides immitis*. These tests are generally sensitive and specific, although pretreatment of isolates with formaldehyde leads to false-negative results. False-positive *B. dermatitidis* Gen-Probe results were obtained with *Paracoccidioides brasiliensis*. The test does not distinguish between the two species of *Coccidioides*.<sup>6</sup>

### Histoplasmosis

Genes coding for specific proteins such as H and M antigens, Hcp-100 and some ITS regions of the rDNA have been used as targets for developing “in house” assays. The most common gene coding for a specific protein used for molecular diagnosis of histoplasmosis has been the gene coding for a specific 100 kDa protein (Hcp100), which had been described as essential for survival of *H. capsulatum* in human cells.<sup>52</sup> Bialek's group reported in 2002 a method using two nested PCR assays that allowed detection of *H. capsulatum* DNA in 100 human formalin-fixed and paraffin-embedded tissues (50 of them positive by stains for *H. capsulatum*).<sup>2</sup> The first of these assays used specific primers targeting the gene coding for the specific Hcp100 that had been described by Porta et al.<sup>52</sup> and the second assay used primers for the 18S rRNA gene. However, products had to be confirmed by sequencing, which in a clinical context would increase costs and delay the reporting of results. Other authors have described the use of the same nested PCR targeting the Hcp100 gene in a variety of human samples including respiratory specimens and fresh biopsies,<sup>39,45,55</sup> reporting sensitivity values varying between 86 and 100% and specificity values between 92 and 100%. When using total blood,<sup>64</sup> sensitivity was reported to be 89% and specificity 96%. A real time PCR assay to detect *H. capsulatum* in formalin fixed paraffin embedded tissues (FFPE) that targeted the specific Hcp100 gene was described, giving an analytical sensitivity of 6 pg/ $\mu$ l of fungal DNA, sensitivity 88.9% and specificity 100%.<sup>32</sup>

The M antigen has been used to detect *H. capsulatum* DNA from culture (31 isolates)<sup>26</sup> with sensitivity and specificity of 100%. A semi-nested PCR assay using a sequence of the gene coding for the H antigen was evaluated in 30 clinical samples (just six of them proven by culture) and the assay reported 100% sensitivity and 92% specificity.<sup>5</sup> The use of these gene targets remains very limited at this time in spite of their promise as targets for diagnosis, and definitely more evaluation is needed. The ribosomal ITS region(s) have been used as targets by different groups. Buitrago et al. designed a real time PCR assay and reported 100% sensitivity in two different studies,<sup>9,11</sup> although the numbers of clinical samples that were proven by culture for *H. capsulatum* were very low (10 samples from 4 patients in the first study and 17 samples from 9 patients in the second study). Martagon-Villamil et al.<sup>38</sup> also tested a real time PCR assay targeting the ITS in 107 cultured fungal isolates and identified *H. capsulatum* in 34 of them. Simon et al.<sup>61</sup> more recently targeted the ITS region and developed also a real time PCR protocol using the TaqMan platform (Roche). They evaluated 348 human samples submitted for routine diagnosis and reported 95.4% sensitivity and 96.0% specificity.

The first approach to compare the “in house” protocols developed by the different groups was conducted recently.<sup>8</sup> A panel containing DNA from *H. capsulatum*, as well as negative and

cross-reaction controls, was sent to the 5 different laboratories. Different PCR protocols were tested, nested PCR ( $n = 3$ , same assay), SCAR220 PCR ( $n = 1$ ), real time PCR ( $n = 2$ , different assays). Overall sensitivity was 86% and specificity was 100%. The SCAR220 protocol, which is based on a unicity target, presented lower sensitivity (43%). The real-time protocols tested were highly reproducible, sensitive, and specific and the authors concluded that real time PCR seems to be a promising tool to efficiently detect *H. capsulatum* in clinical samples.

#### Paracoccidioidomycosis

The number of “in house” tests developed and evaluated so far for the detection of *P. brasiliensis* in cultures and clinical samples is very limited. Amplification and sequencing of rDNA regions, especially 5.8 and 28S subunits, as well as intergenic spacer regions have been successfully developed and enabled discrimination between *P. brasiliensis* and other human pathogenic fungi by PCR using DNA extracted from culture.<sup>44</sup> A real time PCR targeting the ITS 1 region was developed to detect *P. brasiliensis* DNA in both cultures and in 10 patients' clinical specimens. Real time PCR was positive in all the culture strains tested, as well as in clinical specimens (sputum and biopsies), but was less useful for blood. Although this molecular test was evaluated with a low number of patients, the authors reported 100% sensitivity and specificity.<sup>10</sup>

The gp43 is considered the immunodominant antigen for the diagnosis of paracoccidioidomycosis, and the gene coding for this glycoprotein has been used as molecular target to detect *P. brasiliensis*.<sup>3</sup> A nested PCR assay to amplify the gp43 gene was evaluated using an experimental murine model of paracoccidioidomycosis, and the assay was positive in 91% culture-positive lung homogenates from *P. brasiliensis* infected-mice and did not cross react with other pathogens. An assay based on the 5' nucleic acid assay using a fluorescent probe derived from the sequence of the gene coding for the gp43 antigen was developed,<sup>60</sup> sensitivity and specificity were reported as 100% and the assay could detect at least 10 copies of this DNA sequence.<sup>60</sup> A loop-mediated isothermal amplification (LAMP) assay has been tested for its ability to detect the gp43 gene of *P. brasiliensis*,<sup>21</sup> and positive results were reported for DNA extracted from FFPE tissue samples from paracoccidioidomycosis patients.<sup>21</sup> In another study, respiratory samples were evaluated by LAMP, with reported sensitivity and specificity values of 61% and 100% respectively.<sup>62</sup>

#### Blastomycosis

As for paracoccidioidomycosis, the number of “in house” tests developed and evaluated is very limited. In addition to the AccuProbe test, specific probes complementary to the large ribosomal subunit or to the ITS-regions have been successfully used to identify isolates of *B. dermatitidis* showing specificities of 100%.<sup>57</sup> Universal fungal primers ITS1 and ITS4, designed from conserved regions of rDNA, have been used to amplify DNA from *B. dermatitidis* and from other important fungi that display yeast-like morphology; these primers were used together with probes that were found to be highly specific and should prove to be useful in differentiating of *H. capsulatum*, *C. immitis*, *P. brasiliensis*, *Penicillium marneffei* and *B. dermatitidis* in clinical settings.<sup>36</sup> In situ hybridization using a pair of two oligonucleotides complementary to the 18S and 28S rDNA was used to evaluate 98 archived FFPE tissue specimens, all of them proven by culture and including *B. dermatitidis*, *C. immitis*, *C. neoformans*, *H. capsulatum*, and *Sporothrix schenckii*, and gave a sensitivity of 95% and specificity of 100% for *B. dermatitidis* identification.<sup>28</sup> A nested PCR assay targeting the gene encoding the species-specific *B. dermatitidis* adhesion molecule (*BAD-1*), formerly referred to as *WI-1*, was reported to be highly specific, and with the detection

limit of 0.1 fg DNA was comparable to the 18S rDNA PCR.<sup>12</sup> The latter is routinely used as a screening assay, and if positive, the specific nested PCR targeting *BAD-1* is performed to confirm the diagnosis.

#### Coccidioidomycosis

As for the above endemic mycoses the number of “in house” tests developed and evaluated to date is very limited. In addition to the AccuProbe test for the identification of cultures, species-specific primers complementary to the ITS region of *C. immitis* were designed.<sup>25</sup> After testing against members of the order Onygenales and several species of the genera *Penicillium* and *Aspergillus*, primers were found to be specific as they amplified DNA from at least 30 *C. immitis* isolates.<sup>25</sup> The use of in situ hybridization to identify *C. immitis* in FFPE tissue sections and targeting variable regions of the 18S and 28S ribosomal RNA showed a sensitivity of 94.3%, and specificity and positive predictive values of 100%.<sup>28</sup> In addition, a PCR assay for *Coccidioides* amplifying DNA from serum samples has been described.<sup>30</sup> A nested PCR and a real time PCR were developed targeting the genus-specific antigen2/proline rich antigen of *Coccidioides* spp.<sup>4</sup> Both PCR assays correctly identified 120 clinical isolates tested, which had been identified as *Coccidioides posadasii* according to length of microsatellites.

#### Conclusion

Molecular diagnostics brings many challenges to laboratories and clinicians. Rapid and accurate identification to the genus and species levels of fungal pathogens is crucial for correct management of fungal infections. Better understanding of the strengths and weaknesses of currently available diagnostic tools, and further strategies devised to best implement them either individually or in combination, would greatly improve early and accurate diagnosis of endemic and invasive fungal diseases and should improve their successful management. The EAPCRI has made significant steps in developing a standard for *Aspergillus* PCR but recognizes that the process will not be finished until the 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.

#### Conflict of interest

The author declares no conflict of interest.

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