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Diagnosis of Invasive Aspergillosis by a Commercial Real-Time PCR Assay for *Aspergillus* DNA in Bronchoalveolar Lavage Fluid Samples from High-Risk Patients Compared to a Galactomannan Enzyme Immunoassay▽

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Culture-independent molecular techniques such as real-time PCRs offer the potential for early diagnosis of invasive aspergillosis (IA), thereby reducing the disease-associated mortality rate. PCR-based testing is presently excluded from disease-defining consensus criteria due to lack of standardization and clinical validation. A single-center prospective study was conducted to investigate the performance of the commercially available MycAssay *Aspergillus* test for detecting *Aspergillus* DNA in patients with suspicion of IA. To this end, a total of 158 bronchoalveolar lavage (BAL) fluid specimens that were consecutively collected from hematology ($n = 68$) and intensive care unit ($n = 90$) patients were examined. Sixteen of 17 (94.1%) specimens from patients with proven/probable IA were MycAssay positive, and 15 of these 16 patients were also positive by an "in-house" PCR assay. A total of 139 of 141 (98.6%) specimens from patients without proven/probable IA were MycAssay negative. Fifteen of 16 (94.1%) MycAssay-positive patients were also positive for BAL fluid galactomannan (GM) at an index cutoff of ≥1.0 (index range, 1.1 to 8.3), as were 3 patients without IA but with pulmonary fusariosis. Interestingly, in seven of the PCR-positive BAL specimens that tested culture positive for *Aspergillus* species, cycle threshold values were earlier than those of specimens with a culture-negative result. In conclusion, the MycAssay *Aspergillus* PCR appears to be a sensitive and specific molecular test for the diagnosis of IA, and its performance is comparable to that of the GM assay. However, more large studies are necessary to firmly establish its clinical utility in high-risk settings.

Invasive aspergillosis (IA) remains a major cause of morbidity and mortality in immunosuppressed patients, such as subjects undergoing bone marrow or solid organ transplantation and those under treatment for hematological malignancies (23, 36). In addition to affecting traditionally susceptible patients, IA has become important in other hospitalized patients, such as those with chronic obstructive pulmonary disease (COPD), liver cirrhosis, or taking corticosteroids (30).

The diagnosis of IA is challenging due to a lack of sensitive and specific clinical and radiological signs, and tissue biopsy is often precluded (and invasive), especially among patients with thrombocytopenia. In addition, culture and microscopy of respiratory tract samples are relatively insensitive diagnostic tests (17). As early detection of IA is essential to defining specific therapeutic strategies (39) and improved patient outcomes, diagnostic assays targeting fungal biomarkers have been developed (7) that offer the potential for new paradigms in prevention and early treatment of IA (1). Among them, circulating galactomannan (GM) in serum and bronchoalveolar lavage (BAL) fluid (15, 37, 38) and (1→3)-β-D-glucan in serum (29) may be useful diagnostic adjuncts for IA, but false-positive and false-negative results remain problematic with both assays (41), which is also compounded by the limited knowledge of the disease process (43).

Over the last 2 decades, nucleic acid detection by PCR has emerged as a promising tool for early diagnosis of IA (6, 11, 20). Despite a variety of "in-house" specific primers and conditions reported in published studies (32, 42), the lack of clinical validation of the laboratory-developed assays has prevented the inclusion of *Aspergillus* PCR in the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) disease-defining consensus criteria (10), unlike GM and (1→3)-β-D-glucan. In this context, it should be noted that the European *Aspergillus* PCR Initiative (EAPCRI) working group was formed in 2006 under the auspices of the International Society for Human and Animal Mycoses, with the aim of providing optimal standardized protocols for universal acceptance of PCR diagnosis of IA (43).

In the present study, a single-center prospective evaluation was conducted to assess the reliability of the commercially available MycAssay *Aspergillus* PCR for detection of *Aspergillus* DNA in BAL specimens from hematology and intensive care unit (ICU) patients at risk for IA. PCR results were compared with clinical diagnosis and conventional diagnostic tests, such as culture and enzyme-linked immunosorbent assay (ELISA) detection of GM.

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MATERIALS AND METHODS

Specific equipment utilized. To conduct this study, the following equipment was required (provided by Myconostica, Ltd., Manchester, United Kingdom, unless indicated otherwise): a SmartCycler real-time PCR platform (Cepheid, Sunnyvale, CA), a Genie 2 vortex (Scientific Industries, NY), a vortex adaptor plate (Myconostica, Ltd.) to attach tubes to the vortex, a dedicated minicentrifuge, and micropipettes (40 to 1,000 μl) with sterile filter tips.

Clinical setting and materials. Between May 2010 and April 2011, 68 hematology and 90 ICU patients with a clinical syndrome compatible with pneumonia were enrolled if they had at least two of the following criteria: fever refractory to at least 3 days of broad-spectrum antibiotics, pleuritic chest pain or physical finding of pleural rub, pulmonary infiltrates, or dyspnea. In addition, at least one host factor (hematological malignancy, COPD, cirrhosis, cancer receiving chemotherapy, solid organ transplant recipient, HIV positive, steroid use, or recipient of T-cell immunosuppressant) was required. The local institutional review committee approved the study, and informed consent was waived because of the observational nature of this study. Upon inclusion, all patients underwent a standard bronchoscopy with BAL (15), to yield a total of 158 BAL fluid specimens that were processed and then tested as described below.

Patients were classified as having proven, probable, or possible IA, on the basis of the revised EORTC/MSG case definitions (10), with the modification that cirrhosis, COPD, and steroids were added to the host factor section (30, 31) by clinicians who were blinded to both the BAL MycAssay *Aspergillus* and GM results. This is because EORTC/MSG categories of probable and possible invasive fungal infection (IFI) are proposed for immunocompromised patients only (10). Proven IA was diagnosed if patients had histopathological evidence of tissue invasion by septated, acutely branching filamentous fungi or the isolation of *Aspergillus* species from a normally sterile and clinically or radiologically abnormal body site consistent with an infectious disease process. Probable IA was considered the presence of a host factor, clinical features for lower respiratory tract fungal disease (dense, well-circumscribed lesions with or without a halo, air-crescent sign, or cavity on chest computed tomography [CT]), and mycological evidence of *Aspergillus* infection (by cytology, direct microscopy, culture, or serum GM). For ICU patients, clinical criteria also included symptoms of lower respiratory tract infection (e.g., dyspnea), pleural rub, and new infiltrate without an alternative diagnosis (31). The same criteria for probable IA were used to classify as probable IFI cases due to non-*Aspergillus* mold (e.g., *Fusarium* species). Possible IA was defined by the presence of a host factor and a clinical criterion but without mycological support. Detection of GM in BAL fluid was not included as one of the microbiological criteria (10). To enhance the robustness of the analysis, only patients with proven or probable IAs were evaluated as IA cases.

Processing of BAL fluid specimens. Following receipt in the mycology laboratory, BAL fluid specimens were subdivided into aliquots within 1 h of collection after being shaken to ensure their homogeneous mixing. One of them (5 ml) was centrifuged, and the suspended pellet (0.5 ml) was directly inoculated on conventional fungal medium (Sabouraud dextrose agar [SDA] supplemented with chloramphenicol [40 μg/ml] and blood agar), and incubated at 37°C (only blood agar plates) for 48 h and at 30°C for another 12 days. Fungal isolates grown from cultures were identified by morphological and/or molecular methods (2, 8). For direct microscopic examination, a smear from each specimen was made and subjected to a Gomori stain to detect hyphal elements. Two other BAL fluid aliquots were stored at −80°C until DNA extraction and GM ELISA detection were separately performed in batches.

DNA extraction from BAL fluid specimens. Total genomic DNA was obtained from clinical specimens as follows. A minimum of 2 ml of frozen BAL fluid specimen was processed using the MycXtra fungal DNA extraction kit (Myconostica, Ltd.), which utilizes a mechanical disruption step, and in the case of viscous specimens, the processing was preceded by an *N*-acetyl-cysteine–sodium hydroxide treatment. DNA extracts were frozen at −20°C until batch PCR testing was performed.

MycAssay *Aspergillus* PCR. A commercially available *in vitro* diagnostic test, MycAssay *Aspergillus* (Myconostica, Ltd.), was developed for the detection of genomic DNA from 15 different *Aspergillus* species, including the 4 most frequent pathogenic species (i.e., *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger*). This test, which is based on the molecular beacon real-time PCR technology targeting the multicopy 18S rRNA gene (21), includes an internal amplification control (IAC) sequence of plant origin to detect any PCR inhibitory substances in the sample (9). Briefly, PCR assays were performed on the Cepheid SmartCycler apparatus with 10 μl of DNA template in a final reaction volume of 25 μl, according to the manufacturer's instructions (35). PCR positivity was determined using a threshold of 36 cycles that corresponds to a target sensitivity of ≤50 18S rRNA copies, which is approximately equivalent to 1 *A. fumigatus* genome (9).

In-house *Aspergillus* PCR assay. DNA extracts from all BAL specimens were also tested using an iCycler iQ real-time PCR assay, previously developed for the quantitative detection of *Aspergillus*-specific DNA (40), which amplifies a 124-bp sequence in a conserved region of the 18S rRNA genes of *A. fumigatus*, *A. flavus*, *Aspergillus glaucus*, *A. niger*, and *A. terreus*. Briefly, PCRs were carried out in a 50-μl volume containing 10 μl of DNA sample, primers (Asp-fwd, 5′-CCG ATT ACG TCC CTG CCC TT; and Asp-rev, 5′-TTG ACC AAC TTT CCG GCT CTG), a 5′-6-carboxyfluorescein (5′-FAM)- and 3′-TAMRA (6-carboxytetramethylrhodamine)-labeled TaqMan probe (5′-ACA CAC CGC CCG TCG CTA CTA CCG), and 25 μl of the Platinum-quantitative PCR (qPCR) supermix-UDG (Invitrogen, San Diego, CA) and using thermal conditions already described (40). Each sample was tested in triplicate, and the results were averaged to obtain the final cycle threshold (C_T)—i.e., the point at which sample fluorescence rises above the background level. A standard curve constructed by plotting the log of starting concentration of pCR2 TA plasmid, which harbors the *A. fumigatus* 124-bp PCR fragment, versus the C_T was used to determine *Aspergillus* DNA concentrations in specimens (40).

GM Platelia *Aspergillus* assay. The sandwich ELISA for BAL GM detection (Platelia *Aspergillus*; Bio-Rad, Marnes-la-Coquette, France) was performed according to the manufacturer's recommendations for testing serum specimens. As cleared by the FDA in the United States, the results were analyzed using an optical density (OD) index cutoff value of 0.5. However, a positive specimen was defined as one with an OD index of ≥1.0, based on recommendations published recently (7).

Statistical analysis. The sensitivity, specificity, positive predictive values (PPVs), negative predictive values (NPVs), likelihood ratios, and diagnostic odds ratios (DORs), along with their associated 95% confidence intervals (CIs), were calculated according to their definitions. Sensitivity was also assessed for BAL fluid culture and compared to that of BAL PCR for patients with IA, using Fisher's exact test. A paired *t* test was used to compare the MycAssay *Aspergillus* C_T values and GM indexes from BAL fluid specimens between patient subgroups. *P* values of <0.05 were considered significant.

RESULTS

A BAL fluid sample obtained from each of 158 hematology or ICU patients at risk of IA was tested with both MycAssay *Aspergillus* and in-house real-time PCR assays to reveal the presence of *Aspergillus* DNA and with ELISA to detect GM antigen. As shown in Table 1, 17 patients were diagnosed as having proven ($n = 2$) or probable ($n = 15$) IA. Eight patients for whom a diagnosis of possible IA was made were included in the group of no-IA patients ($n = 141$). Twenty-six of 68 (38.2%) hematology patients, 3 of whom had probable IA, were empirically treated with mold-active antifungal therapy (including polyenes and echinocandins) before BAL sample collection, for a median duration of 6 days (range, 2 to 15).

The performance of PCR and GM assays for the diagnosis of IA is shown in Table 2, in which test results from 17 patients with proven or probable IA were compared to those of 141 no-IA patients. The MycAssay *Aspergillus* PCR demonstrated a numerically (but not statistically) higher sensitivity than the in-house PCR assay, while being comparable to that of the GM enzyme immunoassay (94.1%). Sixteen of 17 patients were PCR positive in BAL fluid when tested by the MycAssay *Aspergillus* PCR, and 15 of these 16 patients were also positive by the in-house PCR assay. The patient with a false-negative MycAssay result was also negative by the other PCR assay. Likewise, the BAL GM OD indexes were ≥1 (range, 1.1 to 8.3) in 16 of 17 patients with IA and 0.4 in the remaining patient. Only 2 and 3 patients (the latter diagnosed with pulmonary fusariosis)

TABLE 1. Clinical characteristics, radiological findings, and results of GM and PCR assays for 17 patients with proven and probable IA

Patient no.	Clinical setting	IA diagnosis[a]	Underlying disease/host factor(*s*)[b]	CT finding(s)	BAL fluid result		GM OD index		C_T result	
					Microscopy	Culture	Serum	BAL fluid[c]	MycAssay[d]	In-house PCR
1	ICU	Probable	Cirrhosis	Nodules	Negative	Negative	1.2	1.8	Positive (33.5)	Positive (34.2)
2	HEM	Probable	AML	Nodules	Positive	Positive (*A. fumigatus*)	2.1	5.5	Positive (25.4)	Positive (27.2)
3	HEM	Probable	ALL	Air crescent sign	Negative	Negative	0.9	2.2	Positive (32.5)	Positive (33.4)
4	HEM	Probable	AML	Halo	Negative	Negative	0.8	0.4	Positive (32.5)	Positive (35.1)
5	ICU	Probable	Steroids	Pleural effusion	Negative	Negative	1.8	3.2	Positive (31.5)	Positive (30.9)
6	ICU	Probable	Steroids, COPD	NA[e]	Positive	Negative	2.2	2.9	Positive (30.9)	Positive (31.8)
7	HEM	Probable	AML	Nodules	Negative	Negative	1.5	2.3	Positive (33.3)	Negative
8	HEM	Probable	ALL	Air crescent sign	Positive	Positive (*A. fumigatus*)	3.5	6.3	Positive (24.4)	Positive (26.0)
9	HEM	Probable	AML	Nodules, halo	Positive	Positive (*A. fumigatus*)	2.9	7.0	Positive (27.2)	Positive (27.9)
10	HEM	Proven[f]	AML	Halo	Positive	Positive (*A. flavus*)	1.8	8.3	Positive (21.5)	Positive (24.1)
11	HEM	Probable	NHL	Nodules	Negative	Negative	0.8	1.9	Positive (29.8)	Positive (30.2)
12	HEM	Probable	ALL	Nodules	Positive	Positive (*A. fumigatus*)	1.3	4.2	Positive (25.9)	Positive (27.1)
13	ICU	Proven[g]	Solid cancer	Necrotizing lesions	Positive	Positive (*A. fumigatus*)	2.0	4.4	Positive (22.1)	Positive (24.3)
14	HEM	Probable	NHL	Halo	Negative	Negative	1.9	3.5	Positive (27.1)	Positive (28.1)
15	HEM	Probable	AML	Halo	Negative	Negative	0.9	2.6	Positive (29.0)	Positive (31.1)
16	HEM	Probable	AML	Nodules	Negative	Positive (*A. fumigatus*)	0.8	4.2	Positive (26.3)	Positive (28.1)
17	HEM	Probable	NHL	Nodules	Negative	Negative	0.8	1.1	Negative (37.2)	Negative

[a] IA was diagnosed according to the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group criteria from 2002 (10).
[b] AML, acute myeloblastic leukemia; ALL, acute lymphoblastic leukemia; COPD, chronic obstructive pulmonary disease; NHL, non-Hodgkin's lymphoma.
[c] An optical density (OD) index of ≥1.0 was used as a cutoff value for positive BAL GM (7, 27).
[d] A threshold of 36 cycles corresponding to a target sensitivity of ≤50 18S rRNA copies was used as a cutoff value for positive BAL PCR (9).
[e] NA, not available.
[f] As defined by positive histopathology of lung biopsy.
[g] As defined by positive culture of pleural fluid drain.

had BAL specimens that tested false positive by the MycAssay *Aspergillus* PCR and GM ELISA (index range, 1.1 to 1.7), respectively, giving assay specificities of 98.6% and 97.9% (Table 2). Using an OD index cutoff of ≥0.5, eight patients tested false positive for GM, with an assay specificity that decreased to 92.2% (Table 2). The diagnostic odds ratios (DORs) were typical of *Aspergillus* PCR assays used to test BAL fluid specimens (42). The statistical performance of the MycAssay *Aspergillus* PCR was calculated using the possible cases also as true positives, thereby resulting in a sensitivity, specificity, positive predictive value, and negative predictive value of 68%, 99.3%, 94.4%, and 94.3%, respectively.

Overall, 15 IA patients who were BAL GM positive tested positive by MycAssay *Aspergillus* PCR. One IA patient with a negative BAL PCR result tested positive for BAL GM; conversely, another IA patient who tested negative for BAL GM was PCR positive. As detailed in Table 1, the MycAssay *Aspergillus* test allowed the detection of *Aspergillus* DNA in BAL specimens from the 16 patients with IA, generating C_T values that ranged from 21.5 to 33.5 (mean, 28.3 cycles). Among these 16 patients, *Aspergillus* was isolated in BAL specimens from 7 patients (sensitivity for IA, 41.2%; $P = 0.002$). *Aspergillus* species were identified by culture as *Aspergillus fumigatus* in 6 patients and *Aspergillus flavus* in 1 patient. Interestingly, the mean C_T values for the 16 PCR-positive specimens were 24.6 cycles for patients with a culture-positive BAL result and 31.8 cycles for patients with a culture-negative BAL result (difference, 7.2; $P < 0.001$). Patients with C_T values of ≤27 had the

TABLE 2. Performances of diagnostic tests with 158 specimens of BAL fluid

Parameter for proven and probable IA vs no IA[a]	Test result (95% CI) [no. of specimens with result/no. tested] by:			
	MycAssay *Aspergillus* PCR	In-house *Aspergillus* PCR	GM ELISA	
			Index cutoff, ≥1.0	Index cutoff, ≥0.5
Sensitivity (%)	94.1 (71–99) [16/17]	88.2 (63–98) [15/17]	94.1 (71–99) [16/17]	94.1 (71–99) [16/17]
Specificity (%)	98.6 (94–99) [139/141]	97.9 (93–99) [138/141]	97.9 (93–99) [138/141]	92.2 (86–96) [130/141]
Likelihood ratio[b]				
Positive	67.2	42.0	44.8	12.1
Negative	0.06	0.12	0.06	0.06
PPV (%)	88.9 (65–98) [16/18]	83.3 (58–96) [15/18]	84.2 (60–96) [16/19]	59.3 (38–77) [16/27]
NPV (%)	99.3 (96–99) [139/140]	98.6 (94–99) [138/140]	99.3 (96–99) [138/139]	99.3 (95–99) [130/131]
DOR[c]	1120.0	350.0	746.6	201.6

[a] Of 158 patients studied, 17 were diagnosed with proven or probable IA according to the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group criteria from 2002 (10).
[b] Likelihood ratio positive, sensitivity/1 − specificity; likelihood ratio negative, 1 − sensitivity/specificity.
[c] DOR, diagnostic odds ratio (likelihood ratio positive/likelihood ratio negative).

highest GM OD index values (range, from 8.3 to 4.2) in their BAL fluid ($P < 0.001$). With the exception of 3 patients with fusariosis, none of 141 patients without proven or probable IA gave BAL cultures positive for *Aspergillus* or other fungi.

DISCUSSION

As culture of the fluid collected during BAL typically has a low sensitivity (17, 45), evaluation of the performance of newer molecular and antigen techniques requires alternative diagnostic information, which differs according to the type of specimen analyzed (i.e., blood, serum, or BAL fluid). Although bronchoscopy is an "invasive" procedure for obtaining specimens (42), BAL is a direct sampling of the affected organ and is therefore important in the diagnosis of pulmonary fungal infections (22). Rationally, detection of fungal markers in the lungs (i.e., at the site of the disease) might be more sensitive than that in the serum (4), and in particular, serum GM levels may be lower in patients with airway IA than in those with angioinvasive aspergillosis (16). Also, *Aspergillus* DNAemia is probably only transient, and the most suitable time point for blood sampling for PCR has yet to be defined (18).

Antigen detection in BAL for the diagnosis of aspergillosis was recently confirmed in multiple reports (for a review, see reference 15 by Hage et al. and the references therein) and a meta-analysis (13). In one of these studies evaluating patients with hematological disorders, GM positivity was the only microbiological finding for IA on BAL samples from all 20 case patients studied by Maertens et al. (27), as opposed to 2 of 33 IA patients studied by Bergeron et al. (4), in whom positive GM BAL was the sole microbiological criterion. Additionally, detection of *Aspergillus* DNA in BAL fluid by quantitative real-time PCR has been used to assist in the diagnosis of IA in many institution-based approaches, yielding high sensitivity (66 to 100%) and specificity (75 to 100%) (20, 42).

This prospective study represents the first evaluation of a commercial PCR test, MycAssay *Aspergillus*, with BAL fluid from a mixed population of patients with underlying hematological or critical illness (89.9% were neutropenic). Using a C_T cutoff of $\leq$36, we found that 16 of the 17 patients with proven or probable IA had a positive BAL PCR result, whereas this was the case for only 2 (1.4%) of the 141 patients at risk who had no evidence of IA. The negative predictive value was 99.3%, with corresponding sensitivity of 94.1% and specificity of 98.5%. The performance of this PCR assay compared favorably with that in other studies using the MycAssay *Aspergillus* PCR to test serum (44) or respiratory tract (9) specimens, yielding high likelihood ratios of probable or proven IA for a positive test result. It was also superior to that shown by an in-house PCR assay (40), but not at a statistically significant level (Table 2), or to that reported in a systematic review of PCR assays (42). Similarly, with an index cutoff of $\geq$1, BAL GM testing showed good sensitivity (94.1%) and specificity (97.9%). For IA, the concordance between BAL PCR and GM was 93% (15 of 16 results); one case of IA was detected with PCR but missed with GM, and another one was GM positive but PCR negative. In line with our previous study (40), and as with others (17, 27, 31), the performance of fungal culture on BAL fluid was only moderate for the diagnosis of IA (sensitivity, 41.2%). Conversely, Khot et al. (21) found that BAL fluid culture was more efficient than quantitative PCR in detecting IA, showing sensitivity and specificity of 84.6% and 100%, respectively.

Despite differences in patient populations and study design, our findings agreed with those reported more recently by Nguyen's group (25), who compared the performance of a previously published *Aspergillus* real-time PCR assay with that of the Platelia GM assay (cutoff used, $\geq$0.5) for 150 BAL specimens from lung transplant recipients, including 16 proven or probable cases of IA and 26 cases of *Aspergillus* colonization. While in that study *Aspergillus* PCR identified 1 patient with IA not diagnosed by GM, it was worth noting that, in addition to show 100% of sensitivity in detecting IA, the PCR assay was positive in 50% (13 of 26) of BAL fluid samples colonized with *Aspergillus*, probably reflecting higher fungal burdens from IA patients (12) or the DNA extraction method used being much more efficient for germlings than conidia, which are known to predominate during colonization (25). Also, for those BAL specimens associated with *Aspergillus* colonization, the specificity of GM was higher (92%) than that of the PCR assay (50%). Consistent with these results, BAL GM was negative in hematological patients who were colonized with *Aspergillus* species (4). Nevertheless, as false positivity caused by *Aspergillus* airway colonization and antibiotic therapies complicates the use of the GM assay for diagnosis, we share the opinion that these test results should always be interpreted as part of a full assessment, including clinical and imaging findings (27), or rather should prompt additional investigation to exclude invasive disease, especially in solid organ transplant or hematology patients. In support of this, the search for circulating *Aspergillus* DNA in the first GM-positive result was seen to improve the specificity of the first GM-positive assay result for diagnosis of IA in liver transplant recipients (5).

In addition to clinical factors (e.g., *Aspergillus* colonization), several procedural factors (e.g., due to *Aspergillus* contamination from the environment, fungal PCR product carryover, and cross-reactivity of primers and probes with sequences from non-*Aspergillus* fungi) can be responsible for false-positive results (20, 34). The two false-positive PCR tests in our study were associated with *Penicillium* colonization. In fact, as a result of the close phylogenetic relationship between *Penicillium* and *Aspergillus* (17), the MycAssay *Aspergillus* PCR shows significant cross-reactivity with most species of *Penicillium*, which are rarely involved in opportunistic infections in humans (26). In contrast, although GM can cross-react more frequently with antigens from *Penicillium* or *Paecilomyces* (15), we detected false-positive GM results in 3 patients with BAL fluid culture positive for *Fusarium* (and subsequently diagnosed with pulmonary fusariosis), according to a study described elsewhere (19).

Prior treatment with antifungal agents, a known cause of false-negative antigenemia (3, 28, 33), may also account for false-negative antigen results in BAL fluid (19). Lower sensitivity rates of PCR with blood samples during antifungal treatment have also been reported in both previous and recent studies (18, 24), but at the current time, the impact of antifungal therapy in terms of the recovery of *Aspergillus* and related markers in the lung remains to be clearly defined (17). False-negative results were documented in only two of our patients.

One patient with a negative BAL GM was receiving antifungal treatment at the time of BAL, in spite of 6 patients who had a positive result in both the BAL GM and MycAssay *Aspergillus* tests but were empirically treated with mold-active agents. Similarly, Meersseman et al. (31) found positive BAL GM in 13 of 14 patients with proven IA who were receiving antifungal therapy, and, interestingly, 3 of these 14 patients had negative serum GM. For our other patient, the cause of a false-negative PCR result could not be determined.

It is important to keep in mind that more accurate comparisons among different assay platforms should be possible if investigators in the field would adopt standards for ruling out false-positive and false-negative results, which in certain populations are the main limitations to their use. As recently reviewed by White et al. (43), the efficiency of the *Aspergillus* PCR is limited by the extraction procedure (i.e., low recovery of DNA and/or the presence of PCR inhibitors) and not by PCR amplification. Although real-time PCR should be preferred to conventional or nested formats in terms of workability in the clinical laboratory setting (14), an optimal quantitative PCR assay for the detection of IA in BAL specimens requires a concentrated BAL fluid pellet fraction to be extracted to ensure efficient recovery of the cell-associated *Aspergillus* DNA and an IAC to exclude inhibition as a cause for false negativity (21). In our study, the IAC, included in the MycAssay *Aspergillus* and properly designed to not affect the analytical sensitivity of the PCR assay target, allowed us to detect inhibition in 8.2% (13 of 158) of the BAL samples, but reextraction of DNA eliminated PCR inhibition in all samples without significant losses of DNA (as confirmed by again positive amplification of the human β-globin gene) (data not shown).

In conclusion, we showed that a single PCR-positive result in BAL fluid is associated with a high probability of disease, even though the sensitivity of detecting IA approaches 100% when MycAssay *Aspergillus* PCR is conjunctively used with the BAL GM assay, whereas a single PCR-negative result in BAL fluid may be sufficient to rule out the diagnosis of IA at that particular time. However, large-scale evaluations of the MycAssay *Aspergillus* PCR would be needed to define the true potential of PCR for the diagnosis of IA and, ultimately, incorporation into the standard BAL fluid analysis for high-risk patients.

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