Aspergillus PCR-Based Investigation of Fresh Tissue and Effusion Samples in Patients with Suspected Invasive Aspergillosis Enhances Diagnostic Capabilities

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Although it is a severe complication in immunocompromised patients, diagnosing invasive fungal disease (IFD), especially invasive aspergillosis (IA), remains difficult. In certain clinical scenarios, examining tissue samples for identification of the infectious organism becomes important. As culture-based methods rarely yield results, the performance of an *Aspergillus*-specific nested PCR in fresh tissue or pleural effusion samples was evaluated. Fresh tissue ($n = 59$) and effusion ($n = 47$) specimens from 79 immunocompromised patients were subjected to an *Aspergillus*-specific PCR assay. Twenty-six patients had proven ($n = 20$) or probable ($n = 6$) IFD, according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria, while the remaining patients were classified as having either possible IFD ($n = 30$) or no IFD ($n = 23$). IA was identified as the underlying IFD in 21/26 proven/probable cases. PCR positivity was observed for 18/21 proven/probable and 6 possible IA cases; cases classified as no IA did not show positive signals. Patients with proven IFD ($n = 5$) with cultures positive for non-*Aspergillus* molds also had negative *Aspergillus* PCR results. *Aspergillus* PCR performance analysis yielded sensitivity and specificity values of 86% (95% confidence interval [CI], 65% to 95%) and 100% (95% CI, 86% to 100%), respectively, thus leading to a diagnostic odds ratio of >200 . In this analysis, good diagnostic performance of the PCR assay for detection of IA was observed for tissue samples, while effusion samples showed lower sensitivity rates. PCR testing represents a complementary tool; a positive PCR result strengthens the likelihood of IA, whereas IA seems unlikely in cases with negative results but findings could indicate non-*Aspergillus* IFD. Thus, PCR testing of these specimens enhances the diagnostic capabilities.

Invasive aspergillosis (IA) is a severe, life-threatening complication in immunocompromised patients, with major impacts on morbidity and mortality rates (1). However, definitively diagnosing IA poses a serious problem in clinical practice, as the existing diagnostic tools lack either specificity or sensitivity or both. At present, only histological examination and/or positive culture results from sterile sites (excluding bronchoalveolar lavage [BAL] fluid) provide definitive proof of invasive aspergillosis according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) consensus definitions (2). Although it is a frequent routine clinical practice, basing the diagnosis of IA solely on computed tomography (CT) criteria seems insufficient; after surgical removal of suspected tissue, only 50% of patients with diagnoses of IA following CT criteria were found to harbor IA in microbiological examinations (3).

Obtaining tissue samples via biopsy or surgery presents the possibility of attaining definite proof of IA. However, due to low platelet counts or critical clinical conditions in patients at risk for IA, especially those with hematological conditions, this diagnostic option is rarely utilized. Underlining the potential in a recent pub-

lication, CT-directed percutaneous biopsies yielded a specific diagnosis for 53% of patients with pulmonary infiltrates who had previously undergone bronchoalveolar lavage (BAL) or even transbronchial biopsies that had failed to identify the pathogen; the biopsy findings led to a change in antimicrobial therapy for these patients (4). In addition, in certain clinical settings (e.g., cerebral aspergillosis), performing biopsies or even surgical resection is still performed, as other diagnostic tools are not yet sufficient to rule in or rule out the diagnosis; furthermore, surgical procedures have been shown to affect survival rates, at least in central nervous system (CNS) aspergillosis (5). Finally, a biopsy might be useful to exclude relapse or metastatic disease, in particular for malignant diseases.

Received 29 August 2013 Returned for modification 23 September 2013
Accepted 3 October 2013

Published ahead of print 9 October 2013

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doi:10.1128/JCM.02387-13

Nodular lesions with the halo sign in heavily immunocompromised patients are typical but nonspecific infiltrates for IA. Other infectious organisms are known to cause similar nodules (6), while IA frequently presents with other radiological manifestations (7), which suggests the necessity of identifying the causative infectious agent with more-invasive measures or procedures.

Although biopsies confer the possibility of identifying the etiology of infections, the sensitivity of culture-based methods is as low as 30% (8) and histological and culture results are often not congruent (9); thus, molecular methods have become increasingly important. In a recent retrospective reevaluation of formalin-fixed, paraffin-embedded (FFPE) samples, the original species identification was corrected using molecular techniques (9).

PCR-based assays have shown to be a promising diagnostic approach to detect and to identify *Aspergillus* rapidly and reliably in a variety of clinical samples, e.g., BAL fluid (10), blood (11), cerebrospinal fluid (12), and even tissue (13) samples. In addition to being more sensitive and yielding results much more rapidly than conventional cultures, sequencing the PCR product (14) or using a DNA microarray (15) for identification can help to identify the exact genus. This is of great importance, as early diagnosis and therapy have been shown to influence overall survival rates not only in IA (16) but also in other invasive fungal diseases (IFDs) (17).

When analyzing tissue samples for IFDs with molecular methods, the majority of publications report on FFPE samples (18) and rarely fresh samples. FFPE sampling has the advantage of sample stability, whereas fresh sampling requires significant additional logistic efforts. The disadvantage of analyzing FFPE samples is that the sensitivity decreases with formalin embedding (19), with amplification rates varying from 55 to 86% in a recent publication (20).

In addition, pleural effusion (PE) is a frequent complication in IA and is associated with increased overall mortality rates (21), yet data on biomarkers, especially PCR performance using pleural effusion samples, are limited. Bonatti et al. recently reported on *Aspergillus*-specific PCR in pleural effusion samples from 4 patients with *Aspergillus* pleural empyema, and they observed PCR positivity in the majority of samples (22). Based on the high sensitivity of PCR and the typical subpleural localization of nodular infiltrates in IA, analysis of pleural fluid samples with *Aspergillus*-specific PCR might be a promising tool, as pleural fluid specimens can be obtained safely for the majority of hematological patients, even those not deemed eligible for invasive procedures (23). Therefore, in order to further elucidate the performance of an *Aspergillus*-specific PCR assay in fresh tissue as well as pleural effusion samples for diagnosing IA, we performed this multicenter trial.

MATERIALS AND METHODS

Patients. Fresh tissue (biopsy or surgical resection specimens) and pleural effusion samples submitted to the scientific laboratory of the Third Medical Department of the Mannheim University Hospital (Mannheim, Germany) for the diagnosis of IA between July 1996 and June 2013 were analyzed to elucidate PCR performance in these samples. Immunocompromised patients at high risk for fungal infections were included in this retrospective analysis. Patient data had been deidentified previously. Written informed consent of patients or their legal representatives had been acquired prior to sampling, and analysis was done according to good clinical practice guidelines and in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee (ethics

TABLE 1 Patient characteristics

Characteristic ^a	Data for patients with (n):		
	Proven/probable IFD ^b (26)	Possible IFD ^b (30)	No IFD (23)
Median age (range) (yr)	52 (4–81)	48 (4–79)	50 (4–76)
Gender (no. female/no. male)	9/17	14/16	6/17
Underlying disease (no.)			
AML	9	15	3
ALL	4	3	0
NHL	4	6	4
HD	1	0	1
MDS	1	0	1
Aplastic anemia	1	1	0
Solid tumor	1	1	6
Other	5	4	8
No. after/no. with:			
Allo-HSCT	6	2	3
Auto-HSCT	1	0	1
HIV infection	1	0	0

^a AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; IFD, invasive fungal disease; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease; MDS, myelodysplastic syndrome; allo-HSCT, allogeneic hematopoietic stem cell transplantation; auto-HSCT, autologous hematopoietic stem cell transplantation; other, chronic obstructive pulmonary disease with high-dose steroid treatment, septic granulomatosis, polyserositis, solid-organ transplantation, or intensive care unit patient after thoracic trauma.

^b According to 2008 EORTC/MSG criteria.

committee of the Faculty of Medicine Mannheim, University of Heidelberg, Heidelberg, Germany). The trial was registered at ClinicalTrials.gov (identifier NCT01902030).

Patient characteristics. A total of 106 tissue/effusion samples from 79 pediatric and adult patients at risk for IA were obtained between July 1996 and June 2013 and were analyzed by an *Aspergillus*-specific nested PCR assay described previously (24) and modified for tissue samples (15) (Tables 1 and 2). Tissue ($n = 59$ from 49 patients) and pleural effusion ($n = 47$ from 33 patients) samples were referred from the university hospitals of Mannheim, Würzburg, Heidelberg, Freiburg, Regensburg, Halle (Saale), Bochum, and Cologne, the pediatric departments of the university hospitals of Mannheim, Frankfurt, and Düsseldorf, the Bone Marrow Transplantation Center Wiesbaden, and the general hospitals of Frankfurt/Oder, Ludwigshafen, Eschweiler, and Recklinghausen, all located in Germany. For 4 patients, both tissue and pleural effusion samples were analyzed.

Definitions for classification. Cases were classified based on the recent 2008 EORTC/MSG consensus criteria (2). The histological diagnosis of IA was made when hyaline septate hyphae with dichotomous 45° branching were observed on a background of inflammation or necrosis, whereas non-IA histology showed unspecified or morphologically different hyphae.

Radiological diagnostics. All patients received computed tomography (CT) or magnetic resonance imaging (MRI) scans of organs suspected to harbor IA, in the participating center, according to standardized techniques. Radiological scan results were analyzed by a panel of experienced local radiologists. The radiological results defining the case were interpreted according to 2008 EORTC/MSG criteria and not on the basis of minor radiological signs.

Tissue sampling. Sampling of tissue or pleural effusions was performed as clinically indicated; reasons for sampling were suspected infection or suspected manifestation of the underlying disease. Tissue sampling was not performed solely for study reasons. The samples were shipped immediately, with typical delivery time of less than 24 h. The

TABLE 2 Proven/probable IFD patients and possible IFD patients tested PCR positive

Patient no.	Underlying disease	SCT ^a	IFD detection defining microbiological probability	Classification of mold IFD ^b	Culture result	Evidence of <i>Aspergillus</i> involvement	PCR result	Comments
5	MDS	No	Histology	Proven	None	GM assay not performed	+	
8	ALL	No	Histology	Proven	None	GM assay negative	+	
9	AML	No	Histology	Proven	<i>Rhizopus</i> spp.	GM assay negative	—	
10	Other	No	NA	Possible	None	GM assay not performed	+	Chronic steroid treatment
								Radiological evidence: dense circumscribed lesion in chest CT scan
16	AML	No	NA	Possible	None	GM assay negative	+	Improvement with AFT
								Radiological evidence: disseminated (renal, spleen, and pulmonary) infiltrations
18	Other	No	Histology	Proven	None	GM assay not performed	+	Chronic steroid treatment
21	NHL	Allo	NA	Possible	None	GM assay negative	+	Radiological evidence: pulmonary nodular infiltrate, pleural effusion in vicinity
22	AML	Allo	Histology	Proven	None	GM assay not performed	+	
25	Other	No	Histology	Proven	<i>Mucor</i> spp.	GM assay negative	—	
29	AA	Allo	Serum GM assay	Probable	None	GM assay positive	+	Radiological evidence: dense nodular infiltrates in chest CT scan
31	MM	Allo	Histology	Proven	<i>Rhizopus</i> spp.	GM assay negative	—	
33	HD	No	GM assay, BAL fluid culture	Probable	None	BAL fluid culture: <i>A. fumigatus</i>	+	Radiological evidence: dense nodular infiltrates with halo sign in chest CT scan
38	AML	No	Serum GM assay	Probable	None	GM assay positive	+	Radiological evidence: dense circumscribed nodular infiltrates in chest CT scan
39	ALL	No	Serum GM assay	Probable	None	GM assay positive	+	Radiological evidence: dense nodular infiltrates with halo sign in chest CT scan, abscess-like lesion with ring enhancement in cerebral MRI scan
40	AA	Allo	NA	Possible	None	CSF BDG assay positive	+	Improvement with AFT
								Abscess-like lesion with ring enhancement in cerebral MRI scan
43	CLL	No	Histology	Proven	None	GM assay negative	+	Chronic steroid treatment
44	AML	No	NA	Possible	None	GM assay not performed	+	Improvement with AFT
								Radiological evidence: pulmonary nodular infiltrate, pleural effusion in vicinity
45	Other	No	Histology, BDG assay	Proven	None	BDG assay positive	+	
47	Other	No	Histology, culture	Proven	<i>A. fumigatus</i>	Biopsy sample culture: <i>A. fumigatus</i> ; GM assay positive	+	Chronic steroid treatment
48	AML	Allo	Histology	Proven	None	GM assay not performed	—	Case definition by histology several months earlier, intensive AFT prior to effusion sampling
52	ALL	Allo	CSF GM assay	Probable	None	GM assay positive	+	Solitary abscess in course of ventriculitis in cerebral MRI scan
56	AML	No	Histology	Proven	None	GM assay negative	+	
58	AML	Allo	Histology	Proven	None	GM assay positive	—	Intensive AFT prior to tissue sampling
64	AML	No	NA	Possible	None	GM assay not performed	+	Improvement with AFT
								Radiological evidence: disseminated (pulmonary, hepatic, and spleen) infiltrates, abscesses, pulmonary nodular lesions with halo sign in CT scan
65	AML	No	Histology	Proven	None	GM assay not performed	+	
67	NHL	No	Histology	Proven	None	GM assay not performed	+	
69	ALL	No	Histology	Proven	None	GM assay not performed	+	
70	AML	No	Histology	Proven	<i>Rhizopus</i> spp.	GM assay negative	—	
72	Other	No	Histology	Proven	<i>Mucor</i> spp.	GM assay negative	—	
75	AML	No	GM	Probable	None	GM assay positive	—	Radiological evidence: dense nodular infiltrates with halo sign in chest CT scan
76	NHL, HIV	No	Histology, GM	Proven	None	GM assay positive; sputum culture: <i>A. fumigatus</i>	+	
79	Neuroblastoma	Auto	Histology	Proven	None	GM assay not performed	+	

^a SCT, stem cell transplantation; MDS, myelodysplastic syndrome; CT, computed tomography; MRI, magnetic resonance imaging; BDG, β -D-glucan; GM, galactomannan; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; NHL, non-Hodgkin's lymphoma; AA, aplastic anemia; HD, Hodgkin's disease; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; AFT, antifungal treatment; CSF, cerebrospinal fluid; allo, allogeneic; auto, autologous; NA, not applicable.

^b According to 2008 EORTC/MSG criteria.

specimens were sent at ambient temperature and were freshly subjected to PCR analysis. The diagnostic work-up was done according to the local standards of the participating centers, usually according to the guidelines of the Infectious Diseases Working Party of the German Society for Hematology and Oncology (25).

DNA preparation and PCR analysis. Fresh tissue samples were disrupted in liquid nitrogen before DNA extraction according to a published protocol (15). Tissue was first sheared with a scalpel under sterile conditions. The tissue nuggets were transferred into a tissueTUBE device for processing of the sample in a cryoPREP impactor (Covaris, Woburn,

MA). The tissueTUBE containing the tissue was incubated in liquid nitrogen for 30 to 45 s, until the sample was completely frozen. After freezing, the tube was fitted into the cryoPREP system, where the tissue was pestled. The frozen tissue crumbs were transferred into a sterile 50-ml reaction tube and mixed with 1.5 ml of 1× phosphate-buffered saline (PBS). The tissue-PBS mixture was transferred into a 1.5-ml reaction tube and centrifuged at 13,000 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in 250 µl of 1× PBS.

For PE samples, total DNA was extracted from 1.0 ml PE and processed by an experienced technical assistant (who was not informed of clinical data), according to the DNA extraction and nested PCR protocol published by Skladny et al. (24). The DNA extraction protocol was the same for the extracted tissue material and the PE samples; 100 ng of total DNA per 25-ml PCR mixture was used as the template. In the nested two-step PCR technique, two pairs of oligonucleotide primers (AFU7S and AFU7AS for the first step and AFU5S and AFU5AS for the second step), derived from sequences of the *Aspergillus fumigatus* 18S rRNA gene (GenBank accession no. AB008401) and specific for *Aspergillus* species, were used. A 138-bp PCR fragment of the human glucose-6-phosphate dehydrogenase gene (GenBank accession no. X55448) was amplified in each clinical sample with the primers G6PD1S and G6PD1AS, as an internal control. All PCRs were performed as duplicates. The sensitivity of this nested PCR assay is 1 to 5 CFU per ml of blood. PCR amplification was done as follows. The standard PCR mixture contained 0.5 U of *Taq* DNA polymerase, 6.25 nmol of the deoxynucleoside triphosphates, and 10 pmol of each primer (first step, primers AFU7S and AFU7AS; second step, primers AFU5S and AFU5AS). PCR was performed using the following conditions: for the first PCR step, 2 min at 94°C and then 23 cycles of 40 s at 94°C, 1 min at 65°C, and 1 min at 72°C, with a terminal step of 5 min at 72°C, and then the mixture was held at 4°C; for the second PCR step, 2 min at 94°C and then 35 cycles of 40 s at 94°C, 1 min at 65°C, and 1 min at 72°C, with a terminal step of 5 min at 72°C, and then the mixture was held at 4°C. For the second PCR step, approximately 1 to 2 µl of the first-round PCR product was used. The PCR products were separated by 2.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light. Control samples included all of the constituents in the reaction mixture except genomic DNA. Diluted samples of *A. fumigatus* were used as positive controls, whereas DNA from the human cell line T47D was used as a negative control. The PCR assay used in this study has been shown to detect a minimum of seven *Aspergillus* species, i.e., *A. fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus clavatus*, *Aspergillus versicolor*, and *Aspergillus nidulans*, whereas the PCR assay does not detect other fungal, bacterial, or human DNA. The amplicon was sequenced in the methodological establishment of this assay and was shown to match the corresponding GenBank sequence (24).

Data collection and statistical analysis. Clinical data for the patients were retrieved from electronic records and hospital charts. Sensitivity rates, specificity rates, positive likelihood ratios (PLRs), negative likelihood ratios (NLRs), and diagnostic odds ratios (DORs) were calculated using GraphPad Prism for Windows 5.0 (GraphPad, La Jolla, CA) and Microsoft Excel 2010 (Microsoft, Redmond, CA). Statistical analysis for comparing PCR positivity was performed using the Wilcoxon signed-rank test, and *P* values of <0.05 were considered statistically significant.

RESULTS

Patients. According to the revised EORTC/MSG criteria, 20 patients were classified as having proven IFD, six patients as having probable IFD, and 30 patients as having possible IFD; the remaining 23 patients did not fulfill the EORTC/MSG criteria for invasive fungal disease, due to missing host criteria or missing radiological features, and thus were used as the control population. As the focus of the PCR assay described is invasive aspergillosis, IFD patients were classified as suffering from either IA (due to culture results and septate hyphae morphology, dichotomously branched) or non-*Aspergillus* mold infections (culture results and

nonseptate hyphae morphology) according to the EORTC/MSG criteria. This classification identified 21 patients as suffering from proven/probable IA, and the remaining 5 patients were identified by culture as harboring non-*Aspergillus* mold infections (3 *Rhizopus* spp. and 2 *Mucorales* spp.). For determination of IA PCR performance, all proven/probable IA patients (*n* = 21) were evaluated, while the no-IA patients (*n* = 23) acted as the control population. The patients with possible IFD (*n* = 30) were excluded from the analysis of PCR performance, as the true causes of their lesions could not be elucidated.

Positive PCR signals were detected in 31 samples (23 tissue and 8 pleural effusion samples) from 24 patients. Of these, 13 patients were classified as having proven IA, 5 as having probable IA, and 6 as having possible IA; neither patients with the no-IA classification nor patients with non-*Aspergillus* IFD were found to be PCR positive. The PCR positivity rate was significantly higher for patients classified as having proven/probable aspergillosis than for no-IA patients (*P* < 0.001).

Diagnostic performance of PCR in all proven/probable IA cases (*n* = 21) versus no-IA cases (*n* = 23). For patients with proven aspergillosis (*n* = 15) as well as for the 6 patients with probable aspergillosis, PCR was found to be positive in 18/21 patients. For the other patients, positive PCR signals were observed in 6/53 patients. Of these, all six were classified as having possible IA, whereas no positive PCR signal was found for those who were classified as having no IA. Of these 6 cases, all had imaging results consistent with IA; two were patients with pleural effusion samples and four were patients with tissue samples. In these 6 cases, galactomannan (GM) testing had been performed in only 2/6 cases. These tests had negative results, microbiological cultures were found to be negative for all patients, and autopsies had not been performed. However, 4 of these 6 patients significantly improved clinically while receiving mold-active antifungal treatment (AFT).

In the three patients with proven/probable IA and negative PCR results, one patient's case definition was provided by serum GM positivity (albeit in a low range). The other two cases were found to be proven; however, both patients had received intensive antifungal treatment (≥2 antifungals) prior to clinical sampling for PCR. Observed test performance parameters of PCR with all samples for diagnosing IA (comparing proven/probable cases versus no-IA cases) yielded sensitivity and specificity values of 86% (95% confidence interval [CI], 65% to 95%) and 100% (95% CI, 86% to 100%), respectively, with a positive likelihood ratio (PLR) of >200, a negative likelihood ratio (NLR) of 0.14, and a diagnostic odds ratio (DOR) of >200 (∞) (Table 3).

Diagnostic performance of IA PCR in solely proven/probable IA tissue cases (*n* = 18) versus no-IA tissue cases (*n* = 9). Eighteen patients (21 tissue samples) with proven/probable IA were identified; of these, 16 patients (18/21 tissue samples) were found to be PCR positive. Thus, performance parameters for the *Aspergillus*-specific PCR included sensitivity and specificity values of 89% (95% CI, 67% to 97%) and 100% (95% CI, 70% to 100%), respectively, a PLR of >200, an NLR of 0.12, and a DOR of >200 for tissue samples.

Diagnostic performance of PCR in proven/probable IA pleural effusion cases (*n* = 5) versus no-IA pleural effusion cases (*n* = 15). Five patients with proven/probable IA with effusion samples (*n* = 9) were identified; of these, 2/5 patients (5/9 samples) showed positive PCR signals in effusion samples. When these

TABLE 3 Diagnostic performance of PCR

Cases	Case definition vs no-IA cases (<i>n</i>)	% sensitivity (95% CI)	% specificity (95% CI)	PLR ^a	NLR	DOR
All combined	Proven and probable (21)	86 (65–95)	100 (86–100)	>200	0.14	>200 (∞)
Tissue	Proven and probable (18)	89 (67–97)	100 (70–100)	>200	0.12	>200 (∞)
Effusion	Proven and probable (5)	40 (12–77)	100 (80–100)	>200	0.6	>200 (∞)

^a PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic odds ratio.

patients were compared with no-IA patients (*n* = 15), sensitivity was 40% (95% CI, 12% to 77%), specificity was 100% (95% CI, 80% to 100%), the PLR was >200, the NLR was 0.6, and the DOR was found to be >200.

Two of the three PCR-negative patients with proven/probable IA (2 proven and 1 probable) had been treated with ≥ 2 antifungals prior to effusion sample PCR analysis. For one patient, the biopsy providing the proven definition had been performed earlier in the course of the infection; however, the initial biopsy sample had not been sent for PCR analysis. Finally, for one patient who had both tissue and effusion samples, the biopsy sample was PCR positive while the effusion sample was negative.

DISCUSSION

In our analysis of a large number of fresh tissue and pleural effusion samples from patients at risk of IA, we observed excellent diagnostic performance of our nested *Aspergillus* PCR assay for tissue samples, whereas lower performance was observed for effusion samples. Although several authors reported on the feasibility and possibilities of molecular diagnostic testing of tissue samples for diagnosing IA, the majority of these publications deal with FFPE samples and the performance of IA PCR with a number of fresh samples and pleural effusion samples is rarely reported.

Sampling of tissue or surgical removal in order to obtain a positive histological result and the possibility of a positive culture result is still the gold standard for diagnosing IA according to the 2008 revised EORTC/MSG criteria (2), as only these methods provide the recognition of proven aspergillosis. Despite the availability of biomarkers like GM or β -D-glucan, diagnosing IA and obtaining a valid diagnosis still remain an issue. Based on an autopsy study from 1989 to 2003, only 25% of all IFD cases were detected before death (26). However, the authors found antemortem IFD detection to improve over the years, probably due to increased availability of biomarkers. Nevertheless, even a positive culture or GM result from BAL fluid only presents evidence for a probable diagnosis of IA (2), as contamination might be prevalent. Therefore, tissue samples from a sterile site (e.g., samples from a CT-guided thoracic biopsy of an unclear/suspicious lung infiltrate) might enable definite diagnoses to be obtained. This is not without risks, however, especially in the hematological patient population. Gupta et al. reported about the high diagnostic yield of CT-guided percutaneous lung biopsies, which led to therapeutic changes for the majority of patients, but they found pneumothorax to occur in 39% of all procedures, even though nearly all of the patients could be managed on an outpatient basis and no life-threatening complications were observed (4).

Therefore, these procedures might provide a way of identifying the infectious agent and influencing therapeutic choices. However, even when histological results suggest IA involvement, based on the histological detection of dichotomously branched septate hyphae, cultures yield results in only a minority of cases (8). In

addition, a number of fungal species are histomorphologically similar to *Aspergillus*, including *Scedosporium*, *Penicillium*, and *Fusarium*. To make matters even more complicated, it was shown recently that histologically suspected species and culturally identified species differ in the majority of cases (9). Therefore, using a method with higher sensitivity and specificity such as PCR might improve the diagnostic capabilities, especially as often prevalent necrosis can make histological identification of IFD exceptionally difficult for the pathologist.

In our study, for the majority of patients with histological results suggestive of IA, the pathogen could be detected via PCR, underlining the potential of this method. This is important, as PCR results can be obtained rapidly and therefore early diagnosis and timely initiation of directed therapy are possible; this has been shown to confer improved outcomes in IFD (16, 17). Based on our data, PCR testing of these samples is a promising and complementary tool for identification of the underlying causative pathogen, as a positive tissue PCR result makes IA highly likely, whereas a negative result makes it rather unlikely. Interestingly, 2/3 patients with proven IA found to be PCR negative had intensive antifungal treatment (AFT) (≥ 2 antifungal agents) prior to the biopsy conferring the proven case definition. This might explain the negative PCR results, as we and others saw pronounced decreases in PCR sensitivity with intensive AFT administered prior to sampling (27, 28). One histologically positive but culture-negative patient was found to be PCR negative while receiving intensive combination AFT, although the results of prior serum GM testing were positive.

In animal models of IFD, PCR has been shown to be superior to culture and cytopathological examination (29, 30), but clinical data in humans on this topic are rather scarce. A major hurdle when evaluating a new diagnostic tool in IA is that its performance has to be compared with the gold standard, i.e., histological examination based on the EORTC/MSG criteria. However, a recent report (9) evaluated the diagnostic performance of histopathological examination and found a sensitivity of just 78% for that diagnostic tool, suggesting that aspergillosis can present (e.g., diagnosis determined by autopsy or culture) but can be overlooked by histology alone; thus, a positive PCR result might indicate fungal involvement even when histology shows only diffuse necrosis without hyphae. Supporting this finding, Rickerts et al. found that PCR identified the fungal pathogen in samples with hyphae not identified to the species level or even samples without hyphae from patients for whom systemic aspergillosis was highly likely (e.g., culture results for a sample from a different site and clinical improvement with antifungal therapy) (18).

We found positive *Aspergillus* PCR results for six patients classified as having possible IA. The exact ramifications of these findings are not clear: Histology in these cases showed only diffuse necrosis and culture results were negative, yet 4 of 6 patients improved while on antifungal therapy, indicating IFD involvement;

it may be that these patients did suffer from IA. However, based on the low sensitivity of conventional methods (such as microbiological cultures) that define the probability of IA, upgrading these cases according to the EORTC/MSG criteria was not possible. In contrast, we found no positive signal for patients defined as having no IA, based on a lack of host criteria or missing typical radiological abnormalities, underlining the specificity and potential of the PCR approach.

Nonetheless, a negative *Aspergillus* PCR result with suspected mold infection based on histological results does not exclude the presence of molds. Because our PCR assay is specific for *Aspergillus* species, the most prevalent IFD (31), a negative PCR might indicate the involvement of rarer molds (e.g., *Scedosporium* spp.) and could suggest initiation of treatment regimens aimed at such molds. Indeed, we identified two patients with histological results defined by the pathologist as indicating *Aspergillus*, based on non-specific hypha morphology, who were found to be negative with our PCR but for whom culture results identified zygomycosis (*Mucor* and *Rhizopus* spp.). Therefore, rapidly obtained negative *Aspergillus* PCR results for patients for whom IFD is highly probable could still be of therapeutic relevance, as a publication by Chamilos et al. suggests that delayed therapy of mucormycosis has a negative impact on survival rates (17). Based on this fact and the high sensitivity we observed for our *Aspergillus* PCR assay, positive (*Aspergillus*) PCR results in a highly immunocompromised patient population with typical radiological results should be taken seriously and should prompt adequate therapeutic measures even without positive histological or culture results; this is clearly demonstrated by the high diagnostic odds ratio we found. Furthermore, rapid identification of the fungal pathogen may be possible with molecular methods like our DNA microarray (15) or sequencing methods.

In addition to tissue samples, the performance of the *Aspergillus*-specific PCR was evaluated in pleural effusion samples. We found PCR to show positive signals for patients with proven/probable IA; however, none of those patients had a positive result from PE culture. Although this observation is rather preliminary and is based on a small number of patients, it warrants further investigation. The majority of typical IA manifestations are localized subpleurally. A study including several hundred IFD patients found a high prevalence of pleural effusion, in nearly one-half of all IA patients (21). Paradoxically, although PE seems to be this frequent, data on biomarkers in PE are rare. In a recent report, Bonatti et al. found positive *Aspergillus* PCR signals in pleural fluid samples from four patients with culturally proven *Aspergillus* empyema (22). These findings suggest that analyzing pleural fluid for biomarkers such as *Aspergillus* PCR results might be useful, as has been seen for cerebrospinal fluid (12, 32); however, our data show a lower sensitivity for effusion samples than for tissue samples, representing a possible sample error as a dilution effect might be present.

Nucci et al. (7) hypothesized that giving lower weight to the prespecified radiological findings for patients with positive serum GM test results would improve the definition's diagnostic sensitivity. According to our data, the combination of less-nonspecific radiological signs (e.g., effusion) and positive PCR results shows high specificity and therefore a high diagnostic odds ratio. Thus, specific biomarker (PCR) positivity supports an IA diagnosis. However, it has to be kept in mind that this assumption is based on a small number of patients. Biomarker testing of effusion samples

is not yet recommended in the recent European Conference on Infections in Leukemia guidelines (33); therefore, further studies should examine that issue in more detail.

Our study has limitations that need to be addressed. First, our PCR assay has been shown to reliably detect *Aspergillus* species, but it does not detect other molds. Still, as *Aspergillus* species make up more than 90% of all molds detected (31), the majority of invasive mold infections can be detected with our assay. Nevertheless, *Fusarium* or *Scedosporium*, which are supposed to be increasingly recognized (34), are not detected. Second, we recognize that our results on *Aspergillus*-specific PCR performance are based on a rather small cohort of patients. However, this is in line with other studies investigating tissue samples for invasive molds (e.g., see reference 35), especially when taking into account that (i) the majority of patients included in the trial were hematological patients and (ii) fresh samples were analyzed, in contrast to the majority of other studies. Analyzing FFPE samples has the advantage that samples with definite proven aspergillosis defined by positive histological findings can be retrospectively focused on, thus increasing the positive case population and therefore the statistical validity. However, formalin embedding has been shown to decrease the sensitivity of *Aspergillus* PCR techniques (19), and a recent work by Lass-Floerl et al. could clearly demonstrate the higher sensitivity of fresh samples versus archived samples (13), which is underlined by the high sensitivity we found for our tissue samples.

In relation to the additional clinical information gained with a positive PCR result, the additional cost of approximately \$40 per sample seems acceptable, and inclusion of this technique in daily routine analysis is feasible. In summary, analyzing fresh tissue samples with the *Aspergillus*-specific PCR in addition to conventional methods is a promising approach and might elucidate the underlying pathogen, as a positive result is highly suggestive of IA and should prompt adequate therapeutic measures even when culture or histological examinations are without definite results.

ACKNOWLEDGMENTS

We are indebted to Handan Moerz, Jasmin Ruebel, Natalia Merker, and Silke Will for excellent laboratory technical assistance.

C. P. Heussel has received lecture and consultation fees from Schering-Plough, Pfizer, Boehringer Ingelheim, Novartis, Roche, Astellas, Gilead, Merck Sharp & Dohme, Lilly, Intermune, Fresenius, Essex, AstraZeneca, Bracco, MEDA Pharma, Intermune, Chiesi, Siemens, Covidien, Pierre Fabre, and Grifols, as well as research funding from Siemens, Pfizer, and MeVis. The other authors report no conflicts of interest.

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