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PCR-based diagnosis of human fungal infections

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Abstract

PCR is a very appealing technology for the detection of human pathogens, but the detection of fungal pathogens is particularly challenging. Fungi have cell walls that impede the efficient lysis of organisms and liberation of DNA, which can lead to false-negative PCR results. Conversely, some human pathogens are also ubiquitous environmental saprophytes that can contaminate PCR reagents and cause false-positive results. We examine the quality of PCR-based studies for fungal diagnostics using 42 variables within the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. This review focuses on taxon-directed PCR assays for the diagnosis of invasive aspergillosis, candidiasis and *Pneumocystis* pneumonia. Finally, we evaluate broad-range fungal PCR assays capable of detecting a wide spectrum of human pathogens.

Keywords

aspergillosis; candidiasis; diagnosis; fungal infection; PCR; pneumocystis; review

The PCR method for DNA amplification was developed by Kary Mullis and colleagues in 1984 and was rapidly adapted to detect a variety of infectious agents, particularly viruses. Despite this success, PCR has not been widely adopted to detect fungal pathogens in human infections and has been eclipsed by other technologies such as fungal antigen detection assays. However, the diagnosis of human fungal infections continues to be a challenge. Conventional diagnostic techniques such as radiological imaging, culture and histology fall short in terms of specificity, sensitivity and time to diagnosis. In addition, diagnostic tests based on galactomannan (GM) antigen and glucan do not detect all fungal pathogens and have problems with specificity.

PCR assays offer several features that could overcome current shortcomings for the diagnosis of fungal infections. PCR assays can have detection limits of a few gene copies per reaction, providing the ability to detect a fraction of an organism when targeting genes present in multiple copies per fungal genome. Primers and probes can be designed such that the target can be refined to a specific phylogenetic/taxonomic level; for example, species or genus, or broadened to include most fungi using a consensus sequence PCR approach. Real-time platforms enable

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quantification of fungal load in the clinical specimen, which may provide information about burden or progression of disease. In addition, real-time PCR formats can provide rapid pathogen detection and confirmation when using a taxon-targeted assay with a probe, or when using methods such as broad-range PCR with melt-curve analysis of the amplified product. Multiplexed assays enable the simultaneous detection of numerous pathogens at varying levels of phylogeny/taxonomy. Furthermore, sequence variation within the amplified product can enable accurate species-level identification.

There is no lack of genomic information for developing fungal PCR assays, so why has adoption of PCR-based diagnostic methods been so glacial? The design of appropriate experimental controls for PCR-based diagnostic assays has hindered interpretation of early assays. In addition, the efficient extraction of fungal DNA from complex clinical samples has been a major impediment to improved diagnostic performance, and fungal contamination is a persistently vexing problem in the field. Nevertheless, there have been significant advances made over the last 10 years in fungal PCR diagnostics. In light of the recently published Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [1], we examine the quality of PCR-based studies for fungal diagnostics. Our review analyzes trends over the past decade to gain insights on the impact of analytical variables on diagnostic performance. Our analysis is based on considering 42 variables (BOX 1) for 68 publications (Supplementary Table; see online www.expert-reviews.com/toc/eri/7/10).

Box 1

Study parameters considered for analysis in this review.

Fungal disease	Cross-reactivity testing of primers with human DNA
Patient population	PCR amplification (inhibition) control
Criteria for clinical diagnosis	Method used to confirm or identify PCR positives
Clinical sample	Receiver operating curve analysis (if applicable)
Study design (retrospective or prospective)	Patients or episodes (proven IFD)
Study design (blinded or not)	Patients or episodes (probable IFD)
Fraction of sample used for extraction	Patients or episodes (possible IFD)
Volume of sample used for extraction	Patients or episodes (no IFD)
DNA extraction (kit or fungal cell wall lysis)	Total number of patients or episodes
DNA extraction (purification)	Prevalence based on proven + probable
Positive extraction control (extraction efficiency)	Diagnostic sensitivity
Negative extraction control (contamination)	Diagnostic specificity
PCR format	Estimates of confidence interval (yes or no)
PCR target gene	Positive-predictive value
Probe chemistry (if applicable)	Negative-predictive value
Target pathogens or group (species, genus or broad range)	Other molecular diagnostic techniques
Basis of analytical sensitivity (PCR or extraction + PCR)	Diagnostic sensitivity of other molecular diagnostic techniques
Analytical sensitivity	Diagnostic specificity of other molecular diagnostic techniques
Limit of detection analysis	Conventional diagnostic technique

Use of uracil-DNA glycosylase enzyme	Diagnostic sensitivity of conventional diagnostic technique
Quantification of human DNA in extracts	Diagnostic specificity of conventional diagnostic technique

IFD: Invasive fungal disease.

Search strategy

To focus our review, we excluded studies related to the endemic mycoses, dermatomycosis and keratomycosis, studies with ten or fewer clinically identified cases of invasive fungal disease (IFD), or studies using only a pool of patients with 'suspected' disease without defining a gold standard to compare the PCR assay. In addition, alternative nucleic acid-based tests such as fluorescence *in situ* hybridization and isothermal amplification techniques such as nucleic acid sequence-based amplification were excluded. We searched PubMed for articles published over a period of 10 years between 1 May 1999 and 1 June 2009 using the search criteria "fungal [Title/Abstract] OR aspergillus [Title/Abstract]) OR *Candida* [Title/Abstract]) OR zygomycete [Title/Abstract]) OR mucormycosis [Title/Abstract]) OR pneumocystis [Title/Abstract]) OR scedosporium [Title/Abstract]) OR fusarium [Title/Abstract]) AND PCR [Title/Abstract]) AND diagnosis [Title/Abstract]) AND ('humans' [MeSH Terms] AND English [lang])". The search resulted in 262 items of which 36 were reviews.

We made exceptions to include publications prior to the designated 10-year period or with fewer than ten clinically identified positive IFD cases if they were cited multiple times by the selected group of publications. Based on the inclusion criteria, 68 publications were considered for analysis. Parameters listed in BOX 1 were extracted from these publications to create an Excel-based data table (Supplementary Table. If a specific data element was not explicitly mentioned or could not be inferred from a related reference, we omitted that parameter from the table. Table 1 lists the distribution of publications considered for our review on the basis of disease category targeted by PCR and the number of publications within each category that reported diagnostic sensitivity and specificity.

Shortcomings of PCR-based fungal diagnostics

An ideal clinical study evaluating the diagnostic performance of PCR-based assays would include most variables listed in BOX 1 and meet the MIQE guidelines. Most studies lack this information. Data on these analytical variables are useful for assessing the true performance characteristics of each assay, including rates of false-positive (FP) and false-negative (FN) results. In addition, these data are useful for comparing assay platforms and assessing the contribution of each step towards diagnostic performance. Beyond the necessity to adopt rigorous quality control measures, other parameters requiring optimization include choice of clinical sample type (e.g., blood vs respiratory secretions), fraction of sample to be used for DNA extraction (e.g., serum vs whole blood), DNA extraction method, format of PCR (e.g., end point vs quantitative), PCR target gene, study design, sample size and the statistical analysis of diagnostic performance.

False positives

The factors contributing towards FPs can be differentiated into procedural false positivity and clinical false positivity. Sources of procedural false positivity are contamination from the environment, previously amplified PCR products, or cross-reactivity of PCR primers and probes with nontarget fungi or other organisms. If procedural false positivity is not monitored with rigorous controls then it could lead to a FP result with clinical relevance. On the other

hand, clinical false positivity caused due to colonization of human tissue surfaces with fungi is not a reflection of inadequacies in assay design or protocol because the assay has detected something that it was designed to do. For example, in a host who is at high-risk of developing invasive aspergillosis (IA), the presence of *Aspergillus* within a bronchoalveolar lavage (BAL) fluid sample may have significance whether it is causing disease or not in that instance, because it may indicate the potential for developing disease in the future. This section will focus on procedural false positivity.

False positives due to contamination

Fungal spores are ubiquitous in the air and environment, creating the opportunity for inoculation of reagents and the generation of FP results when performing some PCR assays. Accordingly, measures to reduce exogenous fungal contamination are critical when performing highly sensitive PCR assays that detect fungi that are both environmental saprophytes and human pathogens, such as *Aspergillus fumigatus*. Even though 'no template control' reactions, consisting of all necessary PCR reagents except template DNA, are widely incorporated in PCR assays, there are several other controls that can be used to rigorously monitor contamination.

Contamination can be introduced at various stages of the diagnostic process:

1. During sampling, handling or storage of clinical specimens
2. During DNA extraction, either from exogenous environmental contamination or endogenously from the extraction reagents themselves
3. During PCR setup, again from environmental contamination or from the PCR reagents
4. At any point in the chain of assay implementation due to carry-over contamination from previously generated PCR products present at high concentrations in the laboratory
5. During DNA extraction or PCR setup due to cross-sample contamination when samples with exceedingly high levels of template DNA lead to migration of template into adjacent reactions via aerosol production

1. During sampling, handling or storage of clinical specimens—In retrospective studies where samples have been stored over a period of several years, it is difficult to control for the introduction of contamination during storage. On the other hand, in prospective studies, negative controls can be introduced during the sample acquisition, handling and storage steps to rigorously control for contamination.

2. During DNA extraction—Contamination of DNA extraction reagents with fungal DNA is common [2]. This problem is compounded because most commercial kits do not reveal the exact nature of their components or chemical composition. This lack of information impedes efforts to mitigate contamination using physical and chemical approaches. An ideal extraction protocol would produce high yields of fungal nucleic acid from clinical samples and have reagents that are either free of fungal DNA or amenable to decontamination methods. This is especially important when using PCR assays capable of detecting a few gene copies per reaction – namely those with the highest analytical and diagnostic sensitivity.

Many in-house and commercial protocols for DNA extraction from clinical samples use proteases and cell wall digestion enzymes. Proteinase K, the most commonly used protease, is usually isolated from the fungus *Engyodontium album* and therefore could be a potential source of contamination especially when broad-range PCR is used. Enzymes such as zymolyase (or lyticase) that digest fungal cell walls by hydrolyzing linear glucose polymers with β-1,3-

linkages have been used in many studies, but are also prone to low level fungal contamination [2,3]. As UV irradiation can damage the functionality of most proteins, other contamination control approaches need to be considered before these enzymes are routinely used for DNA extraction, at least in broad-range assays.

In studies diagnosing aspergillosis or a spectrum of IFDs where PCR targets are ubiquitous fungi, episodic contamination can be a serious concern. Only less than half of studies (~15 out of 33) detecting *Aspergillus* species and two-thirds of studies (~9 out of 14) targeting IFDs reported using a negative (fungal free) extraction control to monitor contamination during the extraction stage (Supplementary Table). The negative extraction controls were either 'water-only' or on occasion blood from a healthy donor. Ideally, negative extraction controls that have similar properties to the clinical sample but lack target nucleic acids should be included in the sample preparation pipeline at a sufficient frequency to effectively monitor for contamination events (e.g., 10% of samples per run).

3. During PCR setup—Various methods have been used to decontaminate PCR reagents, including UV irradiation [4,5], ultrafiltration [5–7] and DNase treatment [8]. Decontamination of reagents will not be successful if environmental contamination occurs during PCR setup, thus consideration should be given to using laminar-flow biosafety cabinets for reaction setup.

4. At any point in the chain of assay implementation—All types of PCR assays are vulnerable to carry-over contamination from previous amplification products entering the pre-PCR laboratory and reagents. Several standard laboratory precautions help to mitigate this problem, including the enforcement of unidirectional workflow patterns (pre- to post-PCR), physically separating laboratories for pre- and post-PCR analysis, using aerosol-resistant pipette tips and laminar flow hoods [9]. In addition, using the uracil-DNA glycosylase (UNG) enzyme and dUTP instead of dTTP in the PCR master mix can eliminate this problem by destroying amplicons prior to PCR [10]. The use of UNG enzyme was reported in approximately a quarter (eight out of 33) of studies diagnosing aspergillosis, but only two out of 13 and one of 14 studies targeting *Pneumocystis* pneumonia (PCP) and IFD, respectively (Supplementary Table).

5. During DNA extraction or PCR setup due to cross-sample contamination—Cross-contamination of DNA from samples containing high levels of target to adjacent samples at low levels or no target can be another source of FPs. Particularly in nested PCR assays, the intermediate step between the two PCRs is prone to cross-contamination of samples due to pipetting and transfer of the fairly high copy-number product. This aspect remains the Achilles heel of nested PCR assays except when nested PCR is performed in a single reaction vessel using a variety of methods. Cross-contamination can be detected by interspersing negative extraction controls with clinical samples. To further assess the rate of cross-contamination, positive control plasmids can be processed alongside negative controls, at both the extraction and PCR stages.

False positives due to cross-reactivity of PCR primers or probes with nontarget fungi or human DNA

Although most PCR assays are developed with the intention of being highly specific to the target fungi, cross-reactivity with nontarget fungi or human DNA has to be carefully examined. Analytical specificity testing with other nontarget fungi is generally assessed quite rigorously by most studies. An under-appreciated aspect is the impact of human DNA on the functioning of primers and probes of the fungal PCR assay. Table 2 shows that only approximately half of PCR studies test amplification of fungal DNA in the presence of relevant amounts of human

DNA, and only approximately one-fourth incorporate a separate PCR targeting a human gene or other methods to assess how levels of human DNA impact fungal assay performance.

There are two ways in which human DNA can impact assay performance. The first is by forming nonspecific products and the second is by inhibiting the amplification of target fungal DNA. By testing fungal PCR assays with high levels of purified human DNA, one can ensure that cross-reactivity with human DNA is not a cause of FPs. It should be noted that in our experience, some commercial products of human DNA are contaminated with fungal DNA. Similarly, testing fungal PCR assays with low levels of fungal template DNA and high concentrations of human DNA will help determine if human DNA has an inhibitory effect on detection of fungal targets, thereby producing FN results. An ideal diagnostic PCR assay would include an extraction control PCR assay that quantifies human DNA to monitor the variable levels found in certain clinical samples (e.g., bronchoalveolar lavage fluid) or patient conditions (e.g., blood samples from patients who may have leukocytosis or leukopenia).

False negatives

False negatives due to suboptimal DNA extraction

Efforts to improve diagnostic performance of PCR assays cannot rely on improved PCR detection limits alone because we have already achieved what approaches the maximum analytical sensitivity with PCR technology, namely single molecule detection. Rather, improvements in PCR assay performance will rest on a foundation of improvements in sample manipulation, DNA extraction and target concentration methods prior to the PCR step. This area of investigation has received little attention despite the great potential to impact diagnostic performance. For example, a major limitation of fungal PCR is the small volume of DNA added to each reaction, usually 1–20 μl, and the high proportion of human DNA, leading to limited sampling of tissues for fungal nucleic acids. If a fungal organism is present at one to two genomes per ml of blood then it is unlikely to be detected in most PCR assays, but this low level of fungal DNA may be most informative diagnostically.

Most commercial extraction kits used for fungal PCR diagnostics are designed either for mammalian cell lysis or for fungal cell lysis from pure cultures. The clinical specimen is generally a combination of a few fungal cells or their nucleic acids entrapped in relatively large volumes of tissue or body fluids. Therefore, an ideal extraction method would have high yields to ensure minimal losses of DNA, the ability to concentrate fungal DNA from the large background of human DNA, minimal copurification of PCR inhibitors, and reagents that are compatible with some form of contamination control technology such as UV irradiation or filtration.

Several in-house protocols for extraction of DNA from blood use erythrocyte and leukocyte lysis steps, which could help reduce inhibition by depleting hemoglobin from erythrocytes and reducing the high levels of human DNA that may impede PCR. As fractions of blood are sequentially discarded along the extraction procedure, it should be ensured that fungal DNA is not lost in a fraction that is ultimately not assayed.

It is common in many extraction protocols to use an enzyme treatment step for digesting the fungal cell wall. This step requires optimization because enzymes such as zymolyase (or lyticase) have differential effectiveness in digesting fungal cell walls due to varying amounts of glucan in different fungi [11].

PCR-based fungal diagnostic studies usually use one of two methods to separate DNA from the lysed cellular material, with an almost equal distribution between use of silica-based columns or organic-phase separation with alcohol precipitation. Both approaches have their

advantages and disadvantages. For example, even though silica-based columns are amenable to high-throughput format and are unlikely to copurify PCR inhibitors, in our experience they are more likely to have greater losses of DNA and therefore lower yields. On the other hand, organic-phase separation or alcohol precipitation tend to have relatively higher yields of DNA but may suffer from problems related to copurification of inhibitors, increased toxicity for the user (if phenol–chloroform is employed) and incompatibility with rapid or automated formats.

Several studies have reported the detection limit of their diagnostic assays in terms of organisms per volume of clinical sample but not a single study from the 68 shortlisted have reported an extraction efficiency (or yield) of fungal DNA from the relevant clinical sample using a specific extraction method. For example, it would be helpful to know the percentage loss of fungal and human DNA that occurs during DNA extraction, or conversely the yield of fungal DNA detected when various concentrations of organisms are spiked in a given volume of tissue. This information would be indispensable if the fungal load in the clinical sample is measured by quantitative PCR and used to assess potential differences between colonization and progression of disease. An exogenous positive extraction control, which is phylogenetically unrelated to the target pathogen (to prevent FPs), yet reasonably reflects the passage of target pathogen through the extraction route, would help monitor extraction efficiency in every clinical sample.

False negatives due to PCR inhibition

Inhibition of PCR has a direct impact on FNs. The most ideal format to monitor inhibition is a noncompetitive, quantitative internal amplification control (IAC) used with every target PCR assay. In addition, an IAC should be compatible with any contamination control technology. Table 3 shows that slightly greater than half of the studies have used some form of an inhibition control, also referred to as an amplification control. Apart from a multiplexed IAC, the other two approaches for monitoring inhibition have been spiking with known quantities of target DNA or checking for amplification of human DNA in the extract. Most often, the fungal DNA spiked into the PCR for use as an inhibition control is at a high concentration compared with the detection limit of the assay. A similar scenario of abundant amplifiable DNA exists when targeting human DNA. Both these formats provide an insensitive measure of inhibition. For example, PCR inhibitor copurification can be a problem with DNA extraction protocols that employ an organic phase separation or alcohol precipitation to separate DNA. Inhibitors may substantially reduce the amount of fungal DNA detected in a reaction while still allowing amplification of human DNA that is present at high concentration. Such assays may be reported as free from PCR inhibition when meaningful inhibition exists. Therefore, an IAC should be designed such that it is sensitive enough to reflect a small degree of inhibition capable of affecting performance and detection limit of the fungal PCR. PCR assays with evidence of significant inhibition should be reported as not interpretable.

False negatives due to suboptimal analytical sensitivity of PCR

Current fungal PCR assays, especially those targeting multicopy genes like the ribosomal RNA operons, are capable of reaching the theoretical limit of detection (one to five copies per PCR). With such sensitive assays, PCR can detect DNA from a fraction of a single organism and, therefore, minimize the probability of FNs due to suboptimal analytical sensitivity. Although the copy number of ribosomal RNA (rRNA) operons of some fungal pathogens is approximately known, it was recently shown that there is strain-dependent variation in the copy numbers of the *18S rDNA* subunit for *A. fumigatus* [12]. If strain-dependent variation in the copy numbers of target genes is significant across other fungi then the detection limit of the assay will probably vary accordingly.

There is a need to reach consensus on reporting analytical sensitivity. Ideally, analytical sensitivity for the complete PCR assay (i.e., the extraction plus PCR step) should be reported

along with detection limits of the individual steps. Although some studies report analytical sensitivity in such a manner, it is still not the norm. Another set of variables that impact the overall analytical sensitivity and define detection limits of the assay are the volume of clinical specimen processed for extraction, elution volume of the final DNA extract, volume of DNA added to PCR and volume of the PCR reaction itself. In addition, various combinations of these volumes can alter the impact of inhibitors in PCR. For example, the analytical sensitivity when 100 μl of blood is extracted, eluted in 200 μl of water and 2 μl added to PCR will be 200-times lower than if 1 ml of blood is processed, DNA is eluted in 100 μl of water, and 20 μl is added to the PCR.

Nested PCR assays have been used to enhance analytical sensitivity (and negative predictive value) and are predominantly restricted to the end point PCR format. In nested PCR, one set of primers amplify a larger target (outside primers), followed by a second PCR using a second set of internal primers that amplify a smaller target within the original amplicon. The rationale for using nested PCR assays is to increase PCR sensitivity by overcoming inhibition and enriching for fungal targets in a background of excess human DNA. However, when a non-nested PCR has a detection limit of a few gene copies per reaction (i.e., the theoretical limit of detection) without evidence of inhibition, then no additional benefit is derived from using nested PCR and the problems associated with nested PCR (enhanced potential for contamination) outweigh any benefits.

False-negative results could arise in multiplex PCR formats due to competition between amplification reactions, particularly when there is a large difference in the amount of target DNA for each PCR within a multiplexed reaction. For example, let us consider a multiplex PCR with independent primer sets targeting groups of pathogens within the *Candida* genus, such as *Candida albicans* and *Candida glabrata*. In samples of mixed *Candida* spp. containing non-equivalent amounts of DNA, the species with a lower quantity of DNA may go undetected because the polymerases preferentially amplify the more abundant target, leading to FNs. A similar scenario is possible when broad-range PCRs are used. *Candida* spp., which are commensals in the oral cavity, may be present in the BAL fluid of a patient suspected of IA. When such a broad-range PCR is applied to BAL fluid, the broad-range primers may preferentially amplify the more abundant *Candida* DNA leading a failure to detect lower levels of *Aspergillus* DNA. Therefore, it is important to evaluate the analytical sensitivity (detection limit) of multiplex and broad-range PCRs using mixtures of fungal targets in various proportions.

Other factors affecting PCR assay performance & study interpretation

There are many other factors that can affect PCR assay interpretation, and a comprehensive list is provided in the MIQE guidelines [1]. Here we focus on some critical variables that can profoundly impact analytical and diagnostic performance.

Clinical sample

Optimization of the choice of clinical sample and the appropriate fraction to be assayed has the potential to significantly impact diagnostic performance. If most fungal DNA is cell associated, then a large volume can be centrifuged to obtain a concentrated pellet for extraction. Alternatively, if most DNA is found in the circulating form then concentration with ultrafiltration could be used prior to DNA extraction. The choice of blood fraction for DNA extraction is a good example where optimization is required. Serum and whole blood have almost equally been used for PCR-based diagnosis of aspergillosis, candidiasis or IFDs with no apparent impact on diagnostic sensitivity. However, additional studies are required that specifically address this issue with good experimental data.

PCR format

The real-time quantitative format has been dominant in most recent PCR-based diagnostic studies of fungal infections. Nevertheless, a significant body of work in this field is built on assays in end point format. There are major advantages of the real-time quantitative format, including the ability to quantify fungal load in the clinical sample over a wide dynamic range (>seven orders of magnitude), perform receiver-operating characteristic (ROC) analysis to define optimal detection cut-offs, enhance specificity using the sequence of the probes themselves, rapidly identify species and nonspecific products with melt-curve analysis when dsDNA-binding dyes are used, quality control PCR kinetics by estimating PCR efficiency, and finally multiplex with IAC to monitor inhibition in real-time and accurately quantify the extent of inhibition. Although end point PCR assays require post-PCR analysis with an electrophoresis gel or ELISA-based system, they are a useful option when longer amplicons are needed to precisely identify species. For post-PCR species identification, sequencing remains the gold standard. Recently, an assay based on Luminex microbead hybridization technology was developed for identification of a broad range of clinically relevant fungi (29 species spanning ten genera), which could emerge as a more rapid alternative [13].

Study design

Fundamental aspects about study design that could help place the diagnostic performance in perspective are not routinely reported. Of the studies that reported diagnostic performance in terms of sensitivity and specificity, only approximately half of those mention whether their study was performed prospectively or retro spectively. In addition, only approximately one-third of studies report blinding of the PCR analysis from clinical diagnosis. Blinding would add to the objective evaluation of test results by removing some biases and, therefore, should be mandatory. Most studies for aspergillosis, IFDs and candidiasis after 2001 have used the European Organization for Research and Treatment of Cancer (EORTC)/Mycoses Study Group (MSG) criteria [14] to categorize patients and this is a significant advance in standardization. Furthermore, some studies use PCR diagnostics to rule in or confirm disease, whereas others use PCR as a screening approach to detect early disease, and the performance characteristics will probably vary in these different situations.

Statistical analysis of study design and diagnostic performance parameters

The statistical analysis and power calculations describing the relationship between sample size and the accepted confidence intervals (CIs) of diagnostic sensitivity and specificity are essential for the objective evaluation of a diagnostic test. CIs enable meaningful interpretation when estimating if differences between assays are statistically significant. Of the studies that reported diagnostic sensitivity and specificity, only a minority reported 95% CIs – nine out of 23 for aspergillosis, two out of 12 for PCP, one out of eight for IFDs and two out of six for candidiasis.

Positive and negative predictive values (PPV and NPV), respectively, are an important measure of diagnostic test performance and reflect the probability of a test result accurately predicting whether a condition is present or absent. However, the predictive values depend on the prevalence of disease in the population under study. The dependence of PPV on prevalence can be better understood through this equation:

$$PPV = \frac{(sensitivity \times prevalence)}{([sensitivity \times prevalence] + [1 - specificity] \times [1 - prevalence])}$$

When the prevalence approaches 1 (independent of sensitivity and specificity), then the PPV approaches 100%. Therefore, to obtain a meaningful PPV, the prevalence in the study should closely match that of the disease in the population being considered. In case–control studies,

the PPV and NPV cannot be directly calculated, although these values can be inferred from the prevalence of disease. For case–control studies, the likelihood or diagnostic odds ratios, which are independent of prevalence, should be reported.

PCR assays for diagnosing aspergillosis

Introduction & significance

There is a spectrum of disease produced by fungi in the *Aspergillus* genus, including allergic bronchopulmonary aspergillosis, aspergilloma of the lung and IA. Our focus is on the diagnosis of IA, which usually begins as an infection in the respiratory tract, such as in the lungs or sinuses. Although *Aspergillus* spores are frequently inhaled by healthy humans without people developing disease, they are capable of causing life-threatening infections in the increasing numbers of immunocompromised patients, such as those undergoing cytotoxic chemotherapy for cancer, or those undergoing solid organ or stem cell transplantation [15]. IA has a high mortality despite use of appropriate antifungal therapy, so great emphasis has been placed on achieving earlier diagnoses with more sensitive methods.

Aspergillus fumigatus is the most common cause of human disease; although a variety of *Aspergillus* species have been linked to invasive infection including Aspergillus flavus, Aspergillus terreus, Aspergillus nidulans and *Aspergillus niger*. There are some differences in antifungal susceptibility among *Aspergillus* species. *A. terreus* tends to be more resistant to amphotericin B than other species in the genus. Distinguishing between *A. fumigatus* and *A. terreus* is less critical in the era of voriconazole therapy for IA since most species are susceptible, but remains an issue when polyene therapy is considered. In addition, a recently described species, *Aspergillus lentulus*, has been reported to be resistant to several classes of antifungal agents, including azoles [16]. PCR assays may help detect new and emerging species if appropriate gene targets are employed.

Current diagnostic techniques & shortcomings

Culture-based diagnosis of *Aspergillus* infections is time consuming and often has poor diagnostic sensitivity. Histopathogical analysis is invasive, has low sensitivity, and findings frequently do not distinguish among fungal genera or species [17,18]. Molecular diagnostic tests, such as the GM antigen assay, have been frequently used to diagnose IA and are part of the microbiological criteria considered for clinical diagnosis using the EORTC/MSG criteria. The GM antigen assay is designed to detect only pathogens from the *Aspergillus* genus but is susceptible to FP results due to cross-reactivity with antigens from *Penicillium chrysogenum* and *Paecilomyces variotii* as well as agents of the endemic mycoses [19,20]. In addition, gastrointestinal graft-versus-host disease, mucositis, administration of amoxicillin–clavulanate or piperacillin/tazobactam antibiotics, and early childhood (possibly resulting from absorption of GM antigens from cereal grains) may cause FPs with the GM assay [21–24]. PCR has the potential to offer a diagnostic test for aspergillosis with fewer FPs due to the factors noted above, although new causes of FP results are likely to emerge, which is why we place such great emphasis on the use of experimental controls to monitor laboratory contamination.

Eight out of 23 IA studies listed in Table 4A reported diagnostic sensitivity and specificity of GM antigen assays in patients undergoing stem cell transplantation for comparison with PCR, but the results were variable. One study reported a diagnostic sensitivity of the GM assay that was 45% greater than PCR [25], five studies had GM and PCR within 21% of each other [26–30], while for the remaining two studies PCR was at least 30% greater than GM [31,32]; the statistical significance of these differences is not always clear. The diagnostic specificity values for both PCR and GM in most of these studies were towards the higher end (80–100%).

Large, well-designed studies are required to accurately measure the relative diagnostic performance of PCR and GM assays, and these are currently lacking.

Highlights from current PCR studies

Data from 23 PCR-based studies that reported diagnostic sensitivity and specificity for the diagnosis of IA have been tabulated in Table 4A. Blood and BAL were the two clinical sample types assayed. Blood is the easier sample to obtain and can be used for detection of infection prior to clinically evident disease that prompts bronchoscopy with BAL. BAL fluid is more likely to have a higher burden of organisms. Based on these 23 studies, obvious trends do not emerge in terms of which clinical sample is more likely to be useful for diagnosis of IA. The values of diagnostic performance for blood and BAL fluid are scattered in a similar range (55–100%) and therefore further investigation is warranted to determine the true potential of each sample type with highly optimized assays. In terms of optimizing the volumes of clinical sample that can be assayed, processing large serum volumes [27] and the pellet fraction of BAL [5] were found to offer better diagnostic performance compared with smaller serum volumes and supernatant of the centrifuged BAL, respectively.

Both commercial kits and in-house methods have been used for DNA extraction. The ribosomal RNA operon has been most frequently targeted for developing PCR assays for IA in part due to its multicopy nature offering improved detection capabilities. Although earlier studies frequently used end point PCR, most current studies are in a real-time quantitative PCR format. It is difficult to determine the impact a specific type of DNA extraction and PCR technique may have on the diagnostic performance across studies because there are several other variables that impact performance. There is a slight trend towards lower specificities with nested PCR when compared with single PCR approach, probably because nested PCR assays are prone to carry-over contamination; definite interpretations can be drawn only with studies designed to address this specific issue.

Hebart *et al.* [33], Halliday *et al.* [34] and Florent *et al.* [29] designed blinded studies to prospectively analyze blood samples of stem cell transplant patients for early detection of IA. Their studies are good examples of how estimating CIs can place the diagnostic sensitivity, specificity and predictive values in the right perspective. These studies reported high NPVs showing the utility of PCR assays as a screening test for excluding IA. In addition, Hebart *et al.* [33] and Halliday *et al.* [34] highlighted the importance of PCR-positive results in identifying patients at high risk of subsequent IA.

The study by Raad *et al.* [35] had the highest number of subjects. They analyzed BAL from 50 patients with pulmonary IA (ten proven, 22 probable and ten possible) and 199 control patients who had no radiologic manifestations indicative of pulmonary IA. Their study suggested that the low PPV could be related to the transient colonizing presence of aspergilli in the respiratory tract and that sensitivity correlates with the certainty of the diagnosis based on tissue invasion. However, there were no obvious trends suggesting lower PPV when BAL fluid was assayed (due to colonization of the respiratory tract) compared with blood for the 19 out of 23 studies from Table 4A that reported a predictive value. In general, the PPVs for both BAL and blood were much lower than the NPVs – reflecting the low prevalence of IA in most studies.

Studies by Florent *et al.* [29], White *et al.* [36] and Musher *et al.* [26] that compared GM antigen and PCR assays found that when used together the sensitivity and NPV of diagnosis can be increased. They concluded that complementary use of these tests may reduce dependence on invasive diagnostic procedures and limit the need for empirical antifungal therapy.

Khot *et al.* found culture comparable with PCR and attributed this result to the role culture played as a gold standard in defining cases with positive pulmonary IA [5]. They found that some patients did not meet standard criteria for pulmonary IA, but had consistently high levels of *Aspergillus* DNA in BAL fluid by quantitative PCR. A similar conclusion was reached by the study of White *et al.* who attributed FP PCR results to the limitations of classic microbiological methods for diagnosis of IA, or to the use of overly restrictive consensus clinical definitions employed to classify infection [36]. Nevertheless, use of a gold standard for comparison of PCR results is critical, and many studies lack this basic assessment.

Only two studies in PCR-based diagnosis of IA have reported using an IAC to monitor inhibition [5,37]. The study by Khot *et al.*, which used an alcohol precipitation-based DNA purification step, reported significant inhibition in 7.6% (11 out of 144) of the BAL samples [5]. By using a sensitive IAC they could exclude the possibility of FNs due to inhibition. These two studies also estimated the fungal burden in samples and performed a ROC analysis to determine the optimal detection cut-off to estimate diagnostic performance. Cuenca-Estrella *et al.* processed 2244 serum and blood samples from 83 patients and performed a thorough analysis using Classification And Regression Trees (Salford Systems, San Diego, CA, USA) software in order to establish the predictive ability of the PCR technique with one, two and three positive results [37].

Quantitative extraction control assays targeting the human *18S rRNA* and β-*globin* genes were used in studies by Khot *et al.* [5] and Frealle *et al.* [38], respectively, to monitor the amount of human DNA in every extracted BAL sample. Lower levels of human DNA could indicate either suboptimal DNA extraction or sampling of fewer cells in BAL fluid. The measurements of human DNA in these studies helped provide optimal quality control for interpreting *Aspergillus* PCR results by ruling out a failed extraction or very high quantities of human DNA as cause for a negative result. Although a few other studies used PCR to target human genes such as the *RAS* oncogene [35], β-*globin* [39,40], *G6PD* [41] and *HLA-DR* genes [33] as amplification or inhibition controls, all these were in end point format and, therefore, quantitative data are absent.

Two recent meta-analyses for the diagnosis of IA using PCR applied to BAL fluid [42] and blood [43] show its clinical value and recommend standardization of PCR platforms. Although standardization is helpful for validating diagnostic performance across multiple end users, several questions remain. It is still unclear to what extent improvements in DNA extraction can enhance diagnostic sensitivity. It is also unclear which clinical sample is most useful for diagnosis and what fraction of blood may carry the most fungal DNA. Additional studies should be performed with BAL fluid and blood to evaluate the impact of processing large volumes of sample. Finally, real-time PCR assays with an integrated system for species-level identification based on melt-curve profiles or via additional probe specificity would be useful in saving time and refining the diagnosis of specific infections, allowing for administration of targeted antifungal therapy based on species-level identification.

PCR assays for diagnosing *Pneumocystis* pneumonia

Introduction & significance

Pneumocystis jirovecii is an opportunistic pathogen found in the lungs capable of causing infections in immunocompromised patients such as those with AIDS or those receiving corticosteroids. PCP is the most common clinical presentation of this infection. In HIV-infected patients, the risk of developing PCP closely correlates with decreasing $CD4^+$ lymphocyte count [44]. It is primarily found in the alveoli where it can exist as a trophic form (predominantly haploid but occasionally diploid) or as a cyst that contains two, four or eight nuclei [45,46]. The binding of the trophic forms to the alveolar epithelium is an important preliminary step in

the establishment of infection and is also important in pathogenesis as these organisms block gas exchange in the alveoli leading to hypoxia.

Pneumocystis jirovecii is a unique fungal pathogen in the sense that it cannot be readily cultured in the laboratory, is suggested to have only a singly copy of its rRNA operon [47] and has a plasma membrane that lacks ergosterol [48]. On the other hand, based on rRNA taxonomic analysis it is a fungus, and the presence of β-1,3-D-glucan in its cell wall supports this classification. Trimethroprim–sulfamethoxazole is the drug of choice for treatment and prophylaxis of *Pneumocystis* infections. Pentamidine and atovaquone are alternative therapeutic agents.

Current diagnostic techniques & shortcomings

Due to the inability to propagate *P. jirovecii* in culture, histo-pathological analysis of sputum, BAL fluid or tissue samples is commonly employed for diagnosis. Methods to enhance microscopic visualization of *Pneumocystis* organisms include use of Gomori methenamine silver or Wright–Giemsa stains, direct and indirect fluorescent antibodies, or nonspecific fluorescent stains for chitin (calcofluor white). These techniques have reasonable sensitivity, but perform best when using samples collected with invasive methods, such as bronchoscopy with collection of BAL fluid. Some methods lack optimal specificity. A detailed description of various diagnostic approaches for PCP can be found in a recent book chapter by Orenstein and Masur [44].

Highlights from current PCR studies

Data from 12 PCR-based studies that reported diagnostic sensitivity and specificity for PCP have been tabulated in Table 4B. Clinical specimens used for PCR-based diagnosis are oral wash, sputum (expectorated and induced), transbronchial biopsy and BAL. Most PCR-based studies assaying BAL fluid listed in Table 4B have reported very high sensitivities (96–100%) [49–55] except for Bandt *et al.* [56] and Nuchprayoon *et al.* [57] who reported sensitivities of 80 and 67%, respectively. It is noteworthy that these two studies were different from the other studies in two key experimental variables. Bandt *et al.* targeted the 5.8S rRNA and *DHFR2* genes, both single-copy genes in *P. jirovecii*, which is in contrast with the high-copy-number mitochondrial or major surface glycoprotein (*MSG*) genes targeted by PCR assays in the other studies [56]. Nuchprayoon *et al.* used FTA-filter paper-based DNA extraction technology but provide no data on DNA yields from this approach [57]. The relatively low diagnostic performance of this study may reflect use of this unique form of DNA extraction.

Oral wash samples are particularly attractive alternatives to the collection of samples using more expensive and invasive procedures, such as bronchoscopy with BAL. Two studies from Table 4B, both prospective and blinded, showed the promise of oral washes for the diagnosis of PCP. The first by Fischer *et al.* was conducted on a ‘patient population susceptible to PCP’ using a PCR assay based on the *MSG* gene, resulting in a sensitivity of 91% and a specificity of 94% [58]. The second by Larsen *et al.* was conducted on HIV-infected patients suspected of PCP using a quantitative touchdown PCR format resulting in a sensitivity of 88% and a specificity of 85% [59].

Amplification or inhibition controls have been commonly used in PCP studies, which contrasts with the other diseases considered in this review. Seven out of 12 PCP studies used such controls, of which three [52,58,59] used internal controls to monitor inhibition and the remaining four studies used control PCRs targeting a human gene [51,53,54,56]. In addition, of the studies designed in a real-time quantitative format from Table 4B, three performed ROC analysis to determine optimal detection cut-offs for estimating diagnostic sensitivity and specificity [54,55,59].

The diagnostic specificity for most PCR studies listed in Table 4B is very high (85–100%). It is noteworthy that the two studies with the lowest specificities at 59 and 77% used a nested PCR format [51,60]. A prospective study by Sing *et al*. using nested PCR found a high number of FP results and low PPVs for non-HIV-infected immunocompromised and immunocompetent patients [49]. Another prospective blinded study by Torres *et al*. using nested PCR also observed significant FP cases especially when a lower intensity of PCR band pattern was considered as positive [50]. In addition, none of these four nested PCR studies used the UNG enzyme to minimize carry-over contamination [49–51,60]. The relatively high FP rates of these studies could be a consequence of the contamination problems associated with nested PCR. As we have previously noted, there is no reason to do nested PCR if one has a highly optimized PCR assay with analytical sensitivity in the single-gene copy detection range.

Several questions remain in this field. There are few studies that have directly compared oral washes with BAL fluid as a sample for PCR testing. What role can quantitative PCR data play in understanding the prognosis of PCP? Does higher tissue burden correlate with worse outcome? Some studies suggest that subjects with AIDS may have a higher tissue burden of organisms and this may impact assay sensitivity [45]. Is *P. jirovecii* DNA detectable in blood, and if so, what is the diagnostic sensitivity of this approach? Additional, well-designed large validation studies are required to evaluate the true potential of PCR assays for the diagnosis of PCP.

PCR assays for diagnosing candidiasis

Introduction & significance

Candida species are commensals of the human mucous membranes and the most common fungal pathogens isolated in immunosuppressed and intensive care unit patients. We will not focus on mucocutaneous candidiasis, which usually presents as oropharyngeal plaques (thrush) or vulvovaginitis and can occur in patients with intact immune systems. Rather, we will focus on invasive candidiasis, which is an opportunistic infection that may occur in any part of the body when epithelial barriers are breached or invaded.

Although approximately 50% of infections are caused by *C. albicans*, several other *Candida* species can cause invasive disease including C. parapsilosis, C. tropicalis, C. dubliniensis, C. glabrata, C. krusei and *C. lusitaniae*. Major emphasis is rightfully placed on species level identification of *Candida* due to the varying antifungal susceptibility within the genus. *C. albicans*, *C. parapsilosis*, *C. tropicali*s and *C. dubliniensis* tend to be fluconazole susceptible, and *C. glabrata* is susceptible to the echinocandin class of antifungals but has a high rate of fluconazole resistance. *C. krusei* is resistant to fluconazole. *C. lusitaniae* tends to be amphotericin B resistant. *C. parapsilosis* infections can emerge on echinocandin therapy and tend to be resistant to this class of agents. Failure to initiate antifungal therapy in a timely manner is associated with poor clinical outcomes and mortality. Due to variations in antifungal susceptibility profiles within the *Candida* genus, PCR assays offer an attractive approach for rapid diagnosis and identification down to the species level. Other *Candida* species are less common etiologic agents of invasive candidiasis, but should be detected.

Current diagnostic techniques & shortcomings

The diagnostic sensitivity of detecting *Candida* by blood culture is low in some settings (~50% or less), particularly with hepatosplenic candidiasis [61,62]. It is likely that the fungal burden is low in the systemic circulation, as opposed to the portal circulation in hepatosplenic candidiasis; therefore, there is a need for diagnostic techniques such as PCR that can detect a fraction of an organism when multicopy genes are targeted. In addition, if nonviable

Candida organisms are present in the circulation (such as within macrophages) then culture may fail to detect these fungi and PCR may prove more useful. Other diagnostic approaches for candidiasis based on antibodies, antigens and metabolites have certain advantages but still fall short of the required sensitivity and specificity and will not be covered here.

Highlights from current PCR studies

Data from six PCR-based studies that reported diagnostic sensitivity and specificity for the diagnosis of candidiasis have been tabulated in Table 4C. Since *Candida* species can be commensal organisms in the mouth and upper respiratory tract, specimens from the airways may be contaminated with *Candida* spp. Therefore, isolating *Candida* spp. from respiratory specimens including bronchoscopy and BAL (even by a protected specimen brush) is not considered diagnostically conclusive of infection without biopsy evidence of invasion. On the other hand, high levels of *Candida* detected in BAL fluid may suggest the diagnosis of *Candida* pneumonia, although this is an uncommon clinical entity. Blood has been the sample of choice for PCR-based diagnosis of invasive candidiasis because it is most likely to contain circulating fungal DNA in either extracellular form or within intact organisms. Of the six studies shown in Table 4C, one half assayed serum and the other whole blood, indicating (e.g., in the case of IA) that it is still unclear which fraction of blood contains the most fungal DNA. A recent study [63] associated with the same non-neutropenic patient population described by McMullan *et al.* [64] compared whole blood versus serum fractions from 104 patients and found serum positive in ten out of ten patients with laboratory-confirmed candidemia compared with seven of ten patients when whole blood was processed. Their results suggest that serum could be a better fraction for non-neutropenic patients, although additional studies would be useful to confirm the optimal choice of blood fraction.

Studies by Morace *et al.* [65] and Ahmad *et al.* [66] testing whole blood and serum, respectively, reported that PCR was more sensitive than blood cultures. The study by Ahmad *et al.* reported identifying cases of mixed *Candida* infections by targeting the internal transcribed spacer (*ITS*)2 region of the *Candida* rRNA operon using a seminested PCR format; such dual infections are not likely to be detected by culture especially if one of the species has a significantly higher growth rate [66]. Dunyach *et al.* evaluated two real-time PCR assays, one targeting the ITS region that enabled identification of five *Candida* species based on their melt-curve profiles and the second targeting the *18S rRNA* gene [67]. This second assay had relatively higher sensitivity, which is expected because variability in the length of the ITS amplicon may result in variable analytical sensitivity potentially impacting diagnostic sensitivity.

The study by Maaroufi *et al.*, which included 69 patients with clinically proven or suspected systemic candidiasis, reported diagnostic sensitivity and specificity of PCR equivalent to blood culture [68]. Their PCR assay was designed in a real-time quantitative format with two variations of the hydrolysis probe, the first FAM (6-carboxyfluorescein) tagged probe was specific only to *C. albicans* whereas the second TET (tetrachloro-6-craboxyfluorescein) tagged probe was specific to five major *Candida* species. Although the diagnostic sensitivity of each individual probe was 100%, the diagnostic specificity of the genus-specific probe fell to 72 from 97% for the probe targeting only *C. albicans*. They found significant cross-reactivity of this genus-specific probe with organisms from other genera such as *Aspergillus*, *Saccharomyces* and *Fusarium*. In addition, they observed some cross-reactivity and sequence similarity of this probe with the human rRNA gene. This is a good example demonstrating the challenges and limitations of designing genus-specific *Candida* assays because the *Candida* genus is phylogenetically diverse compared with many other common genera of pathogens. Experimental controls are critical for assessing the potential for primer and probe cross-hybridization.

McMullan *et al.* tested an array of three real-time PCR assays targeting major *Candida* spp. [64]. Their study included 527 serum samples from 157 patients acquired in two stages of the trial. The patients were classified into a proven, probable and unlikely infection category by EORTC criteria. A significant number of the serum samples (491) belonged to the unlikely infection category. Although they mention being skewed towards disease-negative subjects as a limitation of their study, it is a good example of prospective screening with robust sample size and statistics for diagnostic variables. In the data analysis part of their Methods section, they estimate that 500 negative samples would be required to achieve CIs regarding the specificity of 5% or less. By analyzing 491 negative samples, the resulting CIs of specificity and NPV were in a very tight window, estimated to be 100% (99.3–100) and 99.8% (98.9–100), respectively. Their study also discusses the difficulty of including 'probable' cases based on the EORTC criteria as 'truly disease positive' and therefore they estimate diagnostic sensitivity and specificity independent and inclusive of the probable cases in separate analyses.

Moving ahead, such as in the case of PCR-based diagnosis for IA, optimization of specimen type and the fraction of specimen subjected to nucleic acid extraction are critical variables. In addition, it is important to focus on the impact of DNA extraction and design of PCR primers and probes to rapidly identify the sometimes changing spectrum of etiological agents of candidiasis. Large, well-designed prospective studies are needed to validate PCR assays. Because the *Candida* genus is phylogenetically diverse and has species with varying antifungal susceptibility profiles, mixed *Candida* infections are possible (particularly on antifungal therapy) that may not be detected by cultivation due to overgrowth of the dominant species. Multiplexed PCR formats could have a critical role to play in detecting these mixed infections.

Broad-range PCR assays for the diagnosis of invasive fungal infections

Introduction & significance

Not all fungal pathogens can be rapidly propagated in culture, and GM and glucan assays do not detect all fungal pathogens. Therefore, a negative result in these tests does not rule out fungal infection in a high-risk host. For example, a negative GM antigen or glucan test does not exclude mucormycosis in a stem cell transplant recipient. In addition, due to increasing use of antifungal medications for prophylaxis, the spectrum of fungal infections is likely to change. The next generation of diagnostic tests will need to be capable of detecting these emerging pathogens.

Broad-range PCR assays use primers that target highly conserved regions of genes found in all organisms in the taxon being assayed. For instance, broad-range bacterial PCR assays may use primers that anneal with highly conserved sequences in the bacterial 16S rRNA gene or the RNA polymerase gene. These genes are present in all bacteria, and have both conserved regions that are ideal for broad-range primers and variable regions that help identify species. In theory, broad-range fungal PCR assays could achieve detection of most fungal pathogens. If the PCR amplicon spanning the conserved primer sequences is highly variable, then the assay can also offer a high degree of species-level resolution. In addition, a combination of broad-range amplicons could facilitate rapid species-level identification using methods such as melt-curve analysis [69].

Highlights from current PCR studies

Data from eight studies that reported diagnostic sensitivity and specificity for the diagnosis of IFDs using broad-range PCR applied to human clinical samples have been tabulated in Table 4D. The *18S rRNA* gene and ITS of the rRNA operon were employed as PCR targets, with the former more commonly used. To identify clinically relevant fungal pathogens in culture, in addition to ITS, the hypervariable D1–D2 region of the *28S rRNA* gene has been widely used

[70–74]. Recently, the remainder of the *28S rRNA* gene was shown to be useful for development of broad-range fungal PCR assays [75].

Although most studies using broad-range PCR have focused on patient populations with etiological agents in the *Aspergillus* and *Candida* genera, Lau *et al.* tested broad-range PCR on paraffin embedded tissue specimens of patients at risk of IFDs from a diverse range of fungal genera, including species of Candida, Cryptococcus, Trichosporon, Aspergillus, Fusarium, Scedosporium, Exophiala, Exserohilum, Apophysomyces, Actinomucor and *Rhizopus* [76]. In their study, 93.6 and 64.3% were successfully detected and identified by PCR when culture and histology were used as gold standards, respectively. The PCR targeted the ITS1 region of the fungal rRNA operon, and positives were sequenced to confirm species identity.

Most broad-range PCR assays listed in Table 4D were designed in the end point format and none of them were nested. The ability of broad-range PCR assays to detect a wide spectrum of fungi is clearly an advantage. Conversely, amplification of contaminating fungal nucleic acids present in the environment or laboratory can be a drawback. In general, broad-range PCR assays could be useful when applied to a clinical sample as part of a larger array of assays. For example, broad-range PCR assays could detect new pathogens previously unreported, and identify etiological agents in mixed infections, especially when used with other genus-specific assays.

Some important considerations for broad-range assays are testing the performance characteristics (detection thresholds) of the assay in the presence of human DNA and defining analytical sensitivity in terms of all relevant etiological agents. The issue of cross-reactivity with human DNA is particularly critical for broad-range primers or probes because they are designed in highly conserved regions and therefore are likely to be more similar to the counterpart human gene. Some broad-range ‘fungal’ PCR primers noted in the literature have near complete sequence similarity to human gene targets [75]. Of the eight studies in Table 4D, none reported an analytical sensitivity for more than two pathogens. Five out of the eight studies reported testing cross-reactivity of the fungal PCR assay with either human genomic DNA added directly to the PCR, or by spiking blood from a healthy donor, subjecting this to DNA extraction, and performing PCR. However, quantitative data describing the amount of human DNA per PCR and its impact on detection of fungal DNA are mostly lacking.

Since most studies listed in Table 4D target patients with aspergillosis and candidiasis, an inevitable question needs to be asked – what are the pros and cons of using broad-range as opposed to genus-specific PCR for the diagnosis of these fungal diseases? Blood is the most sterile of the clinical samples used for diagnosis by PCR; therefore, it is more useful for applying broad-range PCR assays because a PCR positive provides more convincing evidence of fungal disease. On the other hand, use of broad-range PCR on nonsterile clinical samples, especially BAL fluid, for the diagnosis of IA may result in suboptimal results – low specificity due to cross-reactivity with commensal fungi such as *Candida* spp. present in the oral cavity, and low sensitivity because these commensal fungi may compete with the pathogen for amplification.

The diagnostic performance of broad-range PCR assays for detecting IFDs does not suggest significant advantages over genus or species-specific assays for *Candida* or *Aspergillu*s, which are the main pathogens in these studies. Specificity may be compromised as reflected by a higher FP rate, 70 and 73% reported in the studies of Jordanides *et al.* [77] and Hebart *et al.* [78], respectively. Apart from contamination, a high FP rate could be due to colonization of the sampling site by commensal fungi or the shortcoming of clinical criteria to accurately classify patients as positive for disease. Sequence analysis of broad-range fungal PCR products for species level identification is critical in parsing factors that contribute to FP results.

As the spectrum of human pathogens continues to expand, broad-range PCRs will be indispensable in detecting emerging pathogens or in ruling out fungal infection when genus-specific assays are negative, but FP results are likely to limit the usefulness of these assays in nonsterile tissues. Broad-range fungal PCR assays may be most effective for diagnosis when the PCR product is sequenced to confirm detection of a true pathogen, and when they are used in conjunction with genus-specific assays.

Expert commentary & five-year view

There are two schools of thought about how the field of PCR-based diagnostics will evolve. The first perspective assumes we have already developed most of the knowhow required to diagnose fungal infections by PCR and it is now time for large-scale clinical validation of these assays. The second perspective holds that additional studies are required to optimize the choice of clinical sample, DNA extraction method, PCR platform, and quality controls to maximize diagnostic performance. In either case, the Aspergillus Technology Consortium (AsTeC) and Invasive Aspergillosis Animal Models (IAAM) programs will serve as excellent resources of prospectively collected clinical samples and animal models for comparison of PCR assays with US FDA-cleared diagnostic tests for IA. The former perspective should lead to multicenter validation studies of existing PCR technologies with the goal of promoting standardization of testing for routine clinical use. The challenge will be deciding which PCR assay platforms warrant validation, and this will most likely be decided on nonscientific grounds. Although there are many calls for increased standardization in the field, it is unlikely that a single amplification technology will dominate. The European Aspergillus PCR Initiative (EAPCRI) is one initiative that seeks to establish a standard for PCR, sufficiently robust that it can be incorporated into the next revision of the EORTC/MSG definitions for IA. If PCR is to gain widespread acceptance as a diagnostic test for fungal infections and incorporated into EORTC/MSG guidelines as a microbiological criterion, then validation studies will need to demonstrate favorable performance characteristics. The latter perspective will lead to important improvements in PCR assay analytical performance that will directly enhance diagnostic performance. Specifically, improvements in sample collection and processing, DNA extraction efficiency, contamination control, and rapid identification of PCR targets will make PCR assays of the future more accurate and useful. Additional targets for PCR will be explored, with emphasis on developing an array of assays that can be used in combination for diagnosis, either as separate PCR reactions or in a multiplexed format. Quantitative formats will become the norm for fungal target detection as well as internal amplification and extraction control PCRs. Rapid methods to identify species based on high-resolution melt-temperature analysis could be widely used in genus-specific or broad-range fungal PCR assays. Microbead hybridization technologies such as Luminex xMAP [13] could also see more widespread use for post-PCR species-level identification, as could mass spectrometry of PCR products.

Although nanoscale PCRs have been touted as the magic bullet for overcoming the current technological limitations of PCR-based diagnostics, this technology has its own limitations. In particular, it is difficult to obtain optimal analytical sensitivity when subjecting nanoliter volumes of extracted DNA to PCR. Current microliter-scale PCRs use volumes in the range of 1–20 μl of extracted DNA in a 20–100-μl PCR, and cannot compare with the diagnostic sensitivity of cultivation where a single organism may be detected in 10 ml of blood. There is also an upper limit to the amount of human DNA that can be added to a PCR before amplification is inhibited. Specific concentration of fungal DNA prior to nanoscale reactions could overcome this problem.

In conclusion, the next 5 years will probably see an increase in the number of studies validating existing fungal PCR assays, hopefully with multicenter trials. A few validated PCR assays will emerge with performance characteristics that rival or surpass existing diagnostic technologies.

Simultaneously, advances in DNA extraction and PCR assay design will enhance the analytical and diagnostic performance.

Key issues

- Accurate and timely diagnosis of life-threatening fungal infections, such as invasive aspergillosis, *Pneumocystis* pneumonia, invasive candidiasis and mucromycosis, remains challenging in many settings.
- Although PCR-based technologies are a promising approach to compliment current methods, false-positive and -negative results continue to impede widespread applicability.
- Lack of rigorous experimental controls has hindered interpretation of diagnostic performance and an objective comparison with other assays.
- The Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines are a valuable template to model quality control for future PCR studies.
- Controls to monitor and limit false positives need to account for fungal contamination from the environment, previously amplified PCR products, cross-reactivity of PCR primers or probes with nontarget fungi or human DNA, and colonization of human tissue surfaces with fungi.
- Controls to monitor false negatives need to account for suboptimal DNA extraction, PCR inhibition and suboptimal analytical sensitivity of PCR.
- There is a need for both taxon-directed and broad-range PCR approaches to detect rare pathogens in a background of colonizing fungal microbiota, assess for mixed infections and detect emerging pathogens.
- Selection of optimal tissue types and DNA extraction methods are comparatively poorly studied but critical areas of investigation.
- It is unclear how fungal burden in tissue correlates with progression of disease.
- Large, well-designed studies with rigorous experimental controls and statistical analysis of diagnostic performance are critical for advancing the field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Distribution of publications considered for review on the basis of disease category targeted by PCR.

Fungal disease	Total number of publications	Number of publications reporting diagnostic sensitivity and specificity
Aspergillosis	33	23
Pneumocystis pneumonia	13	12
Candidiasis	6	6
Invasive fungal infections	14	8
Mucormycosis	2	0

Table 2

Frequency of studies reporting cross-reactivity with human DNA, classified on the basis of disease category targeted by PCR.

Fungal disease targeted	Tested cross-reactivity with human DNA*	Incorporated PCR assay targeting a human gene
Aspergillosis	16 out of 33 (49%)	9 out of 33 (27%)
Pneumocystis pneumonia	2 out of 13 (15%)	3 out of 13 (23%)
Invasive fungal disease	10 out of 14 (71%)	4 out or 14 (29%)

*Studies reported testing amplification of fungal DNA in the presence of human DNA, either by quantifying human DNA after extraction or by amplifying fungi extracted from spiked human specimens.

Table 3

Frequency of studies reporting inhibition, classified on the basis of disease category targeted by PCR.

Fungal disease	Number of studies reporting some form of amplification control	Amplification control targeting human gene	Amplification control in the form of a DNA spike	Internal amplification control
Aspergillosis	16 out of 33 (49%)	5 out of 16	9 out of 16	2 out of 16
Pneumocystis pneumonia	8 out of 13 (62%)	4 out of 8	1 out of 8	3 out of 8
Invasive fungal disease	6 out of 14 (43%)	3 out of 6	2 out of 6	1 out of 6

Table 4A

Diagnosis of aspergillosis.

Study (year), Ref.	Clinical specimen[1]	Study design[2]	Extraction (kit or fungal cell wall lysis)	Extraction (DNA purification)	PCR format	PCR target gene	Target pathogens[3]	#[4]	Prv[5] (%)	Sensitivity[6]	Specificity[7]	PPV[8]	NPV[9]
Jones (1998) [39]	BAL/whole	X[10]	Nonionic and polysorbate surfactants, proteinase K	Phenolchloroform, alcohol precipitation	End point	*mt* [11]	*Aspergillus* genus	69	17.4	100	100	100	100
Skladny (1999) [41]	BAL/pellet and blood/whole	X	Enzymatic, anionic surfactant, proteinase K	Phenolchloroform, alcohol precipitation	End point (nested)	*18S rRNA*	*Aspergillus* genus	68	30.9	100	89	69	100
Hebart (2000) [33]	Blood/whole	Prospective/ blinded	Anionic surfactant	Protein precipitation, alcohol precipitation	End point	*18S rRNA*	Broad-range fungal	84	11.9	100 (48–100)	65 (53–75)	27.8 (10–54)	84 (74–91)
Williamson (2000) [79]	Blood/serum	Retrospective/ blinded	Sorbitol buffer, enzymatic, proteinase K, Qiagen tissue kit (Qiagen, West Sussex, UK)		End point (nested)	*28S rRNA*	*Aspergillus* genus	37	35.1	81	100	100	X
Buchheidt (2001) [80]	BAL	Blinded	X	X	End point (nested)	*18S rRNA*	*Aspergillus* genus	67	– [12]	100	92.6	76.5	100
	Blood							218		91.7	81.3	49.3	100
Kami (2001) [30]	Blood (whole or plasma)	Retrospective and prospective	QIAmp Blood mini-kit (Qiagen, CA, USA)		Real-time quantitative	*18S rRNA*	*Aspergillus* genus	122	27.0	79	92	79	92
Raad (2002) [35]	BAL/pellet	Prospective	Anionic surfactant, proteinase K	Phenolchloroform, alcohol precipitation	End point	*mt* and *AP*[13]	*Aspergillus* genus	249	12.9	64[14] (41–83)	93 (39–96)	64 (41–83)	93 (39–96)
Raad (2002) [81]	Blood/ whole	Prospective	Anionic surfactant, proteinase K	Phenolchloroform, alcohol precipitation	End point	*mt* and *AP*	*Aspergillus* genus	54	20.4	57[14]	100	100	92
Sanguinetti (2003) [28]	BAL/whole	Retrospective	DNeasy Plant Mini Kit (Qiagen, Germany)		End point (nested) and real-time quantitative	*18S rRNA* and *18S rRNA*	*Aspergillus* genus	44	45.5	90	100	100	92
Rantakokko-Jalava (2003) [82]	BAL/conc.,break/>whole	X	Enzymatic, bead-beating, phenolether		Real-time semi quantitative	*mt tRNA*	*Aspergillus fumigatus*	66	16.7	72	93	73	95

Study (year), Ref.	Clinical specimen[1]	Study design[2]	Extraction (kit or fungal cell wall lysis)	Extraction (DNA purification)	PCR format	PCR target gene	Target pathogens[3]	#[4]	Prv[5] (%)	Sensitivity[6]	Specificity[7]	PPV[8]	NPV[9]
Musher (2004) [26]	BAL/pellet	Retrospective/ blinded	Master Pure Yeast kit (Epicentre Biotechnologies, WI, USA)		Real-time quantitative	*18S rRNA*	*Aspergillus* genus	100	50.0	67 (52–81)	100 (93–100)	X	X
Buchheidt (2004) [32]	BAL, blood and others	Prospective	Enzymatic, anionic surfactant, proteinase K	Phenolchloroform, alcohol precipitation	End point (nested)	*18S rRNA*	*Aspergillus* genus	205	9.6	63.6[16] (30.8–89.1)	63.5 (53.4–72.7)	X	X
					Real-time quantitative	*mt cb* [15]	*A. fumigatus*						
Kawazu (2004) [25]	Blood/ plasma	Prospective	Lysis buffer, protease, QIAmp Blood minikit (Qiagen, CA, USA) [92]		Real-time quantitative	*18S rRNA*	*Aspergillus* genus	149	7.4	55	93	40	96
Challier (2004) [83]	Blood/ serum	Retrospective	QIAamp DNA minikit (Qiagen, France)		Real-time quantitative	*28S rRNA*	*A. fumigatus*	70	37.1	84.6	X	X	X
Scotter (2005) [31]	Blood/ whole	Prospective/ blinded	Enzymatic, QIAamp DNA mini kit (Qiagen, Germany)		End point	*18S rRNA*	*Aspergillus* genus	25	20.0	100	85	62.5	100
Halliday (2006) [34]	Blood/ whole	Prospective/ blinded	Enzymatic, GenElute Mammalian Genomic DNA kit (Sigma-Aldrich Co., Dorset, UK), proteinase K		End point (nested)	*18S rRNA*	*Aspergillus* genus	95	13.7	100[17] (75.3–100)	75.4 (62.7–85.4)	46.4 (27.5–66.1)	100 (92.3–100)
White (2006) [36]	Blood/ whole	Retrospective	Bead-beating, MagNA Pure LC System (Roche Diagnostics, West Sussex, UK)		End point/ real-time quantitative (nested)	*28S rRNA*	*Aspergillus* genus	203	6.9	92.3[18] (66.7–98.6)	94.6 (89.8–97.3)	60 (38.7–78.1)	99.3 (96.1–99.9)
Florent (2006) [29]	Blood/ serum	Prospective/ blinded	QIAamp DNA minikit (Qiagen)		End point	*mt*	*A. fumigatus* and *Aspergillus flavus*	167	19.8	63.6 (45.1–79.6)	89.7 (82.6–94.5)	63.6 (45.1–79.6)	89.7 (82.6–94.5)
Khot,break/>(2008) [5]	BAL/pellet	Retrospective/ blinded	Bead-beating, Master Pure Yeast kit (Epicentre)		Real-time quantitative	*18S rRNA*	*Aspergillus* genus	94	13.8	76.9 (50–92)	87.7 (79–93)	58	94
Cesaro (2008) [84]	Blood/ whole	Retrospective	DNA Blood kit (Qiagen)		Real-time quantitative	*18S rRNA*	*Aspergillus* genus	62	12.9	63[19]	81	33	94

Study (year), Ref.	Clinical specimen[1]	Study design[2]	Extraction (kit or fungal cell wall lysis)	Extraction (DNA purification)	PCR format	PCR target gene	Target pathogens[3]	#[4]	Prv[5] (%)	Sensitivity[6]	Specificity[7]	PPV[8]	NPV[9]
Suarez (2008) [27]	Blood/ serum	Prospective	MagNA Pure LC DNA isolation kit (Roche Diagnostics)		Real-time quantitative	*28S rRNA*	*A. fumigatus*	124	12.1	100[20]	96.7	81	100
Botterel (2008) [85]	Blood/ serum	Retrospective/ blinded	MagNA Pure total nucleic acid isolation kit (Roche Diagnostics)		Real-time quantitative	*mt*	*A. fumigatus* and *A. flavus*	25	44.0	62	100	100	71
Cuenca-Estrella (2009) [37]	Blood/whole and serum	Prospective	QIAamp DNA minikit (Qiagen, Madrid, Spain)		Real-time quantitative	*ITS1*	*A. fumigatus*	83	14.5	91.6	94.4	73.3	98.5

Please note that the footnotes to this table can be found under Table 4D.

Table 4B

Diagnosis of *Pneumocystis* pneumonia.

Study (year), Ref.	Clinical specimen[1]	Study design[2]	Extraction (kit or fungal cell wall lysis)	Extraction (DNA purification)	PCR format	PCR target gene	#[4]	Prv[5] (%)	Sensitivity[6]	Specificity[7]	PPV[8]	NPV[9]
Sing (2000) [49]	BAL/pellet	Prospective/blinded	Proteinase K, Qiagen Tissue kit (Qiagen, Germany)		End point (single)	*LSU mt rRNA* [21]	334	7.8	100[22]	100	96	100
					End point (nested)				100	97.5	96	100
Torres (2000) [50]	BAL and lung biopsies	Prospective/blinded	X		End point (nested)	*ITS*	47	38.3	72.2[23]	100	81.8	100
Fischer (2001) [58]	Oral washes/pellet	Prospective/blinded	Insta Gene Matrix (Bio-rad) and NucliSens kit (Organon Teknika, Ireland)		End point	*MSG* [24]	175	18.3	91	94	76	98
						LSU mt rRNA			75	96	80	94
Olsson (2001) [51]	BAL and sputum	Retrospective	Wizard DNA Clean-up (Promega, Wl, USA)		End point (nested)	*mt LSU rRNA*	91	27.5	96	59	48	97
Flori (2004) [52]	BAL/pellet	X	QIAamp DNA minikit (Qiagen)		End point	*LSU mt rRNA*	150	7.3	100	87	X	X
					Real-time	*MSG*			100	98.6		
Pinlaor (2004) [53]	BAL/pellet	X	Proteinase K	Phenolchloroform	End point	*5S rRNA*	21	57.1	100	90	91.7	100
	Sputum/pellet						139	42.2	84.6	98.4	84.6	98.4
Larsen (2004) [59]	Oral washes/pellet	Prospective/blinded	NucliSens kit (Organon Teknika)		Quantitative touch-down	*MSG*	113	82.3	88	85	_ [25]	_ [25]
Nyamande (2005) [60]	Oropharyngeal washings	Prospective	Anionic surfactant, proteinase K	Phenolchloroform, alcohol precipitation	Phenolchloroform, alcohol precipitation	*mt LSU rRNA*	35[26a]	45.7	44	79	64	63
							48[26b]	72.9	40	77	82	32
Bandt (2007) [56]	BAL/whole	Retrospective	QIAamp Viral RNA Mini Kit (Qiagen)[27a] and QIAamp DNA Blood Mini Kit (Qiagen)[27b]		Real-time quantitative	*5.8S rRNA*	88	31.8	79	100	X	X
						DHFR2 [281]			82	97		
Nuchprayoon (2007) [57]	BAL/pellet	X	FTA-filter paper-based DNA extraction (Whatman Bioscience, Kent, UK)		End point	*mt 5S rRNA* [29]	56	21.4	67	91	X	X
	Sputum/pellet						106	8.5	67	90		
Fillaux (2008) [54]	BAL/pellet	Prospective	High Pure PCR template preparation kit (Roche Diagnostics, France)		Real-time, semi-quantitative	*MSG*	400	7.8	100	90.5	47	100

Study (year), Ref.	Clinical specimen[1]	Study design[2]	Extraction (kit or fungal cell wall lysis)	Extraction (DNA purification)	PCR format	PCR target gene	#[4]	Prv[5] (%)	Sensitivity[6]	Specificity[7]	PPV[8]	NPV[9]
Huggett (2008) [55]	BAL/whole	Blinded	DNeasy tissue kit (Qiagen, West Sussex, UK) and QIAmp UltraSens Virus kit (Qiagen)		Real-time quantitative	*HSP70* [30]	136	45.6	98 (91–100)	96 (87– 99)	X	X
					End point	*mt LSU rRNA*			97 (89–99)	68 (56–78)		

Please note that the footnotes to this table can be found under Table 4D.

Table 4C

Diagnosis of candidiasis.

Study (year). Ref.	Clinical specimen[1]	Study design[2]	Extraction (kit or fungal cell wall lysis)	Extraction (DNA purification)	PCR format	PCR target gene	#[4]	Prv[5] (%)	Sensitivity[6]	Specificity[7]	PPV[8]	NPV[9]
Morace (1999) [65]	Blood/whole	X	Nonionic and polysorbate surfactants, enzymatic, proteinase K, anionic surfactant	Phenol-chloroform, Sephadex G50 column, alcohol precipitation	End point	*P-450 L1a1* [31]	72	19.4	92.8	X	X	97.5
Ahmad (2002) [66]	Blood/serum	X	Chaotropic agent (guanidine thiocyanate)	Chloroform-isoamyl alcohol, alcohol precipitation	End point (seminested)	ITS2	50	56	12/12 + (proven) and 9/16 + (probable)	10/10 - (possible) and 12/12 - (healthy controls)	X	X
Maaroufi (2003) [68]	Blood/whole	X	Enzymatic, anionic surfactant, QIAmp Tissue kit (Qiagen, Germany)		Real-time quantitative (*C. albicans*)	ITS2	97	62.9	100	97	X	100
					Real-time quantitative (genus)				100	72		100
White (2005) [86]	Blood/whole	Retrospective	Enzymatic, anionic surfactant, proteinase K	Protein precipitation, alcohol precipitation	End point/real-time (nested)	*18S rRNA*	105	19	95 (99.1–76.4)	97 (89.8–99.2)	90.5	98.5
Dunyach (2008) [67]	Blood/serum	X	QIAamp DNA Blood minikit (Qiagen, France)		Real-time quantitative	*18S rRNA*	33	69.7	93 (proven)	66 (proven)	X	X
						ITS			77 (proven)	100 (proven)		
McMullan (2008) [64]	Blood/serum	Prospective	QIAamp DNA minikit (Qiagen), YM-100 (Millipore, Hertfordshire, UK) columns to concentrate DNA		Real-time (nested)	*18S rRNA*, ITS1 and 2	514	4.5[32]	90.9 (70.8–98.9)	100 (99.3–100)	100 (83.2–100)	99.8 (98.9–100)

Please note that the footnotes to this table can be found under Table 4D.

Table 4D

Broad-range PCR for diagnosis of invasive fungal diseases.

Study (year), Ref.	Clinical specimen[1]	Study design[2]	Extraction (Kit or fungal cell wall lysis)	Extraction (DNA purification)	PCR format	PCR target gene	Etiological pathogens[33]	Patient (n)[4]	Prv[5] (%)	Sensitivity[6]	Specificity[7]	PPV[8]	NPV[9]
Einsele (1997) [11]	Blood/whole	Retrospective/ blinded	Heat-alkaline-enzymatic, anionic surfactant, proteinase K	Protein precipitation, alcohol precipitation	End point	*18S rRNA*	*Candidia Aspergillus*	121	24.7	100	98	X	X
Loeffler (2000) [87]	Blood	Prospective/ blinded	Enzymatic, QIAmp tissue kit (Qiagen, Germany)		Real-time quantitative	*18S rRNA*	*Candidia, Aspergillus*	59	15.3	9/9 + (histologically proven)	50/50 - (healthy controls)	X	X
Hebart (2000) [78]	Blood	Prospective/ blinded	Enzymatic, anionic surfactant, proteinase K	Protein precipitation, alcohol precipitation	End point	*18S rRNA*	*Candidia, Aspergillus*	92	9.8	100	73	36.8	100
Jordanides (2005) [77]	Blood	Prospective/ blinded	Heat-alkaline-enzymatic, anionic surfactant	Protein precipitation, alcohol precipitation or Qlamp Tissue kit (Qiagen)	Real-time quantitative	*18S rRNA*	*Candidia, Aspergillus*	125	6.4	75	70	15	98
El-Mahallawy (2006) [88]	Blood/serum	Prospective	Enzymatic, proteinase K, QIAmp minikit (Qiagen)		End point	*18S rRNA*	*Candidia, Aspergillus, Penicillium*	91	30.8	75 (55–87)	92 (80–97)	84 (63–95)	87 (74–94)
Ribeiro (2006) [89]	Blood/whole	X	Anionic surfactant, proteinase K, enzymatic, DNAzol BD reagent (Gibco, Renfrewshire, UK)		End point	*18S rRNA*	*Aspergillus*	193	7.8	75	91.1	X	X
Lau (2007) [76]	Tissue samples[34]	X	Paraffin wax removal, proteinase K, MagNAPure LC DNA isolation kit II Tissue (Roche Diagnostics)		End point	ITS1	Broad-range[35]	74	X	93.6 (culture) and 64.3 (histology)	18/18 – (no IFD controls)	X	X
Badiee (2009) [90]	Blood	Prospective	Enzymatic, proteinase K, QIAmp minikit (Qiagen)		End point	ITS	*Candidia, Aspergillus*	310	10.6	84.6	92.7	75.3	95.8

1. Type of clinical specimen/fraction used for PCR assay; **2.** Study design in terms of 'prospective or retrospective/blinded or not'; **3.** Target pathogens based on primer specificity; **4.** Number of patients or episodes; **5.** Prevalence of disease based on ‘proven + probable’ cases; **6.** Diagnostic sensitivity with 95% confidence intervals if reported; **7.** Diagnostic specificity with 95% confidence intervals if reported; **8.** Positive predictive value with 95% confidence intervals if reported; **9.** Negative predictive value with 95% confidence intervals if reported; **10.** ‘X’ in the tables indicate information not explicitly reported or could not be inferred from a related reference or that variable was not used or estimated in the study; **11.** Mitochondrial; **12.** Could not determine prevalence because classification scheme deviates from EORTC/ MSG criteria; **13.** Alkaline protease; **14.** Sensitivity, specificity and predictive values shown for probable cases (n = 22); study estimated for each IA category; **15.** Mitochondrial cytochrome b; **16.** Sensitivity and specificity values reported for nested PCR targeting 18S rRNA gene; only 21 % of nested PCR-positive samples were positive by quantitative real-time PCR targeting mitochondrial cytochrome b gene; **17.** Sensitivity, specificity and predictive values shown for ‘proven + probable’ IA as true positives and ‘no’ IA as true negatives; using the criteria of ≥2 positive PCR results to classify an episode as ‘PCR positive’; study estimated for several combinations of IA category; **18.** Sensitivity, specificity and predictive values shown when proven/probable cases are true positives, at-risk cases are true negatives, and possible cases are excluded from statistical analysis; study estimated for several combinations of IA category; **19.** Sensitivity, specificity and predictive values shown for ≥2 PCR+; also reported for single PCR+; **20.** Sensitivity, specificity and predictive values shown for large serum volume; also reported for relatively small serum volume; **21.** Large subunit mitochondrial rRNA; **22.** Sensitivity, specificity and predictive values shown for patients with HIV; also reported for other patient groups; **23.** Sensitivity and specificity reported based on band intensity grades on gel (grade 2 shown); **24.** Major surface glycoprotein; **25.** Due to high prevalence (of study design), estimated likelihood ratios instead of predictive values; **26.** Analysis based on two gold standards: a) TBxx (histology) and b) BAL (PCR); **27.** Extraction method for: a) PCP+ and b) PCP- samples; **28.** Dihydrofolate reductase; **29.** Mitochondrial 5S rRNA; **30.** Heat-shock protein 70; **31.** Cytochrome P450 lanosterol-14*α*-demethylase; **32.** Based on proven candidemia; **33.** The dominant genera of pathogens diagnosed in the patient population under consideration; **34.** Specimens (fresh tissue biopsy and paraffin-embedded) were obtained from a variety of body sites, including both sterile and nonsterile locations; **35.** *Candida*, *Cryptococcus*, *Trichosporon*, *Aspergillus*, *Fusarium*, *Scedosporium*, *Exophiala*, *Exserohilum*, *Apophysomyces*, *Actinomucor* and *Rhizopus.*