A review of nucleic acid-based diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays

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Nucleic acid-based assays have good potential to complement and enhance the sensitivity and rapidity of conventional methods used in diagnostic mycology. The majority of molecular tests are polymerase chain reaction (PCR)-based assays focusing mainly on the detection of Candida and Aspergillus spp. from clinical samples. DNA extraction and purification procedures should be standardized and can be facilitated by using commercial extraction kits. In general, protocols that target multi-copy genes provide the greatest sensitivity. Objective endpoint assessments of PCR tests using enzyme-linked immunosorbent assays (ELISA) or commercial quantitative systems are capable of rapidly detecting and identifying Candida and Aspergillus spp. Sequencing of PCR products can be used to confirm the identity of amplicons. In cases of suspected invasive aspergillosis, PCR should be performed on both blood and bronchoalveolar lavage fluid to maximize test sensitivity and the positive predictive value. At least two blood specimens should be tested if PCR is undertaken on blood samples alone. In situ hybridization techniques have been used with success to identify fungi in tissue specimens. The wide application of PCR-based assays relies on the introduction of standardized protocols following their evaluation in multicentre, prospective studies.

Keywords diagnosis, fungi, molecular, polymerase chain reaction

Introduction

Systemic mycoses have emerged as important causes of morbidity and mortality in hospitalized patients and individuals in the community [1,2]. This is a result of advances in medical treatment and increasing numbers of immunocompromised patients, particularly recipients of haemopoietic stem cell transplants (HSCT). Systemic fungal infections include disease caused by opportunistic yeasts and moulds as well as by dimorphic fungi.

In the United States of America, the percentage of nosocomial bloodstream infections (BSIs) caused by fungi has risen from 5.4% in the 1980s to 9.9% in the 1990s, with 8–10% of all BSIs due to Candida species [1,3–6]. Although most episodes of candidaemia and other forms of invasive candidiasis (IC) are still caused by Candida albicans, the proportion of infections caused by non-C. albicans Candida spp. has risen substantially [7–9]. This has important diagnostic implications for the clinical laboratory [7,10].

A recent report on the first population-based estimates of the incidence of invasive mycoses in California, USA confirmed the pre-eminence of Candida, and to a lesser extent Aspergillus among the invasive mycoses [11]. Invasive aspergillosis (IA) is known to occur in 2.6–10.3% of HSCT recipients [12]. The study also provided data on the frequency of infections caused by previously uncommon fungi, which are increasing among immuno-
compromised patients. The causative fungal agents include the zygomycetes, hyaline filamentous fungi (such as Fusarium spp., Pseudallescheria boydii, Scedosporium prolificans), dermatiaceous moulds and the yeast-like Trichosporon spp. [13–15]. These mycoses are often difficult to diagnose and are resistant to many antifungal agents.

Mortality rates attributable to IC range between 38 and 74% [16] and those figures are even higher for mould infections [17,18]. Case fatality rates reported for IA range from 56 to 88.1% [18,19]. Early institution of antifungal therapy in patients with IA is critical to improving outcome [12,20]. Timely diagnosis of systemic fungal disease, however, may be hampered by the limitations of current diagnostic methods.

The modern clinical mycology laboratory has to play a key role in the early and reliable detection of fungi from clinical specimens and in improving existing methods of identification of uncommon fungal agents. It is strategically placed to assist in epidemiological investigations of disease in which rapid diagnosis is critical in determining the source of infection. Conventional histological, culture and sometimes serological-based, diagnostic methods are widely used but are not without limitations. Some of these limitations are outlined in the context of the need to develop alternative diagnostic methods such as nucleic acid-based approaches.

The main body of this review discusses and compares the nucleic-acid based techniques that have been developed, or those which are undergoing development, for the diagnosis of systemic mycoses. A review of the literature reveals that the majority of nucleic acid-based methods focus on the early detection of Candida and Aspergillus spp. from clinical samples as these fungi account for the greatest morbidity among patients. There is comparatively little data on polymerase chain reaction (PCR)-based methods to detect other fungi but it is envisaged that these techniques will be increasing applied for diagnostic purposes. Part A of this review addresses the parameters influencing the success of a PCR protocol and includes aspects of recent technological advances. Part B reviews the major PCR and non-PCR-based methods that have been developed for diagnostic mycology.

**Histological, culture and serological-based methods of fungal diagnosis**

*Histopathology and culture*

Diagnosis of invasive fungal infection is a problem because of the nonspecific clinical and radiological manifestations of the disease. Although, for example, timely computed tomography (CT) scanning of the chest may be useful in the diagnosis of invasive pulmonary aspergillosis (IPA), many patients still require invasive diagnostic procedures. Diagnosis thus relies on histological demonstration of the fungus invading affected tissue and/or its isolation by culture. Because the histological appearances of certain fungi can be nonspecific, definitive diagnosis typically requires culturing the fungus.

Culture methods, however, may be limited to detecting the fungal agent at an advanced stage of disease with some fungi requiring several weeks to grow. In addition, considerable expertise is needed for the correct morphological identification of the fungal agent. Blood cultures, probably the most reliable marker of IC, are estimated to be positive in only 58% of cases. Furthermore, failure to grow Candida spp. in blood from neutropenic patients is not unusual [21,22]. The yield of moulds from blood cultures is highest for Fusarium spp. (40–60%) [23], but Aspergillus spp. are rarely isolated from blood [24]. Certain fungi, such as the zygomycetes, also often fail to grow from clinical specimens. Culture of bronchoalveolar lavage (BAL) fluid is reported to be positive in 30–50% of cases of IPA [25] but, in general, cultures of respiratory secretions from patients with IA are frequently negative for fungi [26,27]. The predictive value of positive respiratory tract cultures for Aspergillus spp. in cases of IPA also varies substantially (40–100%) [28]. Moreover, environmental contamination of specimens with Aspergillus spp. is not uncommon when they are taken from a nonsterile site.

Identification of cultured fungi can be problematic with the expanding range of pathogens. Although significant improvements in commercial biochemical identification systems have been made, these tests are only useful in the identification of yeasts. The review by Freydiere et al. [29] details the merits of these methods.

*Serology*

Despite slow progress in the development of immunodiagnostic methods to detect fungal antigens, antibodies or metabolites in clinical specimens, selected tests have shown promise in the management of patients with fungal infection. For a detailed description of diagnostic serological methods for invasive mycoses, see Walsh & Chanock [30].

Serological tests make an important contribution to the rapid diagnosis of cryptococcal infection. Commercial latex agglutination and enzyme-linked immunosorbent assays (ELISA) kits are available to detect cryptococcal polysaccharide antigen in sera, cerebrospinal fluid (CSF) and other body fluids. These tests are

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highly sensitive and specific (> 95%) and their utility is comprehensively discussed by Casadevall & Perfect [31]. Conversely, the clinical applicability of assays to detect candidal mannan, a component of the candidal cell wall, in the serum of patients with suspected IC has been hampered by poor sensitivity [32,33]. Only one study has found the detection of serum mannan to be useful in identifying patients at increased risk for IC [34]. Tests measuring the metabolite, D-arabinitol, in serum or the determination of the D/L-arabinitol ratio in urine, appeared promising but their clinical utility has subsequently been found to vary substantially between studies [35,36]. Assays to detect anti-mannan antibodies in patients with IC have poor sensitivity and specificity but limited data indicate more encouraging results with a test that detects anti-germ tube antibodies [37].

Measurement of the plasma concentration of (1-3)-β-D-glucan, another component of all fungal cell walls (except zygomycetes) by an ELISA (Fungitec G test, Seikagaku Corp., Tokyo, Japan) is achievable with high sensitivity (90%) and specificity (85–100%) [38,39]. The test detects infection caused by a range of fungi including Candida and Aspergillus spp. but cannot distinguish between disease caused by the different fungi.

The role of Aspergillus galactomannan detection in the management of patients at risk for IA continues to generate much interest. Galactomannan can be detected in body fluids including blood and BAL, and appears to be useful for the early diagnosis of IA. Indeed, testing serum and/or plasma samples in high-risk patients is routinely performed in many European centres, with ELISA supplanting agglutination techniques because of its greater sensitivity. The sandwich ELISA, Platelia Aspergillus (BioRad, Marne La Coquette, France) is widely available and is reported to have a sensitivity of 50–90% and a specificity of 81–93% when used on serum [40,41]. Where serial monitoring was performed, a higher sensitivity (92.6%) and specificity (95.4%) has been observed, with antigen detected prior to the onset of clinical disease in 63% of patients [42]. Others have found the test to be inadequately sensitive [43]. False-negative (8–10% of sera) and false-positive reactions (8%) occur, interlaboratory reproducibility is suboptimal [42,44] and the performance criteria of the test using BAL are undefined. Thus, the precise role of screening asymptomatic patients at risk for IA for galactomannan, and of monitoring treatment responses are yet to be delineated.

With regard to the diagnosis of histoplasmosis, a promising new ‘dip-stick’ test in which the Histoplasma capsulatum-specific ‘M’ antigen is affixed onto a nylon membrane strip has been developed for use in epidemiological surveys [45]. A Western blot test which utilizes the deglycosylated ‘M’ antigen is also undergoing evaluation as a diagnostic tool. In a pilot study, this test identified early infection with a sensitivity and specificity of 100 and 91.2%, respectively [46].

**Nucleic acid-based diagnostic methods. Part A: General considerations**

The limitations of culture, histological and serological techniques, as outlined above, have led to the search for more sensitive, nonculture-based methods. The majority of nucleic acid-based fungal detection and identification systems are PCR based. PCR techniques offer the potential for the rapid and early detection of fungi. Assays may be designed to detect a specific fungus or a range of fungi within a single test.

The parameters influencing the clinical usefulness of a PCR assay include: (i) the method developed to isolate and concentrate fungal DNA from clinical specimens; (ii) the sensitivity and specificity of the assay to rapidly detect and identify DNA in a variety of specimens; (iii) the need for repeated sampling of clinical specimens for PCR in a particular fungal disease; and most importantly, (iv) the relevance of the test result, namely, whether PCR is likely to predict infection before other diagnostic tests.

**Factors affecting performance of a PCR assay**

A general problem with PCR-based fungal detection methods is the lack of standardized, and commercially available systems. Many studies report good performance characteristics with their ‘in-house’ PCR assays. The relative merits of different assays, however, are difficult to assess because few head-to-head comparisons have been made. The lack of standardization limits the broad use of PCR-based methods in clinical practice. For instance, various methods of sample preparation and different DNA targets are often chosen for PCR. Furthermore, different specimens may be submitted for PCR. PCR assays have been applied to purified fungal DNA, stock fungal cultures, blood ‘spiked’ with fungi, or to clinical specimens such as blood fractions (serum vs. plasma vs. whole blood), BAL fluid and tissue. All these factors should be considered when comparing different PCR protocols.

With the above in mind, a standardized approach for the five major components of a PCR assay is recommended: (i) sample preparation; (ii) selection of the ‘target’ region and subsequent primer design; (iii) post-PCR detection methods to identify the amplified product; (iv) standardization of reproducible PCR conditions and format (single step, multiplex or nested
PCR system); and (v) precautions to minimize false-positive and false-negative results.

Sample preparation

The suboptimal recovery of Candida spp. from blood cultures in immunocompromised patients has been alluded to. Despite the angio-invasive nature of many moulds, positive blood cultures are uncommon (< 10% in IA) [21]. Sample preparation has generally posed a challenge to the development of clinical PCR tests. Ideally, the chosen method should be simple and standardized, and must concentrate any fungal DNA present in specimens into an appropriately small volume for PCR, while removing interfering substances. In addition, the manner in which samples are collected and stored prior to performing PCR is important.

Specimen collection and preservation

In practice, whole blood is the commonest specimen submitted for PCR. Blood is usually inoculated into tubes containing a suitable anticoagulant such as heparin or EDTA. Lysis of erythrocytes and leukocytes is then necessary prior to DNA extraction. If the DNA isolation method involves the use of hexadecltrimethylammonium bromide (CTAB; see ‘DNA extraction and purification’), blood for PCR should be collected in tubes containing citric acid/sodium citrate, which, unlike heparin or EDTA, prevents blood from coagulating during the procedure [47].

Blood inoculated into lysis-centrifugation culture kits (Isolator, Wampole Laboratories Inc., Cranbury, NJ, USA) is also suitable for PCR and has the advantage that the lytic process is ‘standardized’ [48,49]. Fungal elements are recovered by a hot, alkali step and further processed. PCR is also possible if blood is incubated for up to 3 h in commercial blood culture bottles. This presumably dilutes the effect of potential inhibitors of PCR in blood and/or allows some natural amplification of the fungus. Blood culture broth is combined with a suitable buffer, boiled for 15 min and shaken with zirconium beads with a sample preparation time of 1.5 h. The method, however, requires 500 cfu ml⁻¹ Candida spp. to generate a positive PCR signal [50] and its ability to detect moulds has not been tested.

Patient sera are easy to acquire and store, and are processed by a protocol similar to that used for whole blood but without the need to lyse blood cells. Whole blood and serum samples should be processed in the shortest possible time after collection but both may be stored at −20 °C (−70 °C if stored for prolonged periods) prior to use. BAL fluid submitted for PCR to detect, for example, Aspergillus spp. should likewise be transported to the laboratory on ice, where possible processed at the earliest convenience or stored at −20 °C (or −70 °C) until used [51].

Importantly, formalin-free containers should be used to collect tissue specimens submitted for diagnostic PCR. This is critical as formalin is highly inhibitory to the action of Taq DNA polymerase. Specimens that require forwarding to a reference facility may be stored at −70 °C prior to PCR. Not uncommonly, samples are submitted only for histological examination, resulting in biopsy material being embedded in paraffin. Extraction of high molecular mass DNA from paraffin-embedded tissue is very problematic. The recent availability of the QIAamp DNA Mini kit (Qiagen Inc, Valencia, CA, USA) and other similar commercial spin columns has made them the current method of choice for extracting DNA from such tissue. The kit provides high-quality DNA for PCR [52]. However, the extracted DNA is usually of low molecular mass and results in a PCR product of not more than ≈ 700 bp. This should be taken into account when selecting the length of a target for PCR.

DNA extraction and purification

The requirements for extraction of fungal DNA vary with the nature of the specimen. The optimum specimen for PCR may also differ with the disease entity. Among blood fractions, although the collection of serum is the most convenient, circulating leukocytes are known to phagocytose fungal hyphae. Therefore, whole blood, plasma oruffy coat layer may result in higher DNA yields [53,54]. One study investigated the detection limit of a PCR assay to detect Aspergillus infection on different patient samples and found that the test had a higher sensitivity when performed on whole blood rather than plasma [55]. In another analysis of whole blood fractions, PCR was performed on centrifuged leukocyte fractions and on supernatants obtained from erythrocyte lysis. Experiments were carried out in quadruplicate. All four erythrocyte supernatants were PCR positive for Aspergillus DNA in contrast to two of four leukocyte fractions [21].

The different DNA extraction protocols described vary in their details but, in general, all involve: (i) disruption of the fungal cell wall; (ii) release of DNA by lysis of the cell membrane; (iii) lysis of white and red blood cells using either commercial or ‘in-house’ reagents and methods, if appropriate; and (iv) purification of DNA. Extraction techniques from all specimens should be standardized as fine variations in detail influence the recovery of DNA [21].

Many earlier protocols used the enzyme Zymolyase (Novozyme 234, ICN Pharmaceuticals, Costa Mesa, CA,
USA; or Mureinase, United States Biochemical Corp., Cleveland, OH, USA) to disrupt the fungal cell wall. This results in the formation of fungal spheroplasts, which have increased osmotic sensitivity [56]. Zymolyase efficiently releases DNA from yeasts such as Candida and Cryptococcus spp. [57], but it is ineffective in disrupting the cell walls of moulds, including Aspergillus spp. [21]. To overcome this obstacle, mechanical destruction with heat-alkali treatment [58] or with a combination of glass beads and repeated freeze thawing using liquid nitrogen [59] are necessary. Recently, another β-1,3-glucanase, lyticase (Sigma), has been shown to effectively generate spheroplasts in moulds [53]. Lyticase has greater activity against the cell wall of moulds [60] and is the preferred method of choice for disrupting the cell wall of moulds.

Lysis of cellular material in whole blood is, in most instances, required to release DNA for PCR. Protocols usually consist of a combined heat-alkali treatment procedure. Cell membrane lysis is then achieved by treatment with one or more of sodium dodecyl sulfate (SDS) and EDTA or proteinase K [53,61]. Alternatively, the QIAamp DNA Mini Kit (Qiagen) can be used. Sample preparation methods for sputum and/or BAL fluid submitted for PCR have also comprised the use of ‘in-house’ erythrocyte and/or leukocyte lysis buffers followed by treatment with SDS and EDTA or proteinase K [62–65]. Protocols for tissue specimens likewise involve a combined heat–alkaline–enzymatic lysis of the fungal cell wall, with or without the use of proteinase K and SDS [66,67].

Earlier PCR methods used standard phenol–choloroform extraction procedures to purify DNA [48,68]. Although reliable, the procedure is time-consuming. The availability of commercial DNA extraction kits has facilitated DNA purification by shortening the duration of the procedure considerably. One study evaluated the isolation of DNA from cultures of C. albicans and A. niger. Five DNA extraction kits and an ‘in-house’ combined heat-alkaline-enzymatic extraction protocol according to the method of Einsele et al. [61], were compared for sensitivity, purity of the DNA they produce, duration of procedure and cost [68]. The kits tested comprised the QIAamp Tissue Kit (Qiagen, now marketed as the QIAamp DNA Mini Kit), GeneReleaser (BioVentures, Murfreesboro, TN, USA), Puregene D 6000 (Genta, Minneapolis, MN, USA), Dynabeads DNA DIRECT (Dynal, Oslo, Norway) and DNAzol (Molecular Research Center, Cincinnati, OH, USA). All kits shortened the extraction procedure (35 h vs. 8 h). The sensitivity was best for the ‘in-house’ and QIAamp Tissue (1–10 cells ml⁻¹) kit, and lowest for DNAzol (1000 cells ml⁻¹). QIAamp Tissue yielded the same purity of fungal DNA as the ‘in-house’ protocol but was more expensive. Purity of DNA was least optimal using GeneReleaser and DNAzol. The QIAamp DNA Mini Kit (Qiagen) has been successfully applied to clinical specimens to purify fungal DNA. Loeffler et al. [69] tested 95 whole blood samples from patients with proven or presumed mycosis; 52 were PCR positive and 43 PCR negative using their ‘in-house’ method; the corresponding numbers using the above kit were 51, and 44, respectively. Many PCR protocols to detect both yeasts and moulds from whole blood, serum and tissue now use the QIAamp DNA Mini kit (Qiagen) as the preferred method for DNA purification with little or no loss in yield or quality [53,64,67–72]. In addition, the QIAamp Blood kit has been used successfully to extract Aspergillus DNA from serum [73].

CTAB, a strong cationic detergent that releases DNA from proteins by solubilizing cell membranes, has been used in another fungal DNA extraction method [47]. The method was first used to isolate yeast and A. fumigatus DNA using ‘spiked’ blood and sputum samples [47]. It has also been tested on vitreous uid from a patient with candidal endophthalmitis, on skin tissue from a patient with confirmed zygomycosis and on 10 blood samples from patients with candidaemia. Amplification of the expected PCR product was obtained in all instances [47]. A method whereby Aspergillus DNA can be extracted from whole blood without lysis of leukocytes or erythrocytes has been developed. This comprises a commercial total DNA capture method (Roche Diagnostics Corporation, Indianapolis, IN, USA) used with the QIAamp DNA Mini Kit (Qiagen) [21]. Application of this technique requires strict adherence to the manufacturer’s protocol, using only the reagents provided. The protocol has been used to detect Aspergillus spp. in blood (see Part B).

Target and primer selection

Factors downstream from sample preparation that influence the outcome of a clinical PCR include the selection of the gene target(s) to be amplified. This determines the design of primers and probes used to identify the particular amplicon. The decision to use a multi- or single-copy gene sequence as the target is probably the most important parameter in determining the sensitivity of any PCR assay.

Ideally, a marker of an infection should be present in all fungal genera, yet contain adequate internal sequence variation to define a particular species. By selecting such a marker, it should be possible to identify any fungus in a clinical specimen. Multi-copy gene targets have high sensitivity because of the larger number of target
molecules detected. By designing species-specific (or genus-specific) probes, selective detection of variable sequences within these genes is possible [48,49,61]. Assays targeting single-copy genes can be highly species specific but may be not be sensitive enough to detect low numbers of fungi [74,75]. The various fungal genes that have been targeted for PCR and the fungal species the tests detect are summarized in Table 1.

**Multi-copy gene targets**

**Ribosomal DNA gene cluster**

The ribosomal DNA (rDNA) gene is a tandem array of at least 50 100 copies in the haploid genome of all fungi. It comprises the small subunit (SSU) rDNA (18S) gene, the 5.8S gene and the large subunit (LSU) rDNA (28S) gene. Separating the 18S and 5.8S, and the 5.8S and 28S subunits are the intergenic transcribed spacer (ITS) regions, ITS1 and ITS2, respectively (Fig. 1). Independent of this cluster, a second repeat unit contains the 5S rDNA gene, flanked by the nontranscribed spacer (NTS) region. Any component of both gene clusters may be selected as a target for PCR. Panfungal primers to amplify any of the above-mentioned parts of the rDNA gene cluster are also shown in Figure 1. Whereas rDNA genes are highly conserved, the ITS regions are highly variable between different fungi [105]. Furthermore, the SSU and LSU rDNA genes comprise highly conserved and variable regions (Fig. 1) [101,102]. This allows the design of universal primers, based on the conserved regions, which will amplify a certain region of the rDNA gene cluster from a large number of fungal species, as well as of species-specific primers/probes, based on the variable regions, which can then be used to identify a single species. For example, by comparing the sequences of the ITS2 regions of several pathogenic fungi, DNA probes for a given fungal species can be devised.

At least 16 Candida and 5 Aspergillus species-specific probes have been designed based on rDNA gene sequences [88,106]. The sequences of these probes are available from the Center for Diseases Control (Atlanta, GA, USA), at cjm.3@cdc.gov. Other parts of the rDNA gene cluster, namely the variable D1 and D2 regions at the 5'-end of the LSU, also allow for species identification through the design of specific primers [94] or through sequencing [107]. PCR assays targeting one or more portions of the rDNA gene cluster have been widely used for the direct detection of a range of fungi from clinical specimens with most protocols aimed at detecting Candida and Aspergillus infection. Design of primers and probes to detect specific regions of the rDNA gene cluster can now be facilitated by accessing the European databases of the SSU and LSU rDNA genes available via the rDNA server at URL http://rRNA.uia.ac.be/ssl/ [108,109]. The databases have the potential to confirm the identity of any fungus by cross-checking with known sequence data.

**Candida-secreted aspartic proteinase gene**

The family of candidal-secreted aspartic proteinase genes (SAPs), which comprises at least nine related members in the *C. albicans* genome [110], is another multi-copy gene that has been used as a target for the

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**Fig. 1**

One repeat unit of the ribosomal DNA gene cluster in fungi with universal PCR primers. The internal transcribed spacers (ITS1) and -2 divide the 18S rDNA (small subunit or SSU gene), 5.8S rDNA and 28S rDNA (large subunit or LSU gene) genes from each other. The intergenic spacers (IGS) separate the repeat units from each other. Lengths refer to *Saccharomyces cerevisiae* Accession nos Z73326, Y13138. Delineation of variable regions V1–V9 follows Kappe et al. [101] and of D1–D12 follows Michot et al. [102]. The other variable regions (nonshaded) are estimated from an alignment of a complete unit of the ribosomal gene cluster for nine fungal sequences (Z73326 *S. cerevisiae*, Z19578 *Schizosaccharomyces pombe*, Z32848 *Schizosaccharomyces japonicus*, AF113174 *Eremothecium gossypii*, AF356652 *Filobasidiella neoformans*, AB026819 *Magnaporthe grisea*, AF218207 *Metharhizium anisopliae*, AI049262 *Verticillium dahliae*, AJ271061 *Mucor racemosus*). Primers for the amplification of the rDNA gene cluster are as follows: SR1R (5’-TACCTGTGGATTTCTCGGAC-3’) [103]; SR6R (5’-AAGTATAAGCTCGTAAACAAGG-3’) (Vilgalys, pers. Commun.); 5.8S (5’-CGCTGCTTGTCCCTCATCG-3’) [93]; LR1 (5’-GGTTGTTGGTTTCTTTCCT-3’) [103]; LROR (5’-ACCGCTGACCTTTAACCC-3’) (Vilgalys, pers. commun.); LR16 (5’-TTCACCCCAAACACCTCAG-3’) [103]; LR12 (5’-GACTTAGGAGGCTTCAG-3’) (Vilgalys, pers. Commun.); ITS1 (5’-TCCGAGGTTGAACCTTGCG-3’) [104]; ITS2 (5’-GCTGGGTTCTTCTGATGC-3’) [104]; ITS3 (5’-GCATCGATGAAGAACGCAGC-3’) [104] and ITS4 (5’-TCCTCCGCTTATTGATGC-3’) [95]. Adapted from H.M. Daniel, PhD thesis.

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Table 1  Gene targets for PCR-based fungal identification

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Fungal species detected</th>
<th>Specimens used</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>rDNA gene cluster as PCR target</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSU rDNA</td>
<td>Aspergillus flavus, A. fumigatus, A. nidulans, A. niger, A. terreus, A. versicolor, C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis</td>
<td>Whole blood</td>
<td>[61]</td>
</tr>
<tr>
<td>SSU rDNA</td>
<td>Aspergillus spp.</td>
<td>BAL</td>
<td>[76]</td>
</tr>
<tr>
<td>SSU rDNA</td>
<td>Fungal DNA (no specific species or genus detected)</td>
<td>Whole blood</td>
<td>[53]</td>
</tr>
<tr>
<td>SSU rDNA</td>
<td>A. fumigatus, C. glabrata</td>
<td>BAL</td>
<td>[77]</td>
</tr>
<tr>
<td>SSU rDNA</td>
<td>A. clavatus, A. flavus, A. fumigatus, A. niger, A. terreus, A. versicolor, Emericella nidulans</td>
<td>Whole blood, BAL</td>
<td>[64]</td>
</tr>
<tr>
<td>SSU rDNA</td>
<td>Aspergillus spp.</td>
<td>Whole blood, BAL</td>
<td>[78]</td>
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<td>Whole blood</td>
<td>[58]</td>
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<td>A. fumigatus, C. albicans</td>
<td>Whole blood</td>
<td>[71]</td>
</tr>
<tr>
<td>SSU rDNA</td>
<td>A. flavus, A. fumigatus, A. nidulans, A. niger, A. terreus, Penicillium marneffei, Paecilomyces variotii</td>
<td>Cultures, BAL</td>
<td>[79]</td>
</tr>
<tr>
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<td>Aspergillus spp.</td>
<td>Serum</td>
<td>[80]</td>
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<tr>
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<td>Aspergillus spp.</td>
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<td>[81]</td>
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<td>Whole blood</td>
<td>[82]</td>
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<tr>
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<td>Aspergillus spp.</td>
<td>Whole blood</td>
<td>[72]</td>
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<td>Cultures</td>
<td>[83]</td>
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<td>SSU rDNA</td>
<td>Cryptococcus neoformans</td>
<td>Cultures, CSF</td>
<td>[84]</td>
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<td>SSU rDNA</td>
<td>Penicillium marneffei</td>
<td>Cultures, CSF</td>
<td>[85]</td>
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<td>Histoplasma capsulatum</td>
<td>Tissue</td>
<td>[86]</td>
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<td>ITS2</td>
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<td>Cultures</td>
<td>[87]</td>
</tr>
<tr>
<td>ITS1, 5.8S rDNA, ITS2</td>
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<td>Spiked whole blood</td>
<td>[48]</td>
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<td>Serum</td>
<td>[89]</td>
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<td>CSF</td>
<td>[90]</td>
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<tr>
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<td>Ocular samples</td>
<td>[91]</td>
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<tr>
<td>ITS1, 5.8S rDNA, ITS2</td>
<td>A. flavus, A. fumigatus, C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, Cryptococcus neoformans</td>
<td>Tissue</td>
<td>[67]</td>
</tr>
<tr>
<td>ITS1, 5.8S rDNA, ITS2</td>
<td>T. asahii</td>
<td>Serum</td>
<td>[92]</td>
</tr>
<tr>
<td>LSU rDNA</td>
<td>A. flavus, A. fumigatus, A. niger, A. terreus, Paecilomyces variotii</td>
<td>Serum</td>
<td>[54]</td>
</tr>
<tr>
<td>LSU rDNA</td>
<td>C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, C. neoformans</td>
<td>Spiked whole blood</td>
<td>[93]</td>
</tr>
</tbody>
</table>

**Other conserved genes as PCR targets**

| Alkaline protease (ALP) | A. flavus, A. fumigatus | BAL | [51] |
| Alkaline protease (ALP) | A. flavus, A. fumigatus | BAL | [65] |
| Actin | C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, Saccharomyces cerevisiae | Serum | [95] |
| FKS | A. fumigatus | Spiked blood fractions | [96] |
| GP43 | Paracoccidioides brasiliensis | Tissue (mice) | [97] |
| GP43 | Paracoccidioides brasiliensis | Sputum | [98] |
| Lanosterol-14-alpha-demethylase (LIA1) | C. albicans, C. glabrata, C. krusei, C. parapsilosis, C. tropicalis, C. neoformans | Whole blood, BAL, body fluids, pus | [74] |
| Lanosterol-14-alpha-demethylase (LIA1) | C. albicans, C. glabrata, C. tropicalis, C. kefyr | Whole blood | [99] |
| Lanosterol-14-alpha-demethylase (LIA1) | C. albicans, C. glabrata, C. krusei | Whole blood, serum | [75] |
| Mitochondria | A. flavus, A. fumigatus, A. niger, A. terreus | BAL | [83] |
| Mitochondria | A. flavus, A. fumigatus | BAL, Skin biopsy, Sinus biopsy, Sputum, | [73] |
| Mitochondria | A. flavus, A. fumigatus | Spiked blood fractions | [96] |
| Mitochondria | C. albicans | Spiked whole blood | [100] |
detection of *C. albicans* by PCR. Any number of unique primers can be designed to target specific homologous regions of the SAP genes. The potential for such a PCR test to detect *C. albicans* in clinical samples [70] is discussed in Part B.

Aspergillus mitochondrial gene
The *Aspergillus* mitochondrial (mt) gene is an alternative multi-copy gene that may be targeted in PCR assays designed to detect *Aspergillus* spp. [62,63,73]. The utility of such assays is also discussed in Part B.

Aspergillus alkaline proteinase gene target
The family of alkaline proteinase enzymes in *A. fumigatus* is known to be responsible for most if not all, of the organism’s extracellular elastinolytic activity [111]. Isolation and sequencing of fragments of the alkaline proteinase (ALP) genes of *A. fumigatus* and *A. flavus* [51] have allowed the design of complementary oligonucleotide primers that have proved useful in diagnostic PCR tests (see Part B). The sequences of these fragments indicate these genes encode serine proteases of the subtilisin family [112].

Single-copy gene targets
A variety of PCR assays that target single-copy genes of yeasts, in particular *Candida* spp., has been tested. The genes targeted include actin [95], chitin synthase [113], heat shock protein 90 [114] and lanosterol-14-alpha-demethylase (*LIA1*) [74,115]. The *LIA1* gene catalyses the essential step in the conversion of lanosterol to ergosterol, the major membrane sterol specific for fungi [116]. The ‘best’ assays are those that can identify all *Candida* spp. commonly involved in candidaemia. There is less data on single-copy gene targeted PCR tests to detect moulds. For the detection of *Paracoccidioides brasiliensis*, the gene encoding a 43 kDa glycoprotein (gp43) has been targeted [97]. In *Aspergillus* spp., copperzinc superoxide dismutase (Cu-ZnSOD) is a well-known potential marker for IA. A recombinant Cu-ZnSOD of *A. fumigatus* has been produced by PCR amplification using homologous primers encoding the N-terminal sequence and the C-terminal extremity of the Cu-ZnSOD cDNA [117]. The protein has been exploited as a diagnostic tool only to develop Western blot and ELISA assays to detect antibody responses in patients with IA. The potential to target this protein in a PCR assay is worthy of consideration.

Detection and identification of the amplified product
Earlier PCR protocols focused on the detection of fungal DNA from a single species or genera in order to retain specificity [63,74,95]. More recent protocols have used universal/panfungal primers that enable detection of a range of fungal pathogens [53,59,61,118]. Panfungal PCR assays, in theory, would be more useful in the screening of patients in the initial stages of infection. Problems, however, are more common with these assays, for example, co-amplification of human DNA and contamination from environmental fungi [53]. Conversely, improvements in the performance of existing protocols, and the development of more sensitive protocols have re-kindled interest in pathogen-specific-PCR tests. The selection of a particular method depends on the aim of the assay.

Panfungal detection methods
Most methods utilize panfungal primer sets designed to recognize conserved regions among most pathogenic fungi such as the rDNA gene cluster, which is the main region targeted [119]. After generation of amplicon(s) by PCR, different genera or species can be distinguished using a number of techniques. The most widely used methods include: (i) restriction fragment length polymorphism (RFLP) [59], (ii) hybridization of amplicons with specific probes employing Southern blotting methods with radioactive (e.g. $^{32}$P) or enzyme-labelled (e.g. digoxigenin) probes [53,61], (iii) single-strand conformational polymorphism (SSCP) [120,121], and (iv) sequencing of the amplified products [91,107].

**RFLP**
Hopfer et al. [59] successfully detected and identified five groups of fungal pathogens in clinical specimens by amplifying a segment of the rDNA gene, followed by digestion of amplicons with restriction enzymes. A similar combined PCR–restriction enzyme analysis (REA) approach using panfungal primers was used by Maiwald et al. [122] but applied only to known cultures of *Candida* spp., *Saccharomyces cerevisiae*, *Trichosporon beigelii* and *C. neoformans*.

**Specific probes**
Exploring the utility of a ‘general’ PCR approach, Einsele et al. [61] amplified the conserved sequence of the SSU rDNA gene and designed several *Candida* and *Aspergillus* species-specific probes to identify the amplified products. The study demonstrated a sensitivity of 88% when applied to one blood specimen, and 100% when applied to two specimens, from small numbers of patients with documented IC or IA. The specificity of the probes was 98%. The panfungal approach developed by van Burik et al. [53] detected a fragment of the SSU rDNA gene in which primers and probes were optimized.
to detect Candida and Aspergillus spp. When tested on blood specimens \((n = 11)\) from patients with IA and IC, the assay was less sensitive than culture. Hybridization techniques are, in general, able to detect as few as 1 cfu ml\(^{-1}\) of organism.

More recently, molecular beacons have been used to identify fungi, for example, Candida spp. in place of conventional nucleic acid probes [123]. Molecular beacons are small single-stranded nucleic acid hairpin probes that fluoresce brightly when bound to their targets [124]. DNA sequence analysis of the ITS2 region from reference Candida strains has been used to develop species-specific beacon probes for rapid, high-fidelity identification of C. dubliniensis and A. albicans following PCR [123].

Another study used RAPD-PCR to screen A. fumigatus DNA for species-specific amplicons. The sequence of the appropriate amplified band was used to design a specific primer pair which, in turn, amplified a single 864 bp fragment from A. fumigatus by ‘touchdown’ PCR. The protocol detected the 864 bp product in 89 of 90 A. fumigatus strains tested and offers promise as a system for the identification of Aspergillus spp. which does not require sample manipulation post-PCR [125].

**SSCP**

SSCP has been proposed as a further method for identifying fungi following a ‘general’ PCR approach. As SSCP displays migration of amplified DNA fragments as a function of their conformational structure as well as size, it can distinguish amplicons that differ by as little as a single base pair. Walsh et al. [120] applied SSCP to the analysis of a fragment amplified from the SSU rDNA gene and were able to distinguish among Cryptococcus neoformans, Pichoydin boydii, Candida, Aspergillus and Rhizopus spp. using known cultures. SSCP has also been successfully applied to the identification of common pathogenic Aspergillus spp. by analysis of amplified ITS1–5.8S rDNA–ITS2 regions [121]. This method has not been attempted in a PCR-based test on human specimens.

**Sequencing**

Sequence analysis is the most promising and reliable technique for the identification of a number of fungal species following PCR, including certain non-C. albicans Candida spp., or fungi that are slow-growing, for example, H. capsulatum. In general, parts of a gene are amplified using panfungal primers. The sequences of the amplified products are then compared with fungal sequences of the same DNA fragment maintained in the public sequence databases for species identification. These may be accessed via the website: EMBL at: www.ebi.ac.uk; GenBank at: www.psc.edu/general/software/packages/genbank/genbank.html; BioloMICS at: www.cbs.knaw.nl and the International European rRNA database (Candida) at: www.rrna.uia.ac.be.Isu. The databases should, however, be used with caution taking into account the inconsistent quality of the deposited sequence data. Examples of the use of sequencing for the identification of Candida spp., in particular, C. dubliniensis and Aspergillus spp. are discussed below.

As conventional methods cannot always reliably distinguish C. dubliniensis from C. albicans, which it closely resembles morphologically and biochemically, molecular approaches are often applied to identify C. dubliniensis. Earlier methods used a variety of probes such as the C. albicans-specific DNA 27A probe to distinguish the two species [126]. Other techniques that are used include PCR fingerprinting [127], REA, and LSU and SSU rDNA sequencing [126]. Analysis of non-rDNA sequences such as the actin (ACT1) gene also has the potential to distinguish C. dubliniensis from other Candida spp. By designing C. dubliniensis-specific PCR primers for both ACT1 intron and exon sequences, the divergence between this yeast’s and the C. albicans ACT1 intron sequences can be exploited for rapid (4 h) identification of the organism [128]. In another study, the structural gene PHRI, was targeted in a PCR-based test to identify C. dubliniensis [129].

Zhao et al. [130] developed a nested PCR test targeting the ITS1 and -2 regions of the A. fumigatus rDNA gene. The PCR correctly identified A. fumigatus but not other common Aspergillus pathogens and was capable of detecting 10–100 fg A. fumigatus DNA from pure culture. The differentiation of fungal pathogens by Genescan analysis after amplification of the highly polymorphic ITS regions has been successfully explored by many others [131].

**Genus/species-specific detection methods**

Many genus- and species-specific PCR assays using carefully designed primer sets to detect a specific fungal genus or species have been developed. Some have been pursued commercially. The utility of these methods for the detection of yeasts and moulds is discussed in Part B.

**Other detection methods**

Traditional methods to detect PCR amplicons including those described above, involve electrophoresis followed by Southern hybridization techniques to increase the sensitivity and specificity of the PCR. These methods are time-consuming and are not optimal for routine laboratory use because of their toxicity, expense and short half-life. This has led to the development of alternative...
detection methods such as PCR-linked ELISA formats and more recently, ‘real time’ PCR.

**Application of PCR-linked ELISA techniques**

PCR-Linked ELISA techniques have the advantage that they allow multiple samples to be processed and provide further amplification without losing the specificity associated with Southern blots [48,89]. Target DNA is labelled with a nonradioactive material such as digoxigenin. The test format involves either the binding of specific biotinylated oligonucleotide probes to strepavidin-coated wells of microtitre plates, or the direct addition of the probes to the liquid phase of the assay. The probes hybridize with the digoxigenin-labelled amplicon following PCR and the complex is trapped on the solid-phase wells. Detection of the amplicons is accomplished, after binding with peroxidase-labelled anti-digoxigenin antibodies, by standard colorimetric immunoperoxidase reactions. These formats have increased the sensitivity of PCR assays and are discussed further in their application to detect, in the main, *Candida* and *Aspergillus* spp. The prototype assay developed by Fujita et al. [48] is available with minor variation, in the form of two commercial PCR-ELISA kits, the PCR-Dig-Labelling, and the PCR-ELISA-Dig-detection kit (Roche Diagnostics Corporation).

**Detection and quantification of PCR products by ‘real time’ PCR systems**

Quantitative ‘real time’ PCR systems, similar to the Amplicor® Monitor (Roche Molecular Systems, Inc., Alameda, CA, USA) used in other areas of microbiology [132], represent the most recent advance in diagnostic mycology. ‘Real time’ PCR has the potential to not only substantially increase the sensitivity of the PCR, but also to provide the means to monitor response to therapy by measuring fungal burden at any given point in time. The main commercial quantitative PCR technologies available are: the (i) ABI PRISM 7700 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) which use TaqMan technology originally developed by Roche Molecular Biochemicals (Mannheim, Germany); (ii) LightCycler Systems (Roche Molecular Biochemicals) trademark of Idaho Technology Inc. (Idaho Falls, ID, USA); and (iii) Rotor-Gene Four Channel Multiplexing System (CR Corbett Research, Mortlake, NSW, Australia). These technologies combine DNA amplification, probe hybridization and signal generation in a single step allowing accurate quantification of target DNA with good reproducibility.

ABI PRISM 7000, ABI PRISM 7700, ABI PRISM 7900HT and GeneAmp® 5700 Sequence Detection Systems from Applied Biosystems are 96- or 384-well format systems that incorporate an oligonucleotide probe, specific to the PCR target sequence, which binds between the forward and reverse primers used. A fluorescent reporter dye (one of three available) and a quencher dye are attached at the 5’- and 3’-ends of the probe, respectively (Fig. 2). When this probe is intact, the proximity of the reporter to the quencher dye results in the suppression of fluorescence. During PCR, the probe hybridizes to the single-stranded template. The probe is cleaved during extension, resulting in separation of the reporter from the quencher dye (Fig. 2). This generates a sequence-specific fluorescent signal and the probe is displaced from the target DNA. Repeated cycles of primer and probe annealing, and cleavage of the probe result in exponential amplification of the PCR product and a corresponding increase of reporter fluorescence. Based on a standard dilution series with known concentrations of target DNA, the amount of PCR product is expressed as an absolute value.

The applications of the various probes and dyes available in different PCR formats are beyond the scope of this review. The reader is referred to the website http://www.appliedbiosystems.com and to the article by Livak et al. [133] for a description of the technological aspects of the system.

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**Fig. 2** Principle of the dual-labelled probe ‘real time’ PCR: a dual-labelled probe, with two fluorescent dyes, reporter and quencher, attached to both ends of the probe, anneals specifically between the forward and reverse primers. During the extension step, the probe is cleaved by DNA polymerase; the reporter dye is separated from the quencher dye, generating a sequence-specific signal. At the end of extension, the probe is completely separated from the double stranded DNA template (reproduced with permission of Applied Biosystems, Foster City, CA, USA).
The LightCycler (Roche Molecular Biochemicals) differs from the ABI PRISM systems in that it uses fluorescence resonance energy transfer (FRET) to detect amplified PCR products. The probe format is based on a pair of differentially labelled probes (‘HYB’ probes), chosen to detect a particular sequence during PCR (Fig. 3A). One probe is labelled at the 3'-end with fluorescein (the donor fluorophore) and the other at the 5'-end with an acceptor fluorophore (LightCyclerRed 640 or LightCycler-Red 705). The system provides for absolute quantification of target DNA with reference to an external standard curve. It is very fast in obtaining ‘real time’ analytical results because of efficient heat transfer to the borosilicate capillaries used as the reaction chambers.

During PCR, the probes hybridize in a head-to-tail arrangement to adjacent sequences of the target DNA. The donor and acceptor dyes are in close proximity resulting in FRET, in which the donor dye emits green fluorescent light and in turn excites the acceptor dye, which then emits red fluorescent light. As primer extension is initiated, the ‘HYB’ probes are displaced resulting in a decrease in fluorescence. The reactions are detected as a ratio of donor fluorescence to acceptor fluorescence during the annealing step (Fig. 3B). FRET technology allows for melting curve analysis which should be taken into consideration when determining fungal load. The latest software includes new efficiency-correction algorithms, to allow for differences in PCR efficiencies depending on the nature of the target, the organism and assay components. The procedure is detailed Heindl et al. [134] and is available through the Roche website at http://biochem.roche.com.lightcycler.

The Rotor-Gene (Corbett Research) four-channel multiplexing system works on the same principles as the other two commercial systems. It differs, however, in that it comprises an open chemistry platform encompassing a centrifugal rotor and a multilfter system that allows the use of all available ‘real time’ fluorescence technology including SYBER Green, dual-labelled probes, FRET probes and molecular beacons. It has four channels to detect fluorophores (at 470, 530, 585 and 625 nm) allowing the fluorescence of up to four different probes to be detected in a single reaction tube. As there is no variation in temperature from sample to sample, internal reference dye standards are not needed. Unlike the LightCycler systems, the principle of quenched FRET is used, in which the decrease in energy of only the donor probe is measured. This has the advantage of using less spectral bandwidth per probe set. Other features include free software upgrades from www.corbettresearch.com and the capacity to use nonspecialized reaction vessels.

When purchasing a commercial quantitative PCR system, the choice between the three systems rests with the laboratory’s preference and resources. Experience with their performance in PCR assays to detect fungi is discussed in Part B.

Standardized and reproducible PCR assays
Evaluation of the various PCR assays to detect fungi in
clinical specimens is necessary in order to determine the optimal method for use in clinical practice. To compare the results of ‘in-house’ assays, identical DNA preparation and purification methods, PCR conditions, PCR design and detection methods should be used. Comparison of ‘in-house’ and commercial systems is more difficult. Exchange of information between laboratories to ensure interlaboratory reproducibility is critical to the evaluation of any clinical PCR assay.

**False-positive and false-negative results**

Contamination leading to false-positive results is an important pitfall in fungal PCR, especially in relation to the detection of moulds. The risk of contamination of PCR reagents by airborne spores or by carry-over is high unless controlled by specific measures [63,135]. Multiple ‘negative control’ specimens from persons without fungal infection as well as reagent controls should be included in every PCR run. Special care must always be taken in the quality control of reagents and in ensuring that storage conditions of all reagents are optimal. Other measures to avoid contamination are well-known: the use of separate rooms and glassware supplies for PCR set-up and products, aliquoted reagents, positive-displacement pipettes and aerosol-resistant tips [136]. Commercial ‘real time’ PCR systems have ‘no enzyme’ and ‘no template’ controls to detect contamination by the fluorophore itself, and some incorporate the use of AmpErase uracil-N-glycosylase (UNG; Roche Molecular Biochemicals) to prevent PCR product carry-over. As reaction tubes do not require opening for post-PCR analysis, the opportunity for external contamination is minimized.

False-negative results may arise if environmental DNA out-competes low-level, true-positive specimens. Therefore, low-copy number positive controls should be included in every PCR run to verify extraction efficiency by ‘spiking’ samples with predetermined numbers of the target fungus. Commercial systems have a low copy number internal control plasmid that is added to the PCR master mix to monitor inhibition. To exclude the presence of polymerase inhibitors and to control the quality of the extracted DNA, a fragment of the human HLA class I gene or the human β-globin gene, amplified in parallel with samples has been shown to be useful [61,62,65,82]. The inclusion of ‘control human genes’ is particularly important in assays designed to detect *Candida* and/or *Aspergillus* infection, because these pathogens account for the majority of systemic mycoses. This section summarizes the experience of PCR and non-PCR-based assays. Protocols developed to detect fungi in clinical specimens are reviewed, as are those tested only on ‘spiked’ samples or known cultures. The latter are important to the establishment of a clinically relevant test.

A few issues are worthy of mention in relation to PCR assays to detect filamentous fungi, such as *Aspergillus* spp. in clinical specimens. This has proved challenging as there appears to be a greater number of obstacles relating to sample preparation compared with yeasts [21,69]. Moreover, there have been few moves to develop a standardized protocol. Given the ubiquitous nature of this fungus, the environment represents a constant potential source of contamination. Most notably, the clinical usefulness of PCR from respiratory tract samples, for example, in cases of suspected IA is limited by false-positive results and low positive predictive values (PPV; see later). For instance, PCR assays on BAL specimens have been associated with a 10–25% rate of false-positive results [76,77,137] due to the inability of the PCR to distinguish between infection, colonization or contamination of samples by *Aspergillus* conidia. PCR on blood samples could potentially be more useful as colonization of blood is unlikely. Contamination should be avoided if PCR tubes are opened under laminar air-flow conditions. Blood also offers the opportunity for repeated sampling. PCR protocols mainly targeting the rDNA or mitochondrial genes have been developed to detect *Aspergillus* spp.

The performance characteristics and clinical utility of some of the published PCR assays that target different regions of the *Candida* genome are summarized in Table 2 and those of assays developed to detect *Aspergillus* infection shown in Table 3.

**Methods employing gel electrophoresis**

These methods analyse PCR products obtained by single-step or nested PCR via agarose gel electrophoresis and ethidium bromide staining followed in some cases by Southern hybridization with the use of specific probes. The rDNA gene cluster has proved a popular target for PCR-based methods to detect in particular, *Candida* spp. Earlier PCR assays targeted the SSU rDNA gene [122] and the 5.8S rDNA gene including the noncoding ITS region [49,138,139]. These assays identified amplicons using Southern hybridization and worked well for cultured *Candida* cells, purified candidal DNA and blood ‘spiked’ with *Candida* spp. One study employed broad-range PCR primers to target a part of the less conserved...
Table 2  PCR for Candida spp. using panfungal and Candida-specific primers

<table>
<thead>
<tr>
<th>Study</th>
<th>Gene target</th>
<th>Primer type</th>
<th>Detection method</th>
<th>Fungus detected</th>
<th>Specimen (no. tested)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Einsele et al. [61]</td>
<td>SSU rDNA</td>
<td>Panfungal (ITS3, ITS4)</td>
<td>Southern blot (32P Phosphorus)</td>
<td>Broad range</td>
<td>Blood (8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 specimen</td>
<td>88</td>
<td>97</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 specimen</td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td>Van Burik et al. [53]</td>
<td>SSU rDNA</td>
<td>Panfungal (ITS3, ITS4)</td>
<td>Southern blot (digoxigenin)</td>
<td>Broad range</td>
<td>Blood (23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wahyuningshi et al. [89]</td>
<td>ITS1, 5.8S rDNA, ITS2</td>
<td>Panfungal (ITS3, ITS4)</td>
<td>PCR-ELISA</td>
<td>C. albicans</td>
<td>Serum (8)</td>
<td>87.5</td>
<td>100</td>
</tr>
<tr>
<td>Guiver et al. [87]</td>
<td>ITS2</td>
<td>Candida-specific</td>
<td>ABI PRISM 7700 Sequence Detection system (Applied Biosystems)</td>
<td>Candida spp.</td>
<td>Candida isolates (24)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Holmes et al. [49]</td>
<td>SS, NTS</td>
<td>C. albicans-specific</td>
<td>Southern blot (peroxidase)</td>
<td>C. albicans</td>
<td>‘Spiked’ blood</td>
<td>*</td>
<td>100</td>
</tr>
<tr>
<td>Fujita et al. [48]</td>
<td>ITS1, 5.8S rDNA, ITS2</td>
<td>Panfungal (ITS3, ITS4)</td>
<td>Candida-specific probes</td>
<td>C. albicans</td>
<td>‘Spiked’ blood</td>
<td>*</td>
<td>100</td>
</tr>
<tr>
<td>Burgener-Kairuz et al. [74]</td>
<td>LIA1</td>
<td>Candida-specific</td>
<td>Southern blot (32P)</td>
<td>Candida spp.</td>
<td>Blood and tissue (80)</td>
<td>71†</td>
<td>95</td>
</tr>
<tr>
<td>Morace et al. [99]</td>
<td>LIA1</td>
<td>Candida-specific</td>
<td>PCR-REA (species-specific)</td>
<td>Candida spp.</td>
<td>Blood (31)</td>
<td>98.2</td>
<td>NA§</td>
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<td>Miyakawa et al. [100]</td>
<td>Mitochondrial</td>
<td>C. albicans-specific</td>
<td>Southern blot (32P)</td>
<td>C. albicans</td>
<td>‘Spiked’ blood</td>
<td>*</td>
<td>100</td>
</tr>
<tr>
<td>Kan [95]</td>
<td>Actin</td>
<td>C. albicans-specific</td>
<td>Southern blot (32P)</td>
<td>C. albicans</td>
<td>Mouse model (intravenous)</td>
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<td>100</td>
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<td>Flahaut et al. [70]</td>
<td>SAP</td>
<td>C. albicans-specific</td>
<td>PCR-ELISA</td>
<td>C. albicans</td>
<td>Variety specimens (156)</td>
<td>100</td>
<td>98</td>
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<td></td>
<td></td>
<td>Blood (124)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Hidalgo et al. [151]</td>
<td>SSU rDNA</td>
<td>C. albicans-specific</td>
<td>Ethidium bromide</td>
<td>C. albicans</td>
<td>Vitreous fluid (4)</td>
<td>100</td>
<td>NA</td>
</tr>
<tr>
<td>Ferrer et al. [91]</td>
<td>ITS1, 5.8S rDNA, ITS2</td>
<td>Panfungal (ITS1, ITS4 and ITS4, ITS86)</td>
<td>Semi-nested and sequence analysis</td>
<td>Broad range</td>
<td>Ocular specimens (11)</td>
<td>45.5</td>
<td>–</td>
</tr>
</tbody>
</table>

**Abbreviations:** SSU, small subunit; ITS, internal transcribed spacer; NTS, non-transcribed spacer region; NA, not applicable; LIA1, lanosterol-14-alpha-demethylase; SAP, secreted aspartic proteinase.

* Sensitivity achieved was 15 cfu ml⁻¹ [48], 10 cfu l⁻¹ [47] and 30 cfu ml⁻¹ [91].
† Sensitivity and specificity for detection of C. albicans were 71 and 95%, respectively.
‡ Sensitivity and specificity for detection of C. glabrata were 100 and 97%, respectively.
§ The negative predictive value was 97.5%.

LSU and identified amplicons with species-specific Candida probes followed by sequencing (Table 2) [93]. The assay correctly identified Candida spp. inoculated into blood but was tested on blood specimens from only two patients with documented candidaemia.

A number of nested PCR assays to detect Candida spp. have also been designed with the aim of increasing the sensitivity of the test (Table 2). Examples of the more comprehensive assays include protocols targeting the structural gene encoding for the candidal lanosterol-14-alpha-demethylase (LIA1) gene. These also worked well for cultured Candida cells or when blood was ‘spiked’ with these cells or purified candidal DNA, being able to detect 100–200 Candida cells per ml of blood [74,115]. Buchman et al. [115] were the first to demonstrate that detection of C. albicans in clinical specimens was also possible by PCR using this gene target. A nested format of the assay correctly detected and identified C. albicans, C. glabrata, C. tropicalis and C. krusei in clinical samples (Table 2) [74]. Chryssanthou...
### Table 3  PCR for diagnosis of invasive aspergillosis (IA)

<table>
<thead>
<tr>
<th>Study</th>
<th>Gene target</th>
<th>Detection method</th>
<th>Study population (no. patients)</th>
<th>Sample tested (no.)</th>
<th>Proven/probable IA II</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verweij et al. [77]</td>
<td>SSU rDNA</td>
<td>Aspergillus-specific PCR-REA</td>
<td>Haematologic malignancy (19)</td>
<td>BAL (19)</td>
<td>7</td>
<td>71.4</td>
<td>84.2</td>
</tr>
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<td>Einsele et al. [61]</td>
<td>SSU rDNA</td>
<td>Panfungal Southern blot</td>
<td>HSCT recipients (134)</td>
<td>BAL (261)</td>
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<td>63</td>
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<td>Skladny et al. [64]</td>
<td>SSU rDNA</td>
<td>Aspergillus-specific Nested PCR</td>
<td>Haematologic malignancy (93)</td>
<td>BAL (65) Blood (250)</td>
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<td>Buchheidt et al. [78]</td>
<td>SSU rDNA</td>
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<td>BAL (105) Blood (907)</td>
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<td>91.7</td>
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<tr>
<td>Jones et al. [62]</td>
<td>Mitochondrial</td>
<td>Aspergillus-specific PCR-ELISA</td>
<td>Haematologic malignancy (69)</td>
<td>BAL (75) Blood (177)</td>
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<td>Hayette et al. [65]</td>
<td>ALP</td>
<td>A. fumigatus and A. flavus-specific Nested PCR</td>
<td>Pulmonary aspergilosis (9)</td>
<td>Serum (1193)</td>
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<td>Tang et al. [51]</td>
<td>ALP</td>
<td>A. fumigatus and A. flavus-specific Southern blot</td>
<td>Haematologic malignancy (21)</td>
<td>Serum (619)</td>
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<td>Yamakami et al. [80]</td>
<td>SSU rDNA</td>
<td>A. fumigatus-specific Nested PCR</td>
<td>Ethidium bromide staining</td>
<td>Serum (175)</td>
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<td>Kawamura et al. [81]</td>
<td>SSU rDNA</td>
<td>A. fumigatus-specific Nested PCR</td>
<td>Ethidium bromide staining</td>
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<td>Hebart et al. [82]</td>
<td>SSU rDNA</td>
<td>Panfungal (ITS1, ITS4) Southern blot</td>
<td>Haematologic malignancy (41)</td>
<td>Serum (281)</td>
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<td>Panfungal (ITS1, ITS4) PCR-ELISA</td>
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<td>Loeffler et al. [71]</td>
<td>SSU rDNA</td>
<td>C. albicans and A. fumigatus-specific LightCycler (Roche)</td>
<td>Haematologic malignancy (9)</td>
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<td>Breitge et al. [73]</td>
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<td>100</td>
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<tr>
<td>Costa et al. [96]</td>
<td>FKS</td>
<td>A. fumigatus-specific ABI PRISM 7700 Sequence Detection system</td>
<td>–</td>
<td>‘Spiked’ whole blood and blood fractions</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Hendolin et al. [67]</td>
<td>ITS1, 5.8S rDNA, ITS2</td>
<td>Panfungal (ITS3, ITS4) Species-specific hybridization and Sequencing</td>
<td>CRS (8) Proven/suspected fungal infection (20)</td>
<td>Nasal polypous tissue (8)</td>
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<tr>
<td>Catten et al. [152]</td>
<td>ITS1, 5.8S rDNA, ITS2</td>
<td>Panfungal (ITS3, ITS4) Aspergillus-specific Bipolaris-specific Ethidium bromide Phosphoimager</td>
<td>CRS</td>
<td>Tissue (25)</td>
<td>25</td>
<td>40</td>
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</tbody>
</table>

**Abbreviations:** IPA, invasive pulmonary aspergillosis; HSCT, haematological stem cell transplant; immunocompromised; non-IC, nonimmunocompromised; CNPA, chronic necrotizing pulmonary aspergillosis; CRS, chronic rhinosinusitis; FKS, gene involved in beta (1-3) glucan synthesis; ALP, alkaline protease; BRN1 (Bipolaris-specific primer targeting the Brn-1 gene sequence found in all Bipolaris spp.).

*Positive predictive value (PPV) = 76.5% for PCR on BAL; PPV = 49.3% for PCR on blood; negative predictive value (NPV) = 98% for PCR on blood.

PPV = 62%.

PPV = 28% and NPV = 100% where one sample tested; PPV = 15.2% and NPV = 100% where two samples tested.

PPV = 42% and NPV = 98%.
et al. [75] compared the value of testing serial serum samples using the nested PCR with measurement of urine d,l-arabinitol ratios and plasma 1,3-β-D-glucan concentrations for the diagnosis of IC. PCR was positive in three of five patients (60%) with proven IC, but suffered from false positives and poor agreement with the other tests. In both studies, the nested assay was insufficiently sensitive for routine clinical application.

The LIAI-based assay has since been refined by making it species-specific using REA [99] (see Table 2). This assay was found to be more sensitive for the detection of candidaemia in neutropenic patients than the BACTEC 9240 blood culture system (Becton Dickinson, Cockeysville, MD, USA; 21.4%). The negative predictive value (NPV) of the PCR-REA was 97.5% [99]. The assay identified five Candida spp. in blood. In another report, the sensitivity of the same PCR-REA was 93% compared with 70% from lysis-centrifugation blood cultures (Isolator) for recovery of Candida spp. from patients with disseminated IC; the NPV was 98% [140].

Other gene targets used in assays to detect Candida spp. include the mitochondrial (mt) and actin genes (see Table 2). The assay developed by Miyakawa et al. (mt gene target) [100] has only been tested on blood ‘spiked’ with C. albicans and C. glabrata (sensitivity 30 C. albicans cells per ml of blood). Kan [95] published a PCR method in which primers were designed to amplify a 158 bp segment of the actin gene using known cultures as well as blood ‘spiked’ with Candida spp. The study demonstrated that PCR on blood was more sensitive (96%) than blood cultures (70%) for the detection of Candida in mice inoculated with C. albicans, yet specific enough to remain negative for colonized animals. In addition, candidal DNA was detected in the serum of 11 of 14 (79%) patients with known candidaemia.

PCR protocols to detect Candida spp. can be performed either on whole blood [70,74] or on serum samples as shown above [75,95]. However, experience using a particular assay under identical conditions is limited. One nested assay has been evaluated in which five specific primers were used to amplify the 5.8S rDNA gene and adjacent ITS regions of five Candida spp. (C. albicans, C. tropicalis, C. parapsilosis, C. krusei and C. glabrata) [68]. Using an intravenous rabbit model of candidaemia, the ability of the assay to detect C. albicans at various times on whole blood and serum was compared. Blood cultures were used as the reference assay. Detection of candidaemia by culture was possible only up to 1 min after injection, whereas PCR was positive in blood for up to 15 min, and in serum for up to 150 min. For 40 culture-negative samples, PCR was more often positive in serum than in whole blood [68].

The results suggest that serum should be used preferentially over whole blood for the diagnosis of IC by PCR but experience is required with head-to-head comparisons using human specimens. Comparative studies targeting other Candida genes would also be useful.

The advantages of nucleic acid-based diagnostic methods to directly detect C. neoformans in clinical specimens have not been fully explored as diagnosis by culture and serological-based methods is not usually problematic. Direct detection of the organism from clinical samples by PCR has been documented for small numbers of patients with pulmonary cryptococcosis and for CSF samples [90,141]. One study evaluated a nested PCR protocol using primer pairs specific for the ITS regions [142] of the C. neoformans rDNA gene. The technique was applied to 40 CSF samples from patients with clinical meningitis of unknown aetiology. Positive PCR reactions were obtained for 21 patients with cryptococcal meningitis (confirmed by conventional methods). The remaining 19 patients, who either had meningitis caused by other organisms or who did not have meningitis, had negative PCR results. PCR was found to be superior to follow-up CSF antigen testing in monitoring the response to therapy and may have a role in demonstrating clearance of the fungus from CSF [90].

Another study applied a SSU rDNA gene-targeted protocol to CSF samples from 27 patients with known cryptococcal meningitis [84]. Amplicons were identified by hybridization with a C. neoformans-specific probe. Positive PCR results were obtained for all 27 samples and the assay was more sensitive than culture.

Early diagnosis of disseminated trichosporonosis is often difficult as blood cultures become positive only after a relatively long period. Although the latex agglutination test used for diagnosis of cryptococcosis may be positive in affected individuals, the two infections cannot be distinguished by this assay [143]. Detection of Trichosporon species in serum has been attempted by nested PCR using two sets of primers derived from the sequence of the LSU rDNA genes of Trichosporon spp. and probes specific for T. asahii and T. mucoides, the predominant agents of disseminated infection [144]. When applied to 11 serum samples, the sensitivity of the nested PCR was 64% (7 of 11 patients with proven disease). Two of the 11 patients had positive cultures in the event of a negative PCR result. More recently, Sugita et al. [92] used a nested SSU rDNA-targeted assay on 11 serum samples from patients with autopsy-confirmed trichosporonosis. Positive PCR results were obtained from 9 of the 11 samples suggesting that PCR may have a role as an adjunctive diagnostic tool.

PCR protocols developed to diagnose mould infec-
tions have largely been directed at detecting *Aspergillus* spp. Assays that detect multiple fungal species in whole blood [53,61] have been discussed briefly in Part A. More specifically, Einsele *et al.* [61] tested their ‘in-house’ PCR on 13 patients with proven/probable IPA prior to initiation of antifungal therapy. The PCR assay was positive for *Aspergillus* spp. in 8 of 13 patients. Importantly, blood samples from neutropenic patients without clinical IA and ‘control’ patients tested PCR negative. The assay was then applied to HSCT recipients who underwent two BAL procedures at the time of transplantation [76]. The ability of the test to predict IPA is summarized in Table 3. In seven patients, BAL samples were positive by PCR, whereas microscopy and culture were negative. Five of the seven patients subsequently developed confirmed IPA. Three patients with negative PCR results developed nosocomially acquired IPA after transplantation.

A number of promising studies involving two-step nested PCR of BAL and blood specimens have since been reported (Table 3). To examine the utility of the assay developed by Skladny *et al.* [64], BAL and blood samples from neutropenic and healthy ‘control’ patients were tested simultaneously. The sensitivity of the assay varied with the specimen tested (Table 3). When PCR was performed on BAL, 9 of 22 neutropenic patients had positive PCR results; 3 had definite IA (positive culture/histopathology) and 6 had probable IA (characteristic high-resolution CT [HR-CT] scan findings + positive culture). Four of the 13 patients with PCR-negative results were diagnosed with definite IA. Blood samples from 20 neutropenic patients were PCR positive. IA was proven in 7 (6 PCR positive) patients and 14 (all PCR positive) had probable IPA. Thus, all 21 patients with proven IA/characteristic HR-CT scan findings had a positive blood and/or BAL fluid PCR result. The combined test specificity using blood and BAL specimens was 89% [64].

Buchheidt *et al.* [78] confirmed the usefulness of the above PCR for early *in vivo* detection of IPA in a similarly designed study (Table 3). When performed on BAL fluid, PCR results were correctly positive (definite IPA) for 13 patients, correctly negative for 50 patients and there were 4 false-positive results. No PCR result was falsely negative. When tested on an average of three blood specimens per patient, *Aspergillus* spp. was detected in 30.7% of immunocompromised patients, half of whom (15.1%) developed IPA; all had ≥1 positive PCR result. For the remaining patients with positive PCR results, no evidence of IPA was found. Overall, only three (2%) patients with negative PCR results developed proven IPA. In the patients with false-positive results, transient fungaemia cannot be excluded as blood was sampled during antifungal therapy in many cases. Conversely, false-negative findings in two of the three patients with proven IA may have been due to the co-administration of antifungal drugs. Testing of both BAL and blood specimens by PCR was required to maximize the sensitivity of the test [64,78].

An important study performed by Williamson *et al.* [43] set out to systematically evaluate PCR-positivity during the natural history of IA. Serum samples from 37 HSCT recipients were retrospectively tested [54]. Six patients had proven, seven probable and three possible IA (case definitions of the European Organization for Research and Treatment of Cancer [EORTC] Mycoses Study Group) [54]. All these 16 patients were PCR positive. Four of 19 patients with no evidence of IA had a single PCR-positive sample. The PPV of the assay was 80%, using the criterion of one ‘positive’ result. If the criterion of two ‘positive’ tests was used, the sensitivity decreased but the PPV (100%) increased (Table 3). Positive PCR results led to early institution of antifungal therapy in 67% of patients.

To further address the potential of prospective PCR screening for diagnosis of IA, Hebart *et al.* [82] performed PCR on blood samples drawn 2–4 times a week from HSCT recipients. Seven patients had newly diagnosed IA. All had PCR-positive results which preceded the diagnosis by a median of 9 days. Performance of the assay, in which a single PCR result is followed by two positive PCR tests are taken as a ‘positive’ result, are shown in Table 3. In patients without a history of IA (n = 69), however, the PPV was 44.4% [82]. In the screening of HSCT recipients for IA, PCR to detect *Aspergillus* infection should be performed on at least two occasions [54,74,82].

Assays targeting the *Aspergillus ALP* gene, designed to detect *A. fumigatus* and *A. flavus*, have also been evaluated [51,65] (see Table 3). Using a nested procedure [65], 10 of 74 immunocompromised patients had positive PCR results (8 had culture-positive samples). All developed proven or probable IPA. BAL fluid from five patients colonized with *Aspergillus* spp. were PCR and culture-positive. The nested PCR accurately diagnosed culture-negative patients with IPA, excluded *Aspergillus* infection in patients at risk for IPA but could not differentiate between infection and colonization.

PCR assays have been performed to confirm suspected cases of various forms of pulmonary aspergillosis, with most studies targeting the rDNA gene cluster. The results of Verweij *et al.* [77] who evaluated a genus-specific PCR–REA in 19 suspected cases of IPA are shown in Table 3. The false-positive rate was ≈15%. The assay performed similarly to the Platelia *Aspergillus*
test (Bio-Rad) when BAL fluid was used but serum antigen was detected in some patients before pulmonary infiltrates were visible radiologically. Two other studies investigated a nested PCR targeting the SSU rDNA for its ability to detect Aspergillus spp. in serum in the same patient population. Their performance is summarized in Table 3 [80,81]. Yamakami et al. [80] found that the highest proportion of positive PCR results occurred in patients with IPA, whereas those with noninvasive pulmonary aspergillosomas had the lowest frequency. Kawamura et al. [81], however, noted that a significantly higher percentage of patients with pulmonary aspergillosomas had positive PCR results. No ‘control’ subjects were tested in either study.

There is relatively less data with respect to PCR-based methods for the direct detection of other filamentous fungi from human specimens. A molecular database for pathogenic zygomycetes has been constructed using sequences from the SSU rDNA and domains D1 and D2 of the LSU rDNA of this family [145]. Access to the sequences may allow the design of taxon-specific (or species-specific) primers, which could be used to develop a potential PCR assay for the rapid identification of zygomycetes in clinical samples.

PCR assays designed to detect dimorphic fungi include a nested assay targeting the H. capsulatum SSU rDNA gene followed by sequencing of the amplified products. This assay was applied to a murine model of histoplasmosis [86]. Intravenously infected ‘in-house’-bred mice (not treated with antifungal drugs) and BALB/c mice (half given amphotericin B and half untreated) were sacrificed after 29 days. Samples of blood and organ homogenates were cultured and examined by PCR. In the ‘in-house’-bred mice, 265 of 319 samples showed concordant results. Seven samples were culture positive but PCR negative, whereas the reverse was obtained with 47 samples ($P < 0.001$). Organ homogenates and blood from spontaneously cured or treated BALB/c mice were PCR negative. The PCR was able to monitor the natural course of murine infection as well as the effect of therapy on infection. Preliminary data show that the assay can also detect DNA of Blastomyces and Paracoccidioides spp. [86].

Specific detection of P. brasiliensis by a nested PCR targeting the gp43 antigen to detect the organism in a sputum sample [97] was briefly mentioned in Part A (see section on target and primer selection). The assay was able to detect 10 cells ml$^{-1}$ of ‘spiked’ sputa with a specificity of 100%. Sputum specimens from 11 patients with proven chronic P. brasiliensis pulmonary infection were PCR positive. Although the gp43 gene is present in low copy numbers per nucleus, P. brasiliensis yeast cells are multinucleated and the number of targets per cell could reach at least 8–16 copies. The protocol was also successfully applied to lung homogenates of experimentally infected BALB/c mice [98]. Finally, a diagnostic PCR based on amplification of the rDNA gene cluster followed by species-specific hybridization has been described [146] but has not undergone clinical evaluation.

Recently, a SSU rDNA gene-targeted assay has been developed to rapidly identify Penicillium marneffei by species-specific hybridization after PCR [85]. The sensitivity of the assay was 0.1 pg µl$^{-1}$ after Southern hybridization. The usefulness of this method as a diagnostic tool requires investigation.

**Methods using PCR-ELISA formats**

PCR-ELISA formats have proved popular because of their superior test sensitivity and their potential for a high throughput of samples.

In the diagnosis of Candida infection, the approach of using panfungal primers and species-specific probes in a PCR-ELISA format was initially attempted by Fujita et al. [48] (Table 2). They were able to detect low numbers of 13 major fungal pathogens inoculated into blood. Probes developed for C. albicans, C. tropicalis, C. parapsilosis, C. krusei and C. glabrata were highly specific but the C. glabrata probe cross-hybridized with S. cerevisiae. The sensitivity of the PCR-ELISA was 10 times greater than that obtained with PCR only [48]. Shin et al. [50] adapted the same PCR to identify 16 different Candida spp. inoculated into BacT/Alert blood culture bottles (Organon Technika Corp., Durham, NC, USA). Blood from 31 patients, with yeasts seen on microscopy, inoculated into the culture bottles also underwent PCR-ELISA. The test was able to detect mixed inoculums and amplicons were detected in 7 h compared with 4.5 days by culture [50].

The PCR-ELISA developed by Wahyuningsih et al. [89] (Table 2) employed a C. albicans-specific probe designed to detect a fragment within the ITS2 region. The specificity of the assay was 100%. Serum samples from eight patients with IC (seven were culture positive) and from a further three of nine patients at risk for IC were PCR positive. Importantly, PCR was negative for sera from 16 ‘control’ and 11 patients with mucocutaneous candidiasis. The study showed that PCR has the potential to identify patients at risk for IC. These studies were extended by Elie et al. [88], who developed several Candida-specific probes to identify the amplified ITS2 regions and by Pontieri et al. [147], who designed a new probe for C. parapsilosis major group I. These probes performed well with known Candida cultures but have not been used in PCR assays on clinical specimens.
An early study had described a PCR assay with *C. albicans* SAP-specific primers based on the amplification of the single-copy *SAP1* gene (the only *SAP* gene described at that time) [148]. Although *C. albicans* was detected in CSF samples, the sensitivity of the assay was not assessed. Flahaut et al. [70] recently evaluated a PCR-ELISA to detect six of the nine *C. albicans* SAP genes. *C. albicans* DNA was successfully detected in blood, BAL, pleural and abdominal fluids, and one skin biopsy specimen (see Table 2). Notably, cell wall lytic enzymes were not required to isolate DNA. Treatment with proteinase K followed by the QIAamp DNA Mini kit (Qiagen) provided adequate amounts of DNA [70]. Prospective studies are in progress comparing the performance of this PCR with culture for the detection of *C. albicans*. Sequence data of *SAP* genes from other *Candida* spp. are also being explored in order to develop a PCR to detect non-*C. albicans Candida* spp.

The applicability of assays to detect *Aspergillus* infection to screen high-risk patients for IA has been discussed earlier. The value of twice-weekly screening in high-risk patients for IA has also been assessed by a PCR-ELISA (Table 3) [72]. A mean of 4.7 blood samples from 121 patients from the haematology service (28 [23%] were PCR positive) and 47 ‘control’ patients (a single PCR-positive sample in 17 [37%] patients) were tested. Sixteen haematology patients were positive only once these patients did not develop IA and were subsequently PCR negative without treatment. Five patients (two had received previous treatment for suspected IA, three had no evidence of IA) had nonconsecutive intermittently positive results. The remaining seven patients were PCR positive on two or more consecutive occasions (five had proven/ probable IA). The PPV and NPV of PCR monitoring for at least two PCR-positive results are shown in Table 3, taking patients with proven/probable IA as the standard for comparison [72]. The study emphasizes that a single PCR-positive result is not necessarily associated with IA. The significance of intermittent positive PCR results is unclear.

Bretagne et al. [63] successfully modified existing assays targeting the *Aspergillus mt* gene to a PCR-ELISA format to detect *Aspergillus* spp. in sera. They studied 22 allogeneic HSCT recipients 6 of whom had proven IA (two PCR positive), 12 had probable IA (7 PCR positive) and 4 had suspected IA (3 PCR positive). All patients without IA had negative PCR tests [73]. A similar PCR-ELISA test using *Aspergillus*-specific primers and an oligonucleotide probe devised from the *Aspergillus mt* gene sequence was tested on BAL fluid in the same patient population [62]. Of 12 patients with PCR-positive results, 3 had definite, and 9 probable IPA (Table 3). The PCR was able to distinguish among *A. fumigatus, A. niger, A. flavus* and *A. terreus* according to amplicon size and has important infection control implications.

**Application of quantitative PCR technology**

Quantitative PCR systems have been adapted to detect and identify primarily *Candida* and *Aspergillus* spp. in clinical samples. TaqMan technology is currently designed only to amplify portions of the fungal rDNA gene cluster.

Reiss et al. [105] reviewed a procedure whereby probes derived from the ITS2 regions of five major *Candida* spp. are labelled with fluorochromes. Three reporter dyes with different emission wavelengths may then be added separately or simultaneously to the master mix. PCR reaction tubes can be arranged to detect the group of *C. albicans, C. tropicalis* and *C. parapsilosis* (each probe labelled with a different fluorochrome); a second PCR tube may be set up to detect the more resistant *C. glabrata* and *C. krusei* with the third option employing an all-*Candida* species probe. This technology has an equivalent sensitivity to PCR-ELISA in detecting *Candida* spp. in blood culture broth but required only 5 h compared with 7 h for PCR-ELISA [105]. More recently, Guiver et al. [87] evaluated an ITS2 region-targeted assay followed by sequencing of the amplified product, for detection of six *Candida* spp. (see Table 2). Probe sets were designed to identify *C. albicans, C. parapsilosis, C. tropicalis, C. krusei, C. kefyr* and *C. glabrata* using the ABI PRISM 7700 DNA Sequence Detection system. No cross-reactivity between candidal and human DNA, or between DNA from other *Candida* spp. was observed.

Loeffler et al. [58] were among the first to adapt their ‘in-house’ rDNA gene-targeted PCR and hybridization protocol to quantitative technology using the LightCycler system [71]. The sensitivity of the assay was 5 cfu ml\(^{-1}\), when performed on blood ‘spiked’ with *A. fumigatus* and *C. albicans* cultures. The specificity and reproducibility (96–99%) of the test were high. When applied to blood samples from patients with proven IA (n = 7) or IC (n = 2), PCR successfully detected and quantified fungal burden in blood. A further protocol for quantification of *A. fumigatus* DNA from known cultures using the ABI PRISM 7700 Sequence Detection System provided an accurate enumeration of *Aspergillus* spp. in environmental samples but has not been applied to human specimens [149].

Recently, two further assays to detect *A. fumigatus* in serum using TaqMan technology have been developed.
The first targeted the mt gene and the second, the single-copy \textit{A. fumigatus} FKS gene (the FKS gene is involved in \textit{\beta}-1,3-glucan synthesis). The latter was able to detect and quantify \textit{A. fumigatus} DNA using blood ‘spiked’ with this DNA. On comparing the effectiveness of the PCR assay on various blood fractions (white cell pellet, plasma and serum), the yield of DNA from plasma was \(\approx 10\) times lower than that from serum or from the white cell pellet [96]. Although the study recommends serum as the preferred specimen, the ‘best’ blood fraction for \textit{Aspergillus} PCR needs to be adequately defined. An earlier report comparing whole blood and plasma using a ‘qualitative’ assay found that the former had a higher sensitivity [67]. Using a DNA capture method (Roche, see Part A) to prepare samples, quantitative PCR can also be performed as used in the Amplicor Monitor (Roche) assays [132]. Results with blood samples ‘spiked’ with \textit{Aspergillus} cultures have indicated recovery rates of 33–100\%. \textit{Aspergillus} DNA can be detected from as few as one conidium [21].

Although the advantages ‘real time’ PCR techniques are many, the prognostic implications of quantifying fungal DNA load are unclear. Einsele et al. [61] demonstrated that the longer patients are on therapy, the less likely they are to remain PCR positive. Others likewise found that patients with persistently positive PCR tests tended to have an adverse outcome [54,72,73,76]. Conversely, some studies have shown that patients who developed radiological improvement on therapy with amphotericin B remained PCR positive, as did patients who had no clinical response [66,80]. A possible explanation for this discrepancy is that antifungal drugs may be responsible for the clearance of \textit{Aspergillus} spp. from the blood but not from the lung. Alternatively, inhibition of the PCR by amphotericin B may be responsible [72,80]. In a mouse model of \textit{C. albicans} infection, however, PCR-positive results were obtained in mice during treatment with amphotericin B suggesting that the drug \textit{per se} does not inhibit PCR [150]. The role of monitoring PCR results in immunocompromised patients receiving antifungal therapy remains to be clarified. No prospective studies have shown that management strategies that incorporate PCR results alter the outcome of patients with IA.

Detection of fungi in tissue specimens by PCR

The protocols described in relation to the detection of fungi in tissue specimens by PCR are summarized below.

Detection of \textit{Candida} spp. in tissue specimens has been mainly studied in the context of \textit{Candida} endophthalmitis. In one study, cultures of ocular vitreous material were negative in two of four patients with suspected \textit{Candida} endophthalmitis, whereas PCR was positive for all four patients [151]. The method used a \textit{C. albicans}-specific primer, CA-1 and amplified a fragment of the SSU rDNA gene [101]. A preliminary study examined the utility of a nested PCR employing two novel panfungal primers, complementary to the SSU rDNA sequences present in \textit{C. albicans}, \textit{A. fumigatus} and \textit{Fusarium solani} [83]. After testing three ocular samples from patients with suspected fungal endophthalmitis, positive PCR results (\textit{C. albicans} only) were obtained for two patients (one was also culture-positive). PCR followed by sequence analysis of the 5.8S rDNA ITS2 region of the fungal genome has also been applied to ocular material [91]. Eleven samples were tested in one study and positive PCR results obtained for six. The fungi detected comprised two isolates each of \textit{C. parapsilosis} and \textit{Aspergillus} spp. and one each of \textit{Alternaria alternata} and \textit{Scedosporium apispermum}. Bacterial DNA was amplified from four of the samples negative for fungal DNA.

Hendolin et al. [67] recently tested a procedure based on PCR and multiplex liquid hybridization for its ability to detect fungi in tissue specimens (Table 3). The method ‘captures’ amplicons with species-specific probes, amplified products are identified by specific hybridization and the identity confirmed by sequencing. Nonhybridized products may also be identified by sequencing. Twelve tissue samples from patients with suspected or proven systemic mycoses, and eight nasal polyposis tissue from patients with chronic rhinosinusitis (CRS) were studied. Of the 20 specimens tested, PCR was positive for 19, of which 10 were hybridization-positive (6 \textit{A. fumigatus}, 4 \textit{C. albicans}). Only seven (35\%) specimens were culture-positive (six had concordant PCR results). Ten specimens were culture- and direct-microscopy negative of which nine (90\%) were positive by PCR. The amplified products were negative by hybridization but sequencing identified an infecting agent in six specimens. Five contained typical pathogens of CRS (\textit{A. fumigatus}, \(n = 2\); \textit{Penicillium} spp., \(n = 1\); and \textit{Epicoccum nigrum}, \(n = 2\)) [67].

The ability of PCR to detect fungi in the nasal mucosa of patients with CRS was also examined by Catten et al. [152] (Table 3). The protocol included the use of four primer sets comprising the primers shown in Figure 1 and a primer that amplifies a sequence of human \(\alpha-1\)-antitrypsin, used to confirm successful isolation of DNA and subsequent PCR. Fungal DNA was detected in 10 of 25 (40\%) patients with CRS and 18 of 42 (42\%) volunteers (‘controls’) but only when the primers ITS3 and ITS4 were used. [152]. None of the fungi could be identified based on amplicon size. Cultures were positive in 0 and 7\%, respectively of patients and ‘controls’.

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Other nucleic acid-based methods to detect fungal pathogens

Reverse line-blot assay to detect Candida DNA
A reverse line-blot assay based on the SSU rDNA gene cluster is available for use in a PCR-based test to detect Candida spp. as reviewed by Reiss et al. [21]. This utilizes a nylon membrane strip to which species-specific probes are covalently linked for simultaneous detection and identification of the amplified product(s). The probes detect Candida species, C. albicans, C. dubliniensis, C. tropicalis, C. viswanathii, C. glabrata, C. kefyr, C. lusitaniae, C. guillermondii, C. krusei and C. rugosa. A probe to distinguish S. cerevisiae (also amplified by the primer pair used) is incorporated as a ‘negative control’. A dilute genus probe is included to ensure detection of low-copy number signals and another, designed to monitor inhibitors of PCR amplification. The assay has a detection limit of one ‘spiked’ C. albicans cell per ml of blood. Results of evaluations of the assay on clinical specimens are awaited.

Detection of RNA from Aspergillus
Detection of organism-specific RNA in clinical samples using nucleic-acid sequence-based amplification (NASBA), an isothermal amplification technique that specifically amplifies RNA using T7 RNA polymerase [153], has been attempted for the detection of Aspergil- lus spp. Loeffler et al. [154] compared the results obtained using such an assay with those obtained by their ‘real time’ PCR protocol using whole blood samples [71]. Using known cultures, the assay had a detection limit of 1 cfu compared with 10 cfu using ‘real time’ PCR [154]. Four blood samples, known to be positive for Aspergillus spp. by PCR, and 73 samples which were PCR negative, were analysed in parallel. All samples showed identical results by both assays [154]. NASBA requires fewer PCR cycles and yet demonstrates similar sensitivity to traditional PCR. Care should be taken, however, to prevent degradation of RNA with the use of appropriate buffers.

In situ hybridization
In situ hybridization (ISH) has the advantage of not requiring DNA extraction techniques and allows visualization of the causative agent against its histological background. The entire process can be automated, making the procedure rapid and simple to perform. ISH has been used with success to detect a 684 bp fragment of the C. neoformans SSU rDNA gene and a 568 bp fragment of the ALP gene of A. fumigatus in tissues of experimentally infected animals [155]. Species-specific probes were prepared by PCR with digoxigenin-labelled dUTP and applied to formalin-fixed or paraffin-embedded tissue. A. fumigatus or C. neoformans DNA can be detected by the observation of dark purple reaction products. Another A. fumigatus-specific probe has also been used to locate Aspergillus DNA in paraffin-embedded lung tissue from patients with IPA [156].

Probe specificity may be confirmed by dot-blot hybridizations with genomic DNA from other fungi, or by ISH using tissue infected with other fungi. Despite its excellent capacity to localize DNA in cells, the sensitivity of ISH is lower than that of PCR-based assays. To increase the sensitivity, the combination of ISH and PCR may be used as in the case of other organisms to amplify localized nucleic acid in tissue by in situ PCR [157].

Conclusion
Given the limitations of some of the traditional diagnostic methods of systemic fungal infections, nucleic acid-based methods have become increasingly important. The manner in which they are implemented into clinical practice, however, requires careful consideration.

In the detection of pathogenic fungi by PCR, the use of panfungal primers directed towards a multi-copy gene target and species-specific probes, appears to provide greater sensitivity and specificity than assays targeting single-copy genes restricted to one or a few species. The rDNA gene cluster is a popular target but the PCR-REA protocol targeting the candidal LIAI gene, and assays that target the candidal SAP gene and Aspergillus mt gene also appear promising.

The application of PCR assays to detect filamentous fungi such as Aspergillus spp. is limited by inherent variation in protocols, lack of standardization and their relatively low PPV. In IA, this highlights the problems in using a sensitive diagnostic test for a pathogen otherwise almost undetectable in blood. Where PCR is performed on BAL fluid, current protocols cannot distinguish colonization from disease or contamination. To maximize the PPV and sensitivity of PCR tests in patients at high risk for IA, the assay should be performed on both BAL and blood specimens. If PCR of blood samples alone are undertaken, at least two samples are required to meet this goal. Consecutive positive PCR results are more indicative of IA than a single positive result. The performances of all assays should be interpreted in the light of the different protocols used, the population studied and the criteria used to define cases of IA. Further experience in the use of PCR protocols for the detection of other moulds is awaited.

Although quantitative ‘real time’ PCR systems are preferred to provide optimum sensitivity for the detec-
tation of fungi, the value of measuring fungal burden is yet to be established. The utility of fungal PCR assays as screening tests requires careful evaluation in large-scale, prospective studies in defined patient populations, with the aim of identifying those patients that would benefit from immediate therapy. This strategy is likely to prove more cost-effective than the current approach of giving prophylaxis to all at risk. As with every surrogate test, the performance of PCR is dependent on the prevalence of disease in a particular population.

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