The Evolution and Evaluation of a Whole Blood Polymerase Chain Reaction Assay for the Detection of Invasive Aspergillosis in Hematology Patients in a Routine Clinical Setting

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(See the editorial commentary by Donnelly on pages 487–9)

Background. Invasive aspergillosis (IA) is associated with high mortality. Successful outcome with treatment is linked to early diagnosis. The utility of classic diagnostic methods, however, is limited.

Methods. To aid in the diagnosis of IA, we retrospectively assessed our diagnostic service, using real-time polymerase chain reaction (PCR) and galactomannan sandwich enzyme-linked immunosorbent assay (ELISA).

Results. A total of 203 patients at risk of invasive fungal infection were screened by PCR, and 116 of the patients were also tested by ELISA. The patient group comprised 176 patients with hematological malignancy and 28 control patients with evidence of invasive candidal infection. Consensus European Organisation for Research and Treatment of Cancer and Mycoses Study Group criteria were used to classify fungal infection, which, by definition, excluded the PCR result. The PCR method was sensitive (up to 92.3% sensitivity) and specific (up to 94.6% specificity) and had good agreement with the galactomannan ELISA (76.7%) and high-resolution computed tomography scan results.

Conclusions. A negative PCR result can be used to rule out IA and to limit the need for empirical antifungal therapy; thus, it has a role in diagnosing IA infections, especially in combination with antigen testing. PCR-positive cases classified as "false positives" regularly reflect the limitations of classic microbiological procedures or restricted use of consensus clinical methods employed to classify infection.

The incidence of invasive aspergillosis (IA) is increasing [1]. Difficulties with diagnosis mean that most invasive fungal infections are proven only at autopsy. Histopathological analysis can establish diagnosis of a filamentous fungal infection but rarely can identify the agent at a species level. High-resolution CT scan of the chest may be a useful adjunctive investigation in diagnosis but cannot confirm infection, and results may be transient [2, 3].

DNA-based methods have shown potential in the definitive diagnosis of invasive fungal infections. Depending on the target gene, assays demonstrate high specificity, with figures of 100% [4–6], 93% [7], and 92% [8] reported. Other factors, including the efficacy of the DNA extraction [9, 10], quality of primers (dimer formation and/or nonspecific amplification), type of PCR reaction (block-based PCR [11], nested PCR [12], or real-time PCR [5]), size of population studied, type of disease [13], and sample type (plasma, whole blood, or bronchoalveolar lavage [BAL] [14–16]) will affect the sensitivity of the assay.

The method of Williamson et al. [12] has been used in our laboratories for investigational diagnosis. Occasional problems with nonspecific amplification hindering the interpretation of results, plus the additional processing time required to perform gel electrophoresis, suggested that the assay could be improved by using an *Aspergillus*-specific probe and the Roche Light Cycler for the second round of nested PCR. The aim of this study was to evaluate the performance of the modified

assay in a clinical setting as an adjunct to diagnosis by accepted European Organisation for Research and Treatment of Cancer and Mycoses Study Group (EORTC-MSG) criteria [17].

PATIENTS AND METHODS

Patient Group

A group of 203 patients at risk of invasive fungal infection were tested by real-time PCR over a 13-month period (November 2003–December 2004). The majority (176) were hematology patients, with 133 receiving remission-induction therapy for acute leukemia (68 patients) or undergoing stem cell transplantation (65 patients) (table 1). The mean age of patients was 48 years (table 1). The EORTC-MSG criteria [17] (outlined in table 2) were used to classify fungal infection (table 1).

Stem cell transplant recipients and patients with acute myeloblastic leukemia received itraconazole prophylaxis, and patients with acute lymphoblastic leukemia received fluconazole prophylaxis. Piperacillin/tazobactam and amikacin were used for empirical treatment of febrile neutropenia, with lipid formulations of amphotericin or echinocandin added for refractory fever after 96 h.

In accordance with proposed standards of care [3], high-resolution CT scans were requested by clinicians but were delayed or refused on some occasions (resulting compliance, 36.9%), and bronchoscopic analysis was performed if an undiagnosed respiratory pathogen was suspected. Nine patients had Aspergillus species cultured from respiratory specimens (7 sputum and 2 BAL specimens). Aspergillus fumigatus was the most common species isolated ($n = 7$), although Aspergillus niger and Aspergillus terreus were also cultured. Contemporaneous samples for PCR and galactomannan ELISA (BioRad) were taken at the decision of the clinicians, who were advised to obtain samples twice weekly (Monday and Thursday) as part of the investigation of febrile neutropenia in high-risk patients, although sampling was not always consistent (compliance, 50%).

The galactomannan ELISA was performed as described by the manufacturer. To comply with European Directive 98/79/EC, only index values of ⩾1.5 were reported as positive, although the absolute values were recorded, and the number of probable cases of IA were not affected by a reduction of the index factor to 0.5.

Retrospectively, patients were assessed for evidence of bacterial or viral infections according to standard diagnostic protocols. Significant pathogens isolated from blood cultures (excluding single isolates of coagulase-negative Staphylococcus), positive cytomegalovirus PCR results, or positive immunofluorescence findings for respiratory viruses resulted in the reclassification of possible IA cases as "at-risk" cases. Probable cases were not reclassified, as the evidence for probable IA retains its weight even if other infections are diagnosed. However, no patients with probable IA had evidence of any alternative sig-

nificant infection. Hematology patients without host factors or with an alternative cause of fever with no clinical or microbiological features of invasive fungal infection were classified as at-risk. In addition, 28 nonhematology, critical-care patients with clinical and microbiological evidence of invasive candidal infections and no evidence of IA were included as a control population to test the specificity of the assay and were classified as at-risk (table 1).

Sample Type

For PCR, twice-weekly 2-mL whole blood samples collected in EDTA vacutainers were requested and were stored at −80°C until testing. Clinicians requesting Aspergillus antigen tests were instructed to collect clotted blood specimens twice weekly, and 2 mL of serum was removed and was stored at −80°C until testing. Samples were stored for up to 7 days before testing.

DNA Extraction

From fungal cultures. Cultures were grown overnight at 37°C in glucose broth. The following day, fungal biomass (1–2 mL of culture) was harvested by centrifugation (at 5300 g for 10 min), and the supernatant was discarded. Fungal DNA was then extracted as described, with the exclusion of the blood lysis steps.

From clinical specimens. Fungal DNA was extracted from blood using a modified version of the semiautomated method described by Loeffler et al. [9]. All manual steps of the extraction procedure were performed in a class 2 laminar flow cabinet. After lysis of the RBCs and WBCs [18], samples were bead-beaten with ∼20 μL of acid-washed glass beads (1180 microns; Sigma) and were pulse-centrifuged before being washed with 200 μL of molecular-grade water. The 200-μL washings were transferred into a MagNA Pure LC sample cartridge and were loaded onto a MagNA Pure platform (Roche), and the extraction was completed using the Total NA serum, plasma, blood kit, and program (Roche). The DNA was eluted in a volume of 100 μL.

In each clinical run, A. fumigatus conidia were quantified using a Fuchs Rosenthal counting chamber, and known levels of A. fumigatus (10–20 colony-forming units [cfu]/mL) were spiked into (i.e., added to) whole blood samples collected in EDTA vacutainers from a healthy donor, to provide a positive extraction control. Nonspiked blood from a healthy donor was used as a negative extraction control.

PCR

Production of standards. By use of primers (AF4 and AR1) and methods described elsewhere [12], a 270-bp PCR product was amplified from A. fumigatus DNA (clinical isolate). PCR products were cloned using the TOPO TA Cloning kit (Invitrogen), incorporating a pCRII-TOPO vector and One

Table 1. Data for the patients ($n = 203$) tested by PCR.

Characteristic	Value
Age group, years	
<10	6
11–20	15
21–30	9
31–40	28
41–50	23
51–60	45
⩾61	75
Not given	2
Diagnosis/condition	
Acute myeloid leukemia	58
Acute lymphoblastic leukemia	10
Chronic myeloid leukemia	2
Chronic lymphoblastic leukemia	3
Other leukemia	2
Lymphoma	22
Myeloma	13
Allogeneic SCT and nonspecified malignancy	65
Nonhematological disorder[a]	28
Galactomannan ELISA performed (%)	116 (57)
No. of samples tested (range per patient [$n = 203$])	401 (1–14)
No. of samples with IA classification	
Proven/probable IA (range per patient [$n = 14$])	70 (2–14)
Possible IA (range per patient [$n = 40$])	117 (2–7)
At-risk (range per patient [$n = 149$])	214 (1–10)

NOTE. Data are no. of patients, unless otherwise indicated.

[a] Nonhematological cases included respiratory failure, esophageal rupture, cystic fibrosis, diabetes, pneumonia, premature birth, renal failure, cirrhosis, or postsurgical complications following cardiac or abdominal surgery. All cases had clinical and microbiological evidence of invasive candidal infections (11 proven candidemia cases and 7 probable and 10 possible cases of invasive candida infection). Proven cases were defined by the isolation of *Candida* from sterile sites (excluding drain fluids and urine) and by histopathological analysis. Probable invasive *Candida* infections were classified by the presence of risk factors (surgery, antibiotic use, prematurity, venous catheters, parenteral nutrition, and immunosuppression), with clinical signs of infection (fever unresponsive to antibacterial antibiotics) and *Candida* colonization at multiple (>2) noncontiguous anatomical sites. Furthermore, in neonates, persistent candiduria was considered significant. Possible invasive *Candida* infections were defined by clinical signs of infection linked to predisposing conditions, such as necrotizing pancreatitis, anastamotic breakdown or recurrent perforation of the bowel, or spontaneous esophageal rupture with no evidence of a clinically significant bacteremia [14]. SCT, stem cell transplant.

Shot Top 10F chemically competent *Escherichia coli*. Plasmids were extracted (S.N.A.P. MiniPrep Kit; Invitrogen) from Luria-Bertani and ampicillin broth cultures and were quantified using a GeneQuant DNA/RNA calculator (Amersham Biosciences). To confirm the identity of the insert, the plasmids were screened with direct PCR, with use of the primers AF4 and AR1, and were digested with the restriction enzyme *Eco*RI.

Oligonucleotide design. *Aspergillus*-specific primers described elsewhere [12] were used. The probe was designed to bind an *Aspergillus*-specific region of the 28S rRNA gene amplified by the primers ASF1 and ADR1. Analogous sequences from 45 strains from 13 species of *Aspergillus* were compared for homology. Sequences from representative *Aspergillus* species were compared with the sequences from 23 species from 10 different genera. A hydrolysis (TaqMan) probe was designed to hybridize to a sequence that was specific to the genus *Aspergillus*. The binding sites for the hydrolysis probe ASP28P (FAM-CATTCGTGCCGGTGTACTTCCCCG-TAMRA) and primers ASF1 and ADR1 on *A. fumigatus* (NCBI Nucleotide Database accession number AJ438345) are at positions 453–486, 392–412, and 560–575, respectively.

Roche Light Cycler assay. Real-time PCR was performed using the *Aspergillus*-specific primers and probes (ASF1, ADR1, and ASP28P). For amplicon detection, the Light Cycler Fast Start DNA Master Hybridisation Probes kit was used. The PCR mix contained 1× Fast Start reaction mix, 0.75 μmol/L of each primer, 0.4 μmol/L of probe, 4 mmol/L of MgCl$_2$, and DNA template, for a final volume of 20 μL. PCR conditions were 1 cycle at 95°C for 15 min followed by 60 cycles at 95°C for 5 s and 60°C for 30 s, while data were acquired at channel F1/F2.

In addition to extraction controls for clinical runs, PCR standards in the form of cloned PCR products (200, 20, and 5 input copies) and duplicate DNA-free PCR controls (molecular-grade water) were included in every run. PCRs with positive results were repeated for confirmation and were considered clinically significant when serial positive results were generated during a single episode, although widespread use of empirical antifungal agents was a possible confounding factor affecting positivity.

To further enhance the sensitivity of the assay, it was necessary to perform a nested PCR. The first round of the nested PCR was done as described elsewhere [12], but primer concentrations were increased to 0.4 μmol/L, and DNA template was increased to 10 μL. On completion of the first round, 10 μL of the amplicon was used as the template for the second round on the Light Cycler, by use of conditions described above. Controls were as described above except for 2 additional DNA-free PCR controls.

RESULTS

***Aspergillus* PCR detection limits, reproducibility, and cross-reactivity.** The detection limit for the assay was determined for both cfu and plasmid copy number. Serial dilutions showed that the lower limit of detection of the Light Cycler assay alone was ~2 input copies, although, for 100% reproducibility, 5 input copies were necessary (figure 1). The standard curve for the *Aspergillus* assay is shown in figure 2.

The single-round Light Cycler assay could reproducibly detect 10 cfu/mL spiked in blood, with a lower detection limit of <10 cfu (figure 1). The nested *Aspergillus* assay improved detection limits to 1 input copy and <5 cfu reproducibly detected.

Four species of *Aspergillus* (*A. fumigatus, Aspergillus flavus, Aspergillus nidulans,* and *A. niger*), a strain of *Fusarium*

Table 2. Criteria used to determine certainty of invasive aspergillosis (IA) infection.

IA infection criteria
Host factors
Antibacterial refractory fever (for >96 h)
Neutropenia (neutrophil count, <500 neutrophils/mm^3 for >10 days)
Fever of unknown origin
Prolonged steroid use (>3 weeks)
Graft-versus-host disease
Microbiological factors
Positive result of culture of sputum, bronchoalveolar lavage, or sinus specimen
Positive microscopic analysis result
Positive galactomannan antigen test result
Clinical factors
Major
CT findings (halo, air crescent)
Nodular skin rash
Minor
New pleural infiltrate (on chest radiograph)
Pleuritic chest pain
Shortness of breath
Cough
Hemoptysis
Pleural rub

NOTE. Proven IA is defined by histological evidence with a concomitant positive culture result. Probable IA is defined by a host factor plus a microbiological factor plus 1 major (or 2 minor) clinical factors. Possible IA is defined by a host factor plus a microbiological factor or 1 major (or 2 minor) clinical factors. Criteria from [17].

solani, and 15 species of yeast (4 genera) were tested for cross-reactivity. All *Aspergillus* species tested were detected by the assay, whereas the 12 *Candida* species *(Candida albicans, Candida dubliniensis, Candida famata, Candida glabrata, Candida guilliermondii, Candida kefyr, Candida krusei, Candida lipolytica, Candida lusitaniae, Candida parapsilosis, Candida rugosa,* and *Candida tropicalis), Cryptococcus neoformans, Rhodotorula rubra, Saccharomyces cerevisiae,* and *F. solani* were not detected by the assay.
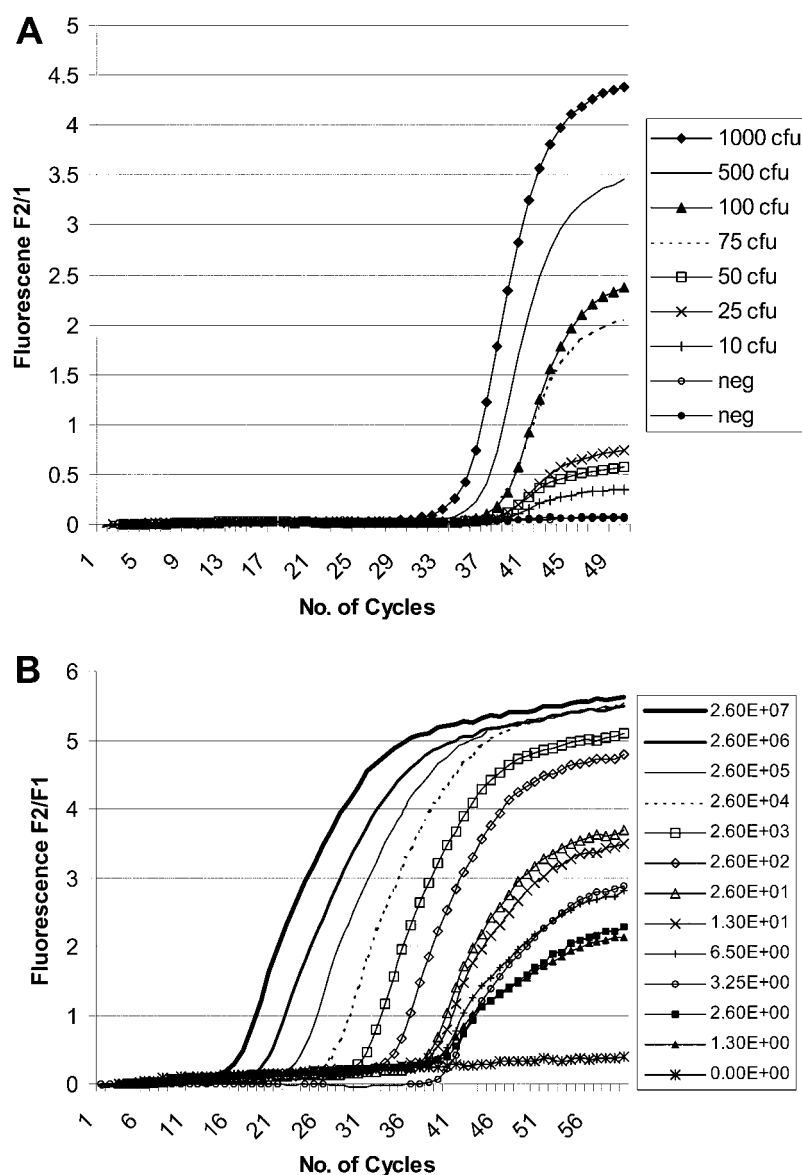
Aspergillus *PCR sensitivity and specificity with clinical specimens.* There were 14 cases (11 with acute leukemia and/or receipt of allogeneic stem cell transplantation [AL/SCT]) of proven/probable IA, 40 cases [13 AL/SCT] of possible IA, and 149 cases (109 AL/SCT) classified as at-risk for IA, generating an incidence rate of 6.9% (9.2% in the AL/SCT population). A total of 401 whole blood samples collected in EDTA vacutainers from 203 patients (range per patient, 1–14 samples) (table 1) were tested by PCR, with 51 patients having at least 1 specimen test positive by *Aspergillus* PCR, but, more significantly, 26 patients (12.8%) had serial positive results (range, 2–10 positive results). Only patients with serial positive results were considered to be PCR positive. Single positive PCR results among multiple samples were considered false-positive results, and single positive PCR results for a specimen with no other

samples were excluded from statistical analysis, although considering them false-positive results had little impact on the assay performance. One patient classified as having probable IA was excluded from analysis because samples were sent 2 weeks after the diagnosis of probable disease. During the intervening period, the patient had received appropriate antifungal therapy.

PCR positivity rates were higher in the proven and probable IA categories and correlated well with radiological markers—10 of the 12 patients with PCR-positive, proven/probable IA had CT scans of the chest suggestive of possible fungal infection. Furthermore, 32 of the 33 patients whose CT scan findings were negative also had negative PCR results. In total, 75 patients underwent a CT scan: 18 scans were suggestive of an invasive fungal infection, 24 were nonspecific, and 33 were negative.

The positivity rate for patients with proven/probable IA with serial positive PCR results was 92.3%, compared with 15.0% for those with possible IA (difference, 77.3%; 95% CI, 48.1%–87.5%) and 5.4% for at-risk groups (difference, 86.9%; 95% CI, 60.9%–93.8%) (table 3). The sample positivity rate by use of serial positives for proven/probable cases was 60.0%, whereas, for possible and at-risk cases, it was 14.5% (difference, 45.5%; 95% CI, 31.6%–57.4%) and 10.7% (difference, 49.3%; 95% CI, 36.6%–64.0%), respectively (table 3).

The statistical performance of the assay was calculated using

Figure 1. The detection limits of the single-round *Aspergillus* PCR using colony-forming units (cfu) *(A)* or plasmid copy number *(B)*

different definitions of disease status [7]. The sensitivity, specificity, positive predictive value, and negative predictive value of the *Aspergillus* PCR are shown in table 4.

DISCUSSION

The value of PCR for diagnosing invasive fungal infections has yet to be determined, and PCR results are not included in the current EORTC-MSG criteria for defining invasive fungal infection. Although PCR methods may lack standardization and optimization across centers, antigen detection provides varied performance in terms of sensitivity (29%–100% [19]). It is included in the consensus criteria because of its commercial availability and good specificity (>85% [19]), despite a lack of agreement regarding the correct cutoff for positivity.

By use of serial positive PCR results, only a slight increase in sensitivity (92.3%) and a slight decrease in specificity (94.6%) were observed when compared with the original method [12]. However, a depreciation of 40% was seen for the positive predictive value by use of the Light Cycler–based assay. This decrease can be explained by the 8 patients with serial positive PCR results who lacked EORTC-MSG clinical or microbiological criteria for IA and were classified as at-risk. Of a median of 5 samples tested per patient (range, 3–8 samples tested per patient), the median number of positive samples per patient was 3. In 4 patients, the positive PCR results spanned up to 4 consecutive weeks, during which the patients remained febrile and neutropenic, with no other cause for their fever. Three of these patients received antifungal therapy, with 2 patients be-
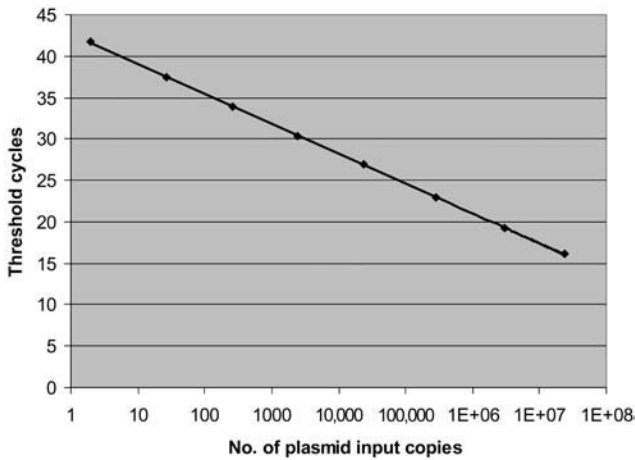
Figure 2. Standard curve for the *Aspergillus* PCR, where $Y = -1.5705\ln(X) + 42.71$. Both the correlation coefficient (r) and the correlation coefficient squared (R^2) were 1.00. The mean ($\pm$ SD) crossing-point for 5 input copies was 38.66 ($\pm$ 0.78) cycles. The maximum variation in crossing-point for 5 input copies was 1.44 cycles (3.7%).

coming PCR negative; no follow-up specimens were available from the third patient. All 3 patients who received antifungal therapy survived. The fourth patient received no antifungal therapy and died, although autopsy was not performed.

Jordanides et al. [20] documented the problem of serial positive PCR results in patients and commented on the difficulty in determining an actual "false-positive" result from an early "true-positive" result, reflecting the fact that PCR is more sensitive than current diagnostic procedures. In clinical practice, other adjunctive procedures required for consensus diagnostic criteria usually are not performed or are performed at the wrong time [3]. It seems inappropriate to use methods that are rarely used antemortem (histopathology), have transient results (CT), or are less sensitive (blood culture) as comparison in an assessment of the performance of a PCR method, unless a structured regimen of testing is in place for all methods. This highlights the difficulties in determining a proven/probable case of IA, and it is not unexpected that only 6% of patients with

IA receive a definite diagnosis [21], which emphasizes the need for improved diagnostic methods [20, 22].

Analysis of specimen positivity rate showed that 60%, 14.5%, and 10.7% of samples for proven/probable, possible, and at-risk cases, respectively, were positive by PCR. The 95% CIs confirm these differences and suggest that, 95% of the time, the sample positivity rate for proven/probable cases would be 37%–64% greater than that for at-risk cases.

Since at-risk cases have low positivity rates for samples, serial positive PCR results during 1 particular episode should be considered indicative of probable/possible IA. Applying these criteria would mean that 7 of the 8 patients classified as at-risk who had serial *Aspergillus*-positive PCR results could be regarded as having possible IA, and it would enhance specificity and positive predictive values to 99.3% and 92.2%, respectively.

The high sensitivity of the assay has implications for initiation and continuation of antifungal therapy. The excellent negative predictive value (99.3%) will allow clinicians to withdraw therapy in cases that have responded. No patients in this study received a diagnosis of other filamentous fungal infections; however, the number of cases of non-*Aspergillus* filamentous fungal infections is rising [23], and other assays [20] are needed to avoid false-negative results of non-*Aspergillus* infections or to withhold therapy.

The optimal specimen for the molecular detection of IA is still not resolved. Some groups prefer whole blood samples [8, 15], others prefer serum samples [24, 25], and some prefer BAL samples [4, 26]. Serum has been reported to be an appropriate sample for the diagnosis of IA [24]. However, only free-circulating *Aspergillus* DNA will be targeted, because hyphae or phagocytosed intracellular fungal fragments will be removed by clot formation and centrifugation.

If fungal fragments are present in blood, they should be detectable by blood culture methods [24], particularly if lytic systems are used [27, 28]. As few as 1–10 cfu of *Aspergillus* conidia inoculated into blood culture bottles can be detected within 24 h [29], but, in the clinical scenario, blood cultures are rarely positive for *Aspergillus* (possibly because phagocytosis

Table 3. Correlation between detection of *Aspergillus* by PCR and the level of confidence in invasive aspergillosis (IA) infection.

Classification	No. of patients with positive PCR		Patient positivity rate, % (95% CI)[a]	No. of samples tested (no. PCR positive)	Sample positivity rate, % (95% CI)[a]
	Single positive result	Serial positive results			
Proven/probable IA ($n = 13$)[b]	0	12	92.3	70 (42)	60.0
Possible IA ($n = 40$)	13	6	15.0 (48.1–87.5)	117 (30)	14.5 (31.6–57.4)
At-risk ($n = 149$)	12	8	5.4 (60.9–93.8)	214 (35)	10.7 (36.6–64.0)

[a] Positivity rates are only for patients with serial positive PCR results, and 95% CIs were generated for differences between proven/probable, possible, and at-risk categories.
[b] One patient was excluded.

Table 4. Performance of *Aspergillus* PCR with use of different definitions of disease status.

Value	Under invasive aspergillosis (IA) definition[a]		
	A	B	C
Sensitivity	92.3 (66.7–98.6)	34.0 (22.7–47.4)	92.3 (66.7–98.6)
Specificity	94.6 (89.8–97.3)	94.6 (89.8–97.3)	92.6 (88.0–95.5)
PPV	60.0 (38.7–78.1)	69.2 (50.0–83.5)	46.2 (28.8–64.5)
NPV	99.3 (96.1–99.9)	80.1 (73.6–85.3)	99.4 (96.9–99.9)

NOTE. Data are percentage (95% CI). Statistics were calculated using serial positive-PCR results. NPV, negative predictive value; PPV, positive predictive value.

[a] Under definition A, proven/probable cases are true positives, at-risk cases are true negatives, and possible cases are excluded from statistical analysis; under definition B, proven/probable/possible cases are true positives, and at-risk cases are true negatives; under definition C, proven/probable cases are true positives, and possible/at-risk cases are true negatives.

of fungal fragments by macrophages renders the fungal component nonviable), whereas DNA can still be detected [30, 31]. Detection of *A. fumigatus* in a murine model with intravenously induced fungemia 3 days after infection was 0% by blood culture and 100% by PCR [30].

Whole blood inoculated with DNA extracted from known amounts of *A. fumigatus* (10–1000 cfu) was reextracted and tested by both single-round and nested *Aspergillus* PCR methods, but no samples tested positive (results not shown). This suggests that the procedure is not suitable for extracting free DNA or that the DNA is degraded by DNAses present in the blood. Results for this method correlate with proven/probable cases of IA. Detecting DNA from nonviable or dead *Aspergillus* hyphae circulating in the blood is a possible explanation.

BAL samples are reported to give higher sensitivities and specificities than blood samples [32]. Although PCR sensitivity may be improved by testing BAL samples, improved specificity must be questioned. Indeed, 25% of BAL samples from healthy donors are PCR positive through inhalation of airborne *Aspergillus* spores [33]. Environmental contamination of blood specimens is minimal, and blood is easier to obtain for initial and repeated sampling [21].

In summary, we describe the evolution of a manual DNA-extraction, nested-PCR method into a semiautomated real-time PCR assay that has improved sensitivity without a major reduction in specificity. Like other groups [34], we believe that if a patient has both PCR and galactomannan ELISA positive results, then it is likely that the patient has fungal disease, and we believe that both tests should be used to screen at-risk patients, with the concurrent use of both assays allowing confirmation of diagnosis.

Acknowledgments

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