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PCR Diagnostics in Medical Mycology

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12.1 Introduction

Recent advances in the use of immunosuppressive therapies to treat cancer and enable solid organ or bone marrow transplants, together with advances in the development of broad spectrum antibiotics, have created an increasing population of immunocompromised patients. In addition, the HIV pandemic has created a large increase in the number of immunosuppressed individuals. These patients are at significant risk from systemic fungal infections. The incidence of invasive fungal infection in bone marrow transplant patients has been reported to be as high as 50%, and the subsequent mortality rates are generally around 80% (Tang and Cohen, 1992). The three major opportunistic fungal pathogens in the UK are *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* but many other fungi have also been reported as pathogens in these clinical settings. These include non-*albicans* *Candida* spp., especially *Candida krusei* and *Candida glabrata*, *Aspergillus flavus*, *Pneumocystis carinii*, *Fusarium* spp., *Trichosporon beigelii*, *Rhizopus arrhizus*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Coccidioides immitis* (Bodey, 1988; Anaissie *et al*., 1989). With the exception of the cryptococcal antigen latex agglutination test, established methods for diagnosing systemic fungal infections are often time-consuming and insensitive; e.g. blood cultures are nearly always negative in cases of invasive aspergillosis (Richardson and Warnock, 1993). If appropriate therapy is to improve the prognosis of the immunocompromised patient with systemic fungal infection, early diagnosis is required (Burch *et al.*, 1987).

Antibody responses in immunocompromised hosts are generally too weak to form the basis of a diagnostic test. However, a large research

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investment has been made in the development of antigen detection-based immunoassays for the diagnosis of various systemic mycoses including both invasive candidosis and aspergillosis, disseminated histoplasmosis, coccidioidomycosis and paracoccidioidomycosis. While these tests have the advantages of being both quick and relatively straightforward to perform, they have not gained general acceptance due to low sensitivity and/or lack of commercial availability (De Repentigny *et al.*, 1994). Extracellular galactomannan (GM) circulating in patients with invasive aspergillosis can be detected in serum samples with a commercially available latex agglutination test (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, Marnes-La Coquette, France) which uses a monoclonal antibody (raised against *A. fumigatus* GM) to detect antigen. However, reports on the utility of this diagnostic test vary (Van Cutsem *et al.*, 1990; Kappe and Schulze-Berge, 1993; Haynes and Rogers, 1994; Manso *et al.*, 1994; Verweij *et al.*, 1995). In contrast with other fungal infections, diagnosis of cryptococcosis by the cryptococcal antigen latex agglutination test is accepted as the standard method of diagnosis, with a high sensitivity and specificity. Most equivalent tests are not sensitive enough to exclude diagnosis on the basis of a negative result.

Commercial biochemical tests are available for identifying clinical yeast isolates, e.g. Vitek Yeast Biochemical Card (bioMerieux Vitek Inc., Hazelwood, Missouri) and the API 20C identification system (bioMerieux Vitek Inc.); both are reliable for the identification of common yeasts but problems of non-specificity have been encountered with more unusual species (Fenn *et al.*, 1994).

Inability or delay in diagnosing fungal infection defers the administration of appropriate therapy. This has grave implications for the prognosis of the patient: reliable and rapid diagnostic tests for systemic mycoses are imperative to improve rates of patient survival (Haynes and Rogers, 1996).

In attempting to overcome low sensitivity, poor specificity and delay, molecular approaches to the diagnosis of fungal infection have been adopted, particularly the polymerase chain reaction (PCR). PCR can be very sensitive and, depending on primer design, very specific. Several species-specific PCR-based diagnostic methods for detection of various common fungal pathogens have been described. This discussion will concentrate solely on the diagnosis of systemic fungal infection; however, many of the principles described could easily be adapted to the diagnosis of superficial or subcutaneous mycoses.

12.2 Strategies for Designing PCR-based Diagnostics

When developing a PCR-based diagnostic method various factors can be adapted to optimize both sensitivity and specificity.

12.2.1 Target

The specificity of a diagnostic PCR can be engineered to suit the purpose for which it is being used by selecting an appropriate target sequence for amplification. The specificity is determined by the degree of homology shared between the target sequence and DNA sequences of different genera or species. For example, a highly conserved region from a gene universally shared by all fungi is a suitable target sequence for a PCR protocol designed to indicate whether an infection is bacterial or fungal in origin. This strategy has been adopted by several groups who have selected highly conserved, or 'universal', regions within fungal ribosomal RNA (rRNA) genes as target sequences for amplification. Various amplicon detection methods can be used to identify the fungal source of DNA to genus or species level. The rRNA genes are a popular choice for this strategy as they contain highly conserved regions which can be used to amplify all DNAs, adjacent to highly variable regions which can be used for subsequent species identification. Another advantage is that rRNA genes are generally present at a high copy number which increases the sensitivity of the PCR. Makimura's group (1994) developed a PCR based on conserved regions within the fungal 18S rRNA genes which successfully amplified DNA from 78 strains of 25 medically important fungi. Haynes *et al.* (1995) described a PCR in which one pair of primers corresponded to the V3 region of the *S. cerevisiae* large subunit rRNA gene sequences universally conserved within the fungal kingdom. Species-specific forward primers were then designed which could be used in tandem with the universal reverse primer to specifically amplify DNA from *A. fumigatus*, *Cr. neoformans* and *C. albicans*.

Hopfer *et al.* (1993) used a previously described set of primers based on an rRNA gene sequence present thoughout the fungal kingdom (White *et al.*, 1990) to amplify a 306–311 bp target in 42 different fungi. The amplicons were subjected to restriction enzyme analysis; PCR products were digested with *Hae*III and separated by electrophoresis through agarose gels. The resulting patterns were used to place the sources of fungal DNA into five broad groups including *Candida* spp. and related yeasts, and *Aspergillus* spp. plus related septate moulds.

Alternatively, a highly variable region would give greater specificity, enabling the detection of a particular genus or species. Generally, the more variable the target region the more discriminatory the PCR (Mitchell *et al.*, 1994b). Tang *et al.* (1993) described a PCR to detect the alkaline protease genes of *A. fumigatus* and *A. flavus*; the species were distinguished by the size of the amplified fragment. This PCR was shown to detect both *A. fumigatus* and *A. flavus* in four bronchoalveolar lavage (BAL) samples of four patients with either proven or possible invasive pulmonary aspergillosis; only one of 18 BAL samples from immunosuppressed patients with no clinical indication of fungal infection was positive by PCR.

12.2.2 Sample choice and preparation

As part of the developmental process for a diagnostic PCR protocol, the choice of clinical material and the method of DNA extraction from that material is critical. Ideally, the presence of target DNA in the chosen specimen will be clinically significant; the detection of *Candida* must distinguish pathogen from commensal, the detection of *Aspergillus* must distinguish pathogen from colonization or environmental contamination. Most PCR protocols for the diagnosis of *Candida* infections involve the examination of blood samples as detection of the fungus in blood is regarded as clinically significant. The diagnosis of invasive aspergillosis is more complicated as the fungus is rarely isolated from blood cultures and a reproducible PCR method for detecting *Aspergillus* DNA in blood remains elusive. This problem is complicated further as the significance of detection of this ubiquitous organism in respiratory specimens is questionable (see Section 12.3.2). Often a positive PCR result will need to be interpreted within the clinical setting to determine its relevance. As so many patients with systemic or pulmonary fungal infections are immunocompromised, the invasiveness of the procedure required to obtain the specimen must also be considered, particularly where sequential samples may be required.

Potentially suitable specimens for the diagnosis of systemic fungal infection are blood and/or serum, particularly as these may be obtained by relatively non-invasive procedures. The organism load may be very low or transient and a 5 ml blood sample is unlikely to be fully representative of the body's 4–5 l. Therefore in order to decrease sampling error as much as possible, consecutive samples may need to be taken over a short period of time.

The DNA extraction method used must achieve two particular aims: to efficiently purify fungal DNA with minimal loss, and to remove inhibitors of PCR from the sample, e.g. haem. Breaking open the fungal cell wall is difficult but can be achieved either enzymatically or mechanically. Digestion with lytic enzymes such as Zymolyase (ICN Biomedicals Ltd, Thame) or NovoZym 234 (Calbiochem-Novabiochem Ltd, Nottingham) can aid DNA extraction, although mechanical means such as breaking open cells with glass beads have been successful, particularly for penetrating the thick polysaccharide capsule of *Cr. neoformans* (Tanaka *et al.*, 1996). Yields of extracted DNA may also be improved by using commercially available kits to purify DNA from proteins released by cell lysis (e.g. QIAamp Tissue Kit, Qiagen, Crawley). They are usually quicker to perform than established methods and avoid the use of phenol and chloroform, but they do add significantly to the cost of protocols.

12.2.3 Amplicon detection and analysis

The method used to detect or display amplicons resulting from PCR can increase both sensitivity and specificity. The simplest method is ethidium

bromide staining of PCR products separated by electrophoresis through agarose gels. This has a detection limit of approximately 20 ng per amplified band, and in addition yields information on the size of the PCR product. An increase in sensitivity can be achieved if a nested PCR is performed. Yamakami *et al.* (1996) designed a nested PCR to detect *Aspergillus* species in serum of patients with invasive aspergillosis; the second (nested) round of PCR increased the sensitivity of detection by gel staining from 50 pg to 50 fg target DNA. Southern analysis increased the sensitivity another ten-fold to 5 fg.

Specific oligonucleotide probes have been developed for identification of PCR products from individual fungal species by Southern hybridization. Sandhu *et al.* (1995) used PCR to amplify a conserved region within the 28S rRNA genes from 50 fungal isolates (including common pathogens and saprophytes). Variable regions within 21 of these 50 isolates were used to design species-specific probes. When labelled with ^{32}P each probe hybridized only to homologous DNA. In order to avoid the use of radioisotopes, fluorescein can be used to label probes; fluorescein labelling of an internal probe followed by chemiluminescence (ECL detection system) of Southern blots allowed the confirmation of products from a PCR that amplified a region within the 18S rRNA gene universally conserved in all fungi (Polanco *et al.*, 1995).

Alternatively, probes can be used as part of an enzyme immunoassay detection system. Species-specific probes are bound to the wells of a microtitre plate and denatured PCR products labelled with digoxigenin are hybridized to the probes; the products are visualized using anti-digoxigenin antibody conjugated to horseradish peroxidase with an appropriate chromogen substrate system, e.g. 3,3′,5,5′-tetramethylbenzidine (Sigma, Poole) and hydrogen peroxide. A colour reaction indicates the presence of target DNA identified by the specific probe. Probes are often captured on to the well surface by biotin-labelling the probe and precoating the wells with streptavidin, but dry-adsorption (evaporating a probe solution in the wells overnight at 37°C and heating for 2 h at 60°C) has been reported to be more efficient and negates the need for biotinylating probes (Hirayama *et al.*, 1996). Fujita *et al.* (1995) described a PCR-immunoassay that generated amplicons with universal primers based on the internal transcribed spacer (ITS) region of fungal DNA; species-specific probes derived from this region were used to identify amplicons from *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. krusei*. Using the biotin–avidin capture system and the *C. albicans*-specific probe, a detection sensitivity of two yeast cells per 0.2 ml blood was achieved.

Another approach to amplicon analysis is to look at single-strand conformational polymorphism (SSCP). Minor sequence changes in highly conserved regions of DNA will cause subtle morphological changes in DNA tertiary structure resulting in changes in a fragment's mobility. Differences of only one base pair can be resolved by separating the DNA products

electrophoretically under non-denaturing conditions on a high-resolution acrylamide gel, allowing discrimination between species or even strains (Yap and McGee, 1994). Walsh *et al.* (1995) developed a universal PCR based on the 18S rRNA gene combined with SSCP detection to identify a variety of opportunistic fungal pathogens. The SSCP gel patterns enabled discrimination between *Candida* spp., *Aspergillus* spp., *Cr. neoformans*, *Pseudallescheria boydii* and *R. arrhizus*.

12.3 Diagnosis of Systemic Fungal Infections by PCR

Much research investment has been made into the development of PCR as a diagnostic tool in the field of medical mycology. The major developments in PCR-based diagnosis of invasive candidosis, invasive aspergillosis, *P. carinii* pneumonia, cryptococcosis and infections due to dimorphic fungi are discussed below.

12.3.1 Invasive candidosis

Candida spp., *C. albicans* in particular, are the most common fungal pathogens of immunocompromised hosts, causing a variety of infections from cutaneous disease of skin and nails to deep-seated systemic infection and candidaemia. Diagnosis is relatively straightforward for superficial infections but remains a problem for the more invasive forms of disease (Dupont, 1990). While most attention was initially paid to developing detection methods for *C. albicans*, as the primary pathogen of the genus, it has become apparent that other species of the genus may also cause disease. In a retrospective study of blood culture isolates from cancer patients approximately half (28 of 55) of the *Candida* isolates were *C. albicans* while the rest comprised of *C. tropicalis* (seven), *C. glabrata* (seven), *C. krusei* (five), *C. parapsilosis* (three), *C. guilliermondii* (three) and two mixed infections of *C. albicans* and *C. glabrata* (Meunier *et al.*, 1992). Many of the PCR protocols published therefore describe strategies to detect both *C. albicans* and other species.

One of the first PCR methods for diagnosing fungal infection to be published was that by Buchman *et al.* (1990); the gene coding for *Candida* spp. cytochrome P_{450} lanosterol-14α-demethylase (L1A1) was chosen as the target for amplification due to its involvement in the process of ergosterol biosynthesis. The sensitivity of the PCR was not fully assessed but was estimated in spiking experiments to be approximately 10^2–10^3 c.f.u. ml^{-1}. Clinical samples including urine, sputum, wound fluid and blood from six patients that had all undergone surgical trauma were tested by PCR; 15 of these gave positive results. However, the specificity of this PCR was not evaluated and the implications of the PCR-positive clinical samples was not discussed.

A sequence within a duplicated region of *C. albicans* mitochondrial DNA (mt DNA), EO3, was chosen by Miyakawa *et al*. (1992) as the target for a *C. albicans*-specific PCR. Primers, designed from partial sequencing of the region, generated a 1.8 kb fragment from 40 strains of *C. albicans* (type A and B) but not from any other of 38 isolates including seven non-*albicans Candida* spp., *Cr. neoformans*, *Saccharomyces cerevisiae*, three bacterial isolates and a human cell line. The sensitivity of the PCR was determined by spiking serial dilutions of yeast cells into both saline and human urine; the detection limits were two to ten cells and 100 cells in saline and urine respectively on ethidium bromide-stained gels. This was improved to two to ten cells for both saline and urine by Southern hybridization analysis. The authors conceded that such a large amplicon may be impractical when trying to detect small amounts of DNA present in clinical specimens such as blood, but suggested that smaller *C. albicans*-specific fragments (400–500 bp) within EO3 may amplify more efficiently.

Niesters *et al.* (1993) used a nested PCR in conjunction with a number of detection methods to identify various species of *Candida*. Sequences from the small subunit (SSU) rRNA genes of *C albicans*, *C. glabrata* and *S. cerevisiae* were used to design primers which amplified the entire gene. Products were also obtained from *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. pseudotropicalis*, *C. stellatoidea* and *C. tropicalis*. Species-specific fingerprints generated by direct sequencing, Southern hybridization with species-specific probes, restriction enzyme mapping and interrepeat PCR (with repeat-oriented primers) were all able to identify these eight *Candida* isolates to species level. Restriction mapping provided the most rapid means of identification (1 day), but none of the methods were used to test clinical material.

In contrast to the high copy number gene targets described above, a PCR for diagnosing candidaemia was reported based on amplification of a 158 bp segment from the *C. albicans* actin gene (Kan, 1993). Amplicons were hybridized in the PCR reaction tube with a radiolabelled 30 bp internal probe before electrophoresis on acrylamide gels which were then exposed to autoradiography film. The sensitivity was reported as 25 fg of DNA or ten yeast cells in spiked samples. The specificity testing showed the PCR to be genus-specific, with the exception of *S. cerevisiae*. Preliminary evaluation of the PCR was carried out using immunocompetent mouse models and patient samples. Blood samples from a murine candidaemia model were cultured and plasma samples were pooled and tested by PCR. All samples were consistently positive by culture and PCR over a period of 4 days after initiation of infection. In the second model, localized infection in the thigh was induced and blood and plasma samples were taken every 3 days up to 15 days after inoculation; no samples tested positive either by culture or PCR. A similar pattern was seen in blood cultures from patients. Serum samples were collected from 14 patients who had previously had *Candida* isolated from at least one blood culture; 11 of these were PCR positive while sera from 12

patients with active oral thrush and 17 healthy volunteers were all PCR negative. This protocol was the first described that could detect and identify numerous species of *Candida* and was shown to work with samples from both mice and patients. More importantly the PCR could detect candidaemia without false positives generated by the presence of *Candida* either as a commensal or in a non-disseminated infection.

Crampin and Matthews (1993) also used a low copy number gene for PCR amplification, heat shock protein 90 (*HSP90*). Primers designed from the *C. albicans* gene generated amplicons with *C. albicans* DNA. In addition, non-specific products were seen in samples 'spiked' with *C. glabrata*, *C. parapsilosis*, *C. guilliermondii*, *C. krusei* and human DNA, although the authors stated that these were not seen in PCR performed on any clinical samples. Southern analysis allowed detection of 100 c.f.u. *C. albicans* ml^{-1} broth or 5 pg genomic DNA. An investigation of 100 clinical specimens (including swabs, urines, peritoneal fluids, pus and blood/serum) tested by routine culture, extended culture and PCR, gave positive results with 23%, 31% and 37% of samples respectively. The clinical relevance of detecting *C. albicans* in these samples was not discussed.

One of the *C. albicans* chitin synthase genes (*CHS1*) has also been exploited as a target for PCR based on the rationale that *CHS1* is not present in any mammalian genome and could therefore help to prevent false-positive results (Jordan, 1994). The single pair of primers described produced different sized amplicons from *C. albicans* (122 bp), *C. parapsilosis* (311 bp), *C. tropicalis* (519 bp) and *C. glabrata* (535 bp); these four species are responsible for 90% of cases of neonatal candidaemia. This was the first published method that was capable of detecting different medically important species of *Candida* in just one step. Species-specific probes were designed for use in Southern hybridization which gave a detection limit of 10 c.f.u. ml^{-1} blood. Comparing PCR and blood culture in 27 pairs of blood samples showed concordance in 26 pairs; blood samples from 29 high-risk neonates with no signs of *Candida* infection and five bone marow transplant recipients with mucosal colonization with *Candida* were all negative by PCR. While this protocol was not shown to be more sensitive than blood culture in this case, it had the advantages of speed and easy species differentiation.

Meanwhile, the rRNA genes remain a popular target for PCR. Holmes *et al.* (1994) described two sets of primers based on the 5S rRNA gene and the adjacent non-transcribed intergenic spacer (IGS); one pair amplified a product of 105 bp from *C. albicans* and five non-*albicans* species, while the second set amplified a 684 bp product from *C. albicans* only, with a sensitivity comparable to the PCRs already described of approximately 15 c.f.u. ml^{-1} blood.

Similarly, the V4 region within the SSU rRNA gene was the basis for a PCR which, in conjunction with Southern blotting and a species-specific probe, could specifically detect *C. albicans* candidaemia in neutropenic mice

with a sensitivity of 10–15 c.f.u. ml^{-1} blood (van Deventer *et al.*, 1995). Gastrointestinal colonization was established in immunocompetent mice and blood samples were tested by the same method to demonstrate that the PCR would only detect invasive fungal cells and not commensals. A comparison of PCR with blood culture demonstrated a large increase in the number of positives when PCR was used (89–100% compared with 44–100% for culture). Only *C. albicans* was investigated in this report but the authors indicated the possibilities for adapting this method to other species using different probes.

Fujita *et al.* (1995) reported a universal PCR that was capable of detecting any fungal species with primers based on sequences from the 5S rRNA gene and adjacent ITS region; specific probes designed from the ITS2 region (found between 5S and 26S) were used in an enzyme immunoassay to specifically detect PCR products from *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. glabrata*. Using colorimetric detection instead of ethidium bromide staining increased the sensitivity ten-fold from 10^2 to 10 cells ml^{-1}.

Haynes *et al.* (1995) and Haynes and Westerneng (1996) used a reverse primer based on a sequence from the large subunit (LSU) rRNA gene, universally conserved within the fungal kingdom, in tandem with four different forward primers, each specific for a medically important species of *Candida*. The four PCRs used the same temperature cycles and so could run concurrently, and were shown to correctly identify nine isolates of *C. albicans*, 18 of *C. glabrata*, 13 of *C. parapsilosis* and 18 of *C. krusei* in a blind study. The testing process, including DNA extraction from single colonies, was estimated to take approximately 3 h, although no testing was performed on clinical samples.

Recently a PCR based on the *C. albicans* L_1A_1 gene was described which was capable of amplifying DNA from a variety of yeasts including *C. albicans*, ten non-*albicans Candida* species, *S. cerevisiae* and *Blastoschizomyces capitatus* (Morace *et al.*, 1997). The PCR had a sensitivity of 200 fg *Candida* DNA and seven different species were identified by restriction enzyme mapping. This method was applied to a small number of blood and BAL samples from patients considered likely to have fungal infection; 15 of 21 bloods and six of 20 BAL samples gave PCR-positive results and this was considered to correlate well with culture results. The authors emphasized that this protocol must be used to analyse a much larger number of patient specimens before any conclusion can be made. These results were encouraging but a much simpler extraction method would be neccessary for this protocol to be used on a routine basis.

12.3.2 Invasive aspergillosis

Members of the genus *Aspergillus* are ubiquitous saprophytes commonly found in soil and decaying vegetation. *Aspergillus* conidia account for

0.1–22% of total airborne spores and are found in air and dust within the hospital environment (Anassie, 1992). There are at least 200 species of *Aspergillus* but few are associated with disease in humans; the majority of invasive disease is due to *A. fumigatus* and can arise from colonization or *de novo* infection (Rinaldi, 1983). The common route of entry into the body is via the respiratory tract. Conidia are smooth and, in the case of *A. fumigatus*, only 2–3 μm in size and can therefore easily reach the alveolar airspace; this may be directly linked to *A. fumigatus* being the primary pathogen of the genus (Kwon-Chung and Bennett, 1992). In predisposed (e.g. neutropenic) patients the infection rapidly becomes invasive; pulmonary disease disseminates haematogeneously through the body to other major organs and the associated morbidity and mortality are very high (Cohen, 1990). Early administration of antifungal therapy is essential for improvement of prognosis in cases of invasive disease (Burch *et al.*, 1987), however, early diagnosis remains notoriously difficult. Routine microbiological investigation involves culture and cytological examination of respiratory specimens such as BAL fluid and induced sputum, procedures that have been evaluated and reviewed by several groups in a number of retrospective studies. Nalesnik *et al.* (1980) suggested that even a single *Aspergillus*-positive sputum culture must be treated as potentially significant in patients with haematological malignancy. Yu *et al.* (1986) confirmed this, adding that in neutropenic cases positive respiratory specimens were virtually diagnostic. Cytological examination of BAL specimens has been considered to be of a higher predictive value than culture (Levy *et al.*, 1992), although in patients with acute leukaemia, BALs have been reported to be of more use for diagnosing *P. carinii* pneumonia than invasive aspergillosis (Saito *et al.*, 1988). The majority of respiratory specimens are negative for *Aspergillus* by cytology and culture, and the predictive value of immunological diagnosis has yet to be proven. The gold standard remains cytology and culture of biopsy specimens, but open surgery is contra-indicated in critically ill patients.

In order to avoid the invasive procedures of obtaining specimens such as BAL fluid or bronchial washings, Reddy *et al.* (1993) investigated the possibility of using PCR to detect *A. fumigatus* in urine samples. The primers were based on an *A. fumigatus* partial protein sequence that shared a high degree of homology with ribotoxins of *A. restrictus* and with alpha sarcin of *A. giganteus*. The PCR amplified *A. fumigatus* and *A. restrictus* DNA only, with a detection limit of 0.6 pg by Southern analysis. Prospective screening of 13 urine samples from patients undergoing bone-marrow transplantation revealed two positives; however only one was from a proven case of IA.

Spreadbury *et al.* (1993) were able to specifically detect *A. fumigatus* DNA in three of three culture-positive respiratory specimens from patients with clinically diagnosed IA by using a PCR that amplified part of the 26S intergenic spacer region from the *A. fumigatus* rRNA gene complex. However, as *Aspergillus* conidia are inhaled by the general population on a day-to-day basis, the significance of finding *Aspergillus* in the respiratory

tract of an individual is still controversial. To assess the significance of a positive PCR result, culture-negative samples from immunosuppressed patients at high risk and from immunocompetent patients with known lung disease (other than IA) were analysed by PCR: two of ten of the former patient group and two of seven of the latter patient group gave positive results. It was suggested that results based on Southern hybridization may be too sensitive – the two positives from the former patient group were not detected by electrophoresis alone – but that the high level of correlation between culture, clinical data and PCR results supported PCR as a valid method of diagnosis of IA.

These findings were corroborated by further work from the same group; Tang *et al.* (1993) used primers based on sequences obtained from the alkaline protease (*Alp*) genes of *A. fumigatus* and *A. flavus* to detect *Aspergillus* DNA in four BAL samples from four patients with proven or probable aspergillosis. One patient with possible aspergillosis was PCR positive, as were one of 18 (6%) immunosuppressed patients with no evidence of fungal infection and five of 28 (18%) immunocompetent patients, the latter most likely resulting from colonization.

Verweij *et al*. (1994) investigated a larger group of low-risk patients and found a similar level of prevalence of detectable *Aspergillus* colonization. 72 BAL specimens from 70 non-neutropenic patients were tested by culture and genus-specific PCR: 11 of the 72 samples (15%) were PCR positive. In a separate study the validity of the PCR was assessed in a mouse model of IA and in patient BAL samples. The primers were derived from sequences obtained from the 18S rRNA genes of *A. fumigatus*, *A. flavus*, *A. terreus*, *A. nidulans* and *A. niger* so that other species that have been reported as opportunistic pathogens could also be detected (Cohen, 1990). Strong cross-reactions were observed with *Paecilomyces variotii*, *Penicillium marneffei* and *Penicillium chrysogenum* but these were differentiated from *Aspergillus* by Southern analysis and restriction enzyme digestion. Sensitivity was increased from 1 pg to 10 fg when rRNA was transcribed into cDNA prior to amplification, although this was deemed unnecessary as no further samples were identified as positive using this extra step. Five of six mice with experimental IA were PCR positive, as were nine of 18 BAL samples from immunosuppressed patients; none of the control mice or 14 BAL samples from low-risk patients were positive. In comparison only one BAL sample from four patients with documented IA grew *Aspergillus*.

A competitive PCR was described by Bretagne *et al.* (1995) which used an internal control to indicate false-negative results due to inhibition of the PCR. This was achieved by spiking each sample with a fragment of M13mp18 phage DNA flanked at each end by the *A. fumigatus*-specific primers; successful amplification would generate a 135 bp product from *A. fumigatus* DNA and a separate smaller amplicon from the control sequence. False-positive results due to carry-over contamination from previous amplifications was avoided by the substitution of dUTP for dTTP and digestion

with uracil-*N*-glycosylase. However, it should be noted that this is not necessarily sufficient to prevent false positives due to contamination. In a study of PCR protocols used in seven centres for *Mycobacterium tuberculosis* detection, this substitution method was employed by the centre that had the highest rates of false-positive results (Vaneechoutte and van Eldere, 1997). Of 55 BAL samples tested with this PCR, 15 were positive, 37 were negative and three were negative due to inhibition and discounted. Three patients had proven IA and another three were high risk, but the remaining nine positive samples were from two groups of patients, none of whom developed IA. As the three patients with aspergillosis had already been clinically diagnosed and given empirical antifungal therapy, the authors concluded that PCR was of little value in diagnosing cases of IA where clinical symptoms were overt; routine methods were sufficient and the question of the predictive value of PCR diagnosis in high-risk patients remained unanswered. Verweij suggested that optimized techniques and sampling schedules are required if PCR is to prove useful in the early diagnosis of IA (Verweij *et al.*, 1996).

Recently, Yamakami *et al.* (1996) reported a genus-specific nested PCR based on 18S rRNA genes of *Aspergillus* which was evaluated using serum from experimentally infected mice and from patients with IA. Southern analysis increased the sensitivity of the PCR to 5 fg *Aspergillus* DNA. Serum samples from animals sacrificed over a period of 4 days post-infection were tested by PCR and Pastorex *Aspergillus* latex agglutination; these tests gave five of eight and three of eight positives respectively and all the controls were negative. However, mice were inoculated with viable conidia through a surgical incision over the trachea, possibly allowing direct inoculation of the bloodstream, in which case this would not be an appropriate model of IA. Serum samples (100 µl) from 20 patients with documented IA were also tested, giving 14 positive samples by PCR and 12 positive samples by Pastorex. Sera from 20 healthy volunteers were all negative. These results are promising but care would need to be taken when designing sampling schedules, especially when sample volumes are so small.

A test proven to be sensitive and specific that can be performed on clinical material obtained without invasive procedures is still needed to improve the *ante-mortem* diagnosis of IA.

12.3.3 Pneumocystis carinii *pneumonia*

Interest in the ubiquitous and, until recently, taxonomically ambiguous *P. carinii* (Pixley *et al*., 1991; Stringer *et al*., 1992) was re-awakened in the early 1980s when the advent of AIDS caused a significant increase in the incidence of *P. carinii* pneumonia (PCP) to such an extent that PCP became a diagnostic feature of AIDS and was a major cause of morbidity and mortality in AIDS patients (Cushion *et al.*, 1994). The major obstacle yet to be overcome in the development of simple, rapid diagnostics for this infection is the lack of an *in vitro* culture system, primarily due to the organism's

fastidious nature (Wakefield *et al.*, 1990). Conventional diagnostic procedures remain: cytochemical staining of BAL or, less frequently, induced sputum samples, either non-specifically (Giemsa, methenamine silver) or with a specific immunofluorescent monoclonal antibody stain (Cartwright *et al.*, 1994). However, small numbers of organisms are difficult to visualize and a more sensitive method is needed to attempt diagnosis at an earlier stage of infection. Many research groups have therefore explored the possibilities of using PCR as a diagnostic method for PCP.

The first PCR reported for detection of *P. carinii* in clinical samples amplified a sequence from the large subunit mitochondrial rRNA gene (mt rDNA) and although sequences were derived from a rat isolate, the PCR also amplified *P. carinii* DNA from human isolates (Wakefield *et al.*, 1990). The products were verified using Southern analysis with a probe specific for human *P. carinii*. The PCR appeared to be at least as sensitive as staining when detecting *P. carinii* in BAL, but apparently false-positive signals were observed on blots from three immunosuppressed patients with no evidence of PCP and from bronchoscope washings from an unspecified source.

While some groups have designed novel PCRs to detect *P. carinii*, the mt rDNA primers have since been used by many other investigators. Eisen *et al.* (1994) found this PCR to be more sensitive than Toluidine Blue-O staining and immunofluorescence in sputum samples from 20 HIV-positive patients. In another study an enzyme immunoassay was developed as a potential replacement for Southern analysis (Cartwright *et al.*, 1994). This assay detected *P. carinii* in all induced sputa from documented PCP cases, compared with 78% by immunofluorescence, and the entire procedure could be performed in a single day. This PCR assay was considered sensitive enough to detect *P. carinii* in induced sputa from patients with a low organism burden, possibly saving them from the invasive procedure of bronchoscopy. However, no advantage over conventional methods for routine detection of *P. carinii* in BAL was evident. These findings are similar to those reported by other groups (Lipschik *et al.*, 1992; Tamburrini *et al.*, 1993; Roux *et al.*, 1994).

Honda *et al.* (1994) described a comparison of conventional and capillary PCR in which both protocols used the mt rDNA primers to detect *P. carinii* in sputum and BAL samples from immunosuppressed patients. The volume of the reaction mix in capillary PCR is only 10 µl (compared with 25–100 µl in conventional PCR) and thermocycling is performed in sealed glass capillary tubes; this allows much faster and more efficient temperature transfer. However, savings made in reaction constituents have to be balanced against the purchase of a specialized thermocycling machine. The capillary PCR was 1000-fold more sensitive than conventional PCR (detecting 3×10^{-2} fg DNA compared with 30 fg) and took only 20 min to complete. The authors suggested that the high sensitivity of the capillary protocol enabled detection of *P. carinii* carriage and subclinical infection and allowed for earlier initiation of antimicrobial therapy.

In an attempt to monitor organism loading during treatment of *P. carinii* infection in immunosuppressed rats, O'Leary *et al.* (1995) incorporated the mt rDNA primers into a semi-quantitative PCR (SQPCR). Rat β-globin was used as the internal standard and assay results were expressed as *Pneumocystis* mt rDNA/globin signal ratios. The SQPCR results correlated strongly with cyst counts in BAL and lung homogenates. Furthermore the PCR was capable of detecting *P. carinii* DNA during the early phase of infection when few cysts could be visualized by staining of pulmonary tissue and none were seen in BAL fluid.

Alternative gene targets for *P. carinii* PCR include those encoding the 5S rRNA (Kitada *et al.*, 1991), 18S rRNA (Lipschik *et al.*, 1992), dihydrofolate reductase (DHFR) (Schluger *et al.*, 1992) and thymidylate synthase (TS) (Olsson *et al.*, 1993) genes. These were all included in a comparison of the efficacy of different PCR methods, along with the mt rDNA PCR and a nested PCR which amplified the ITS of the rRNA genes (Lu *et al.*, 1995). When used to analyse 50 BAL specimens, the two nested PCRs (ITS and 18S) achieved 100% sensitivity after the second round (53% and 50% respectively after the first), the mt rDNA PCR 87%, the 5S rDNA PCR 33%, the TS PCR 60% and the DHFR PCR 23%. The 18S and TS PCRs demonstrated false-positive results with *S. cerevisiae* and *C. albicans*, plus *Cr. neoformans* and *C. albicans* respectively, leading to the conclusion that the nested ITS PCR was the most sensitive and specific of the methods tested.

In order to avoid the invasive nature of sampling methods such as bronchoscopy or open lung biopsy, non-invasive samples such as serum and blood have been investigated as attractive alternatives for potential routine specimens when diagnosing *P. carinii* infection. Preliminary experiments by Kitada *et al.* (1991) detected *P. carinii* DNA in three of five mice with experimentally induced *P. carinii* infection; reduced sensitivity compared with performance of PCR with lung biopsy specimens was attributed to low organism load in the blood. Schluger *et al.* (1991) used the DHFR PCR on serum samples from AIDS patients with PCP; *P. carinii* DNA was detected in five of 14 experimental rats and seven of 18 AIDS patients. However, four patients had documented extrapulmonary PCP, two of which were not detected by PCR. Tamburrini *et al.* (1993) successfully detected *P. carinii* in spiked samples down to two organisms μl^{-1} serum or whole blood, but this could not be reproduced in serum and blood specimens from HIV-positive patients with documented PCP. The conclusion was that, even allowing for haematogeneous dissemination, the presence of *P. carinii* in the bloodstream is transient and PCR detection is not applicable to serum samples.

12.3.4 Cryptococcosis

Cryptococcal meningitis, caused by *Cr. neoformans* var. *neoformans* or *Cr. neoformans* var. *gattii*, is a major cause of morbidity in individuals infected with HIV (Kwon-Chung *et al.*, 1994). Before the cryptococcal antigen latex

agglutination (LA) test became widely available, cryptococcal meningitis was diagnosed by India ink preparations of cerebrospinal fluid; this gave a positive result in approximately 50% of cases. Detection of capsular polysaccharide by LA has a reported sensitivity and specificity of 99% and immunological diagnosis appears to be reliable (De Repentigny *et al*., 1994).

The reliability of the LA test as a method of diagnosing cryptococcosis has obviated the need for diagnostic PCR. However, a number of protocols have been described. Mitchell *et al*. (1994a) designed primers from the 5.8S rDNA and adjacent ITS region of *Filobasidiella neoformans*, the teleomorph of *Cr. neoformans*. These primers were able specifically to amplify DNA from 37 strains of *Cr. neoformans*; amplification from clinical material was suggested but not attempted. A nested PCR based on the *Cr. neoformans URA5* gene was developed to diagnose non-HIV related pulmonary cryptococcosis (Tanaka *et al.*, 1996). Sixteen respiratory specimens were analysed in order to evaluate both the PCR and the glass bead-based extraction method. Of the five which were positive by culture, four were PCR positive and no culture-negatives were PCR positive. While this protocol performed well on this small number of samples, the advantages over culture were unspecified.

12.3.5 Dimorphic fungal infections

The dimorphic fungi *H. capsulatum*, *B. dermatitidis*, *Co. immitis* and *Pa. brasiliensis* are primary human pathogens. However, the incidence of each has increased as the number of immunosuppressed patients has risen. The route of infection for these is generally via the respiratory tract; they cause primary pulmonary disease in competent hosts. *H. capsulatum* causes chronic pulmonary disease in those with structural lung defects and disseminated disease in immunocompromised hosts. *Co. immitis* is asymptomatic in 60% of cases and rarely disseminates. *Pa. brasiliensis* manifests more as a disseminated infection than a pulmonary one while *B. dermatitidis* infection is initially pulmonary but can disseminate and mimic other diseases such as tuberculosis (TB) (Bradsher, 1996).

While the incidence of these fungi is increasing, particularly in the Americas where they are endemic, diagnosis using molecular methods is not well documented and little diagnostic PCR research has been published. Diagnosis relies mostly on cytological staining of BAL fluids and sputa with Papanicolaou stain and methenamine silver; TB is excluded by the acid Schiff test (Lemos *et al.*, 1995). Visualization of the fungus gives a firm diagnosis for histoplasmosis and blastomycosis as colonization with these organisms does not occur (Bradsher, 1996).

Molecular methods other than PCR have been successfully used; a chemiluminescent DNA probe kit (GenProbe, San Diego, California) has been shown to have sensitivities of 87.8–100% and specificities of 100% when identifying different isolates of *B. dermatitidis*, *H. capsulatum*, *Co.*

immitis and *Cr. neoformans* (Stockman *et al.*, 1993). The probes, based on rDNA sequences, were found to shorten the diagnostic process significantly, and the preparation required was much less than for exoantigen testing. These probes were recommended by the author to supplement diagnosis by *in vitro* conversion of hyaline mould to yeast forms; false-negative results that occasionally occurred with the *B. dermatitidis* probe could thus be avoided and morphologically similar organisms could be differentiated, such as the yeast form of *H. capsulatum* and *C. glabrata*.

12.4 Future Prospects for PCR Diagnostics in Medical Mycology

Commercially produced PCR-based protocols are now widely available for the diagnosis of various infectious diseases, e.g. TB, viral hepatitis. Notwithstanding the huge research investment that has been made in the development of diagnostic PCRs in the field of medical mycology, there are no such protocols available for the diagnosis of fungal infections. The most likely contender for a successful PCR-based protocol is the detection of *P. carinii* for the diagnosis of PCP, but the advantages of PCR over established diagnostic methods for this disease are debatable. Until simple, rapid and reproducible methods are developed for the extraction of fungal DNA from clinical material, the routine diagnosis of systemic fungal infections by PCR remains unlikely.

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