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Multicenter Comparison of Serum and Whole-Blood Specimens for Detection of *Aspergillus* DNA in High-Risk Hematological Patients

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Samples from patients at high risk for invasive aspergillosis (IA) were prospectively collected and analyzed for the presence of molecular markers of fungal infection. Serum specimens were screened for galactomannan and *Aspergillus* DNA, and whole-blood specimens were screened only for *Aspergillus* DNA. Fungal infections were categorized according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria. Forty-seven cases (proven and probable IA) and 31 controls (no evidence of IA) were selected retrospectively for this case-control study, comprising 803 samples, in order to determine the performance of whole-blood PCR, serum PCR, and serum galactomannan testing. Although no single assay was able to detect every case of IA, a combination of different assays provided the best performance. There was no significant difference between the use of whole-blood and serum specimens for PCR-based diagnosis of IA, but there was a trend for whole blood to be more sensitive (85% versus 79%) and to yield an earlier positive result (36 days versus 15 days) than for serum. However, DNA extraction from serum specimens is easier and faster than that from whole-blood specimens, and it allows the same specimen to be used for both galactomannan and PCR assays. In conclusion, the appropriate sample type for DNA extraction should be determined by the local requirements and the technical platforms available at each individual center. A combination of biomarker tests offered the best diagnostic utility for detecting IA.

Invasive aspergillosis (IA) is a major complication in immunocompromised patients, particularly individuals with acute leukemia or those receiving allogeneic stem cell transplantation (1). Mortality rates remain high, at up to 89% (2, 3); this is linked to difficulties in diagnosing IA due to nonspecific and late clinical signs and to the insensitivities of conventional laboratory diagnosis methods (4, 5). Consequently, empirical therapy is frequently used, at a great cost, and it exposes patients to unnecessary drug side effects and toxicity. Early diagnosis is paramount, and sensitive molecular assays have the potential to improve diagnosis and patient outcome by providing alternative preemptive strategies.

PCR testing can be used as a screening tool to exclude IA (6). Therefore, high-frequency sampling is required, which favors the use of easily obtainable specimen types, such as blood. The fungal load is very limited in blood, and a high analytical sensitivity is essential for reducing the number of false-negative results (7, 8).

Different blood fractions have been evaluated for molecular tests, but direct comparisons of PCR performance are limited. DNA extraction from whole blood (WB) is technically demanding, requiring greater standardization than DNA extraction from serum (or plasma). Guidelines for DNA extraction from WB and serum specimens and for DNA detection were published by the European *Aspergillus* PCR Initiative (EAPCRI) (9, 10). Extraction efficacy was influenced by sample type, the amount of clinical material, and the protocol used (11).

The exact origin of *Aspergillus* DNA in blood is unclear since the fungus is rarely recovered from the bloodstream in cases of IA. Recent studies in murine models of IA and *in vitro* have indicated that cell-free fungal DNA is released by actively growing mycelia (12). Additionally, cell-free fungal DNA in blood might be a by-product of phagocytosis or the result of antifungal therapy. Cell-bound fungal DNA is thought to be linked to free or phagocytosed hyphal elements in the circulation (13).

Despite the existence of standardized methodologies for testing both serum and WB specimens, there have been only limited comparisons of their performance. In this case-control study, we describe a multicenter approach (University Hospitals of Cardiff and Wuerzburg and St. James's Hospital Dublin) comparing DNA detection of *Aspergillus* in serum and WB specimens obtained from patients at high risk for IA. Both specimen types were collected in parallel; WB was analyzed prospectively by PCR, whereas serum was prospectively tested by galactomannan (GM) enzyme-linked immunosorbent assay (ELISA) and stored at −20°C prior to retrospective serum PCR testing. Patients were selected as either cases (diagnosed with proven or probable IA categorized according to the current European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, National Institute of Allergy and Infectious Diseases Mycoses Study Group [EORTC/MSG] criteria [14]) or controls (categorized as no evidence of fungal disease). All DNA

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extraction methods were compliant with published EAPCRI recommendations (9, 10).

MATERIALS AND METHODS

Study design. Allogeneic stem cell transplant recipients (alloSCT) and patients receiving myelosuppressive chemotherapy with an expected duration of ≥10 days of neutropenia (leukocyte count, <1,000/μl) were included in the study. Between 2006 and 2011, blood and serum samples were taken twice weekly from patients at high risk for invasive fungal disease. Signs and symptoms of IA were collected together with other microbiological data from patient charts in order to categorize the onset and type of fungal infection according to the revised EORTC/MSG criteria (14). WB specimens were analyzed prospectively. In parallel, serum samples were used for galactomannan quantification. PCR results were not subject to EORTC/MSG classification.

In accordance with EORTC/MSG classification criteria, cases (proven or probable IA) and controls (unclassified patients with no signs of IA) were retrospectively selected. Frozen samples from these patients were thawed and used to extract DNA from serum.

The study was approved by the local ethics committees of the University Hospitals of Wuerzburg and St. James's Hospital Dublin. In Cardiff, samples were prospectively tested as part of routine diagnostic care. PCR testing of serum samples was performed as a retrospective anonymous evaluation of the diagnostic service and needed no ethical approval. Results from all assays were anonymized by the referring center prior to retrospective testing.

DNA extraction from WB. All DNA extraction steps were performed in a class II laminar-flow cabinet and were compliant with EAPCRI recommendations (9).

WB was extracted as described previously (15, 16). Briefly, red and white cells from 3 ml of EDTA blood were lysed and centrifuged. The pellet was bead beaten to lyse fungal cells. DNA was isolated by a commercially available kit (High Pure PCR template preparation kit; Roche). The elution volume was 100 μl in Wuerzburg and 65 μl in Dublin and Cardiff. In each extraction procedure, at least one negative control was included. In Cardiff and Dublin, positive extraction controls were also included.

DNA extraction from serum. All steps for extracting serum from DNA were performed in a class II laminar-flow cabinet and were compliant with EAPCRI recommendations (10).

In Wuerzburg and Dublin, DNA from 1 ml of serum was extracted using the QIAamp UltraSens virus kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The elution volume was adjusted to 35 μl. In each extraction procedure, at least one negative control was included. Extraction performance was monitored by testing both blinded and known simulated positive-control specimens.

In Cardiff, 0.5 ml of serum was used. DNA was purified by using the High Pure PCR template preparation kit (Roche) (17). Briefly, 0.5 ml of serum was mixed with 0.4 ml of binding buffer and 80 μl of recombinant proteinase K (Roche), and the mixture was incubated at 70°C for 10 min. Two hundred microliters of isopropanol was added and mixed by pipette before the entire specimen was applied to the spin column by centrifugation at 8,000 × *g* for 1 min. Other than these modifications, the kit was used in accordance with the manufacturer's instructions, using an elution volume of 65 μl. Positive and negative serum extraction controls were included in every run.

DNA amplification and detection. All PCR methods were validated by testing different EAPCRI panels and showed comparable performance in detecting the thresholds set by the EAPCRI (9, 10).

In Wuerzburg, an *Aspergillus*-specific real-time PCR assay targeting the internal transcribed spacer 1 (ITS1)-5.8S rRNA gene region (16, 18) was used to detect fungal DNA. Briefly, 21-μl reaction mixtures contained 0.3 μM primer Asp fum_F degen, 0.6 μM primer Fungi 5.8_R, 0.15 μM hydrolysis probe ITS-PF, 10 μl TaqMan gene expression master mix (Applied Biosystems), and 10 μl template DNA. Asp fum_F degen was used instead of Asp fum_F to increase sensitivity for non-*fumigatus* species of *Aspergillus*.

Amplification was carried out in a StepOnePlus machine (Applied Biosystems) with the following steps: 50°C for 2 min, 95°C for 10 min, and 55 cycles of 95°C for 15 s, 54°C for 30 s, and 72°C for 30 s. Negative and positive PCR controls were included in each run. Samples were analyzed in triplicate (WB) or duplicate (serum). PCR efficiency was 92.5% for *A. fumigatus*, 90.3% for *A. terreus*, and 90.1% for *A. flavus*. No cross-reactivity with other fungi or human genomic DNA was observed (18). This assay detected all clinically relevant *Aspergillus* species (16).

The assay in Dublin was a modified version of the previously described protocol of Wuerzburg (16). Briefly, 20-μl reaction mixtures contained 0.25 μM primer Asp fum_F, 0.25 μM primer Fungi5.8_R, 0.375 μM hydrolysis probe ITS-PF, 10 μl TaqMan gene expression master mix (Applied Biosystems), and 7 μl template DNA. Amplification was carried out on a 7500 real-time PCR machine (Applied Biosystems) with the following steps: 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 30 s). Sensitivity was 94% for *A. fumigatus*, which was the only *Aspergillus* species detected. No cross-reactivity was observed for other fungi and human DNA (12, 16, 19).

In Cardiff, the *Aspergillus* real-time PCR test was performed as a single-round assay using a Corbett Rotor-Gene 3000 instrument targeting the 28S rRNA gene, modified to allow greater template DNA input (15 μl), in a final reaction mixture volume of 50 μl (6). Extraction controls, PCR controls in the form of cloned PCR products (300, 30, and 3 input copies), and no-template molecular-grade water were included to monitor PCR performance. PCR positivity was determined using a threshold of 45 cycles. PCR efficiency was ~90% when testing DNA extracts from both serum and WB samples. The assay was specific to *Aspergillus* and *Penicillium* species and did not cross-react with other fungal, yeast, or human DNA.

At all centers, duplicate and triplicate (for serum and WB samples, respectively) PCR testing allowed different interpretations of test positivity. In this study, one positive PCR replicate was considered significant.

Galactomannan ELISA. In all three centers, GM was quantified in serum and bronchoalveolar lavage (BAL) samples using the Platelia *Aspergillus* GM ELISA (Bio-Rad) according to manufacturer's instructions, with a cutoff optical density of 0.5. Bronchoscopy was performed whenever necessary and if the general condition of the patient allowed. GM testing of BAL samples from Dublin patients was not performed.

Data analysis. Patients with proven or probable IA according to the EORTC/MSG criteria (14) were classified as true positives, and those diagnosed as unclassified were classed as true negatives. Diagnostic performance parameters were calculated using previously described methods (20). Two-sided *P* values were calculated using Fisher's exact test. Paired and unpaired 95% confidence intervals were calculated using the method of Newcombe (21, 22).

RESULTS

Patient details. According to the EORTC/MSG criteria (14), there were 47 cases (7 proven and 40 probable IA) and 31 controls (no evidence of IA) selected on the basis of the availability of serum samples for additional molecular testing. Controls were selected randomly but in temporal correlation to patients with proven and probable IA.

These 78 patients provided 808 samples (500 from cases, 308 from controls), with a mean of 10.4 specimens per patient (range, 3 to 32). Patient demographics are shown in Table 1.

Performance of molecular assays. (i) Sample positivity. Of the 808 clinical samples collected, 803 samples were available for GM ELISA testing, 790 samples for WB PCR, and 754 samples for serum PCR testing (Table 2). In total, GM yielded 146 positive results (18.2%), WB yielded 145 positive results (18.4%), and serum yielded 123 positive results (16.3%).

TABLE 1 Patient demographics

Patient characteristic[a]	Value(s)
No. of patients	78
Male/female ratio	53:25
Median age (yr) of males (range)	53 (18–75)
Median age (yr) of females (range)	51 (20–72)
Mean no. of specimens per patient (range)	10.4 (3–32)
No. of AML patients	36
No. of ALL patients	10
No. of patients with other underlying diseases[b]	32

[a] AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia.
[b] Includes chronic lymphoblastic leukemia (CLL) ($n = 7$), chronic myeloid leukemia (CML) ($n = 5$), chronic eosinophilic leukemia (CEL) ($n = 1$), Hodgkin's lymphoma ($n = 5$), non-Hodgkin's lymphoma ($n = 5$), multiple myeloma ($n = 2$), myelodysplastic syndrome ($n = 3$), immunocytoma ($n = 1$), and myelofibrosis ($n = 3$).

Analysis of IA cases for their positivity showed that all three biomarker assays generated a high number of positives and there were no significant differences in sample positivity between assays (Table 2). In contrast, in the control group, very few samples tested positive for GM (Table 2). Interestingly, only one sample showed concordant positivity for WB and serum testing. The potential false-positive rate in WB PCR was greater than the rates generated by both GM and serum PCR; for all assays, the sample positivity rate in cases was significantly greater than that generated by the controls. The mean number of samples detected in patients with proven or probable IA by each assay was 3.1 (range, 0 to 12) by GM, 2.7 (range, 0 to 8) by WB PCR, and 2.5 (range, 0 to 10) by serum PCR. The percentage of these patients with more than one positive assay result was 57.4% (9), 63.8% (13), and 59.6% (6) for GM, WB, and serum, respectively. From 27 patients with a GM positive result, two could not be confirmed by WB PCR and three by serum PCR. From 30 patients with a WB PCR positive result, five could not be confirmed by GM or by serum PCR. From 28 patients with a serum PCR positive result, seven could not be confirmed by GM and two by WB PCR.

The individual sample concordance (observed agreement [kappa]) for GM versus WB PCR, GM versus serum PCR, and WB versus serum PCR was 76.7% (95% confidence interval [CI], 72.9 to 80.5) and kappa, 0.22 (95% CI, 0.13 to 0.31); 81.3% (95% CI, 77.4 to 84.6) and kappa, 0.35 (95% CI, 0.26 to 0.44); and 77.9% (95% CI, 73.8 to 81.6) and kappa, 0.23 (95% CI, 0.14 to 0.32), respectively.

(ii) Patient positivity. Using a single positive assay, patient positivity in the case group ($n = 47$) was 80.9% for GM, 85.1% for WB, and 78.7% for serum PCR. In the control group ($n = 31$), 3.2% of the patients were positive by GM, 16.1% by serum, and 35.5% by WB (Table 3). There were nine cases not detected by GM, seven by WB, and 10 by serum. Twenty-eight cases were detected by all three biomarker assays, 12 by two assays (five by GM and WB assays, five by WB and serum assays, two by GM and serum assays), and seven by only one assay (two serum, two WB PCR, three GM). The four cases detected by only one PCR assay were classified using a positive GM from a BAL sample or *Aspergillus* culture from sputum with the EORTC/MSG microbiological criteria. No cases were negative by all tests. As mentioned before, if only one test was performed, positivity in some patients was not detected.

TABLE 3 Patient positivity using individual and combinations of biomarker assays

Assay	Positivity results[a]				
	Cases ($n = 47$)		Controls ($n = 31$)		
	No. positive	% positive (95% CI)	No. positive	% positive (95% CI)	*P* value[b]
GM	38	80.9 (67.5–89.6)	1	3.2 (0.6–16.2)	0.0001
WB PCR	40	85.1 (72.3–92.6)	11	35.5 (21.1–53.1)	0.0001
Serum PCR	37	78.7 (65.1–88.0)	5	16.1 (7.1–32.6)	0.0001
Combination testing[c]					
GM/GM	23	48.9 (35.3–62.8)	0/31	0 (0–11.3)	0.0001
GM/WB	32	68.1 (53.8–79.6)	0	0 (0–11.3)	0.0001
GM/serum	28	59.6 (45.3–72.4)	0	0 (0–11.3)	0.0001
WB/WB	22	46.8 (33.3–60.8)	2	6.5 (1.8–20.7)	0.0002
WB/serum	28	59.6 (45.3–72.4)	1	3.2 (0.6–16.2)	0.0001
Serum/serum	24	51.1 (37.2–64.7)	0	0 (0–11.3)	0.0001

[a] Positivity in cases was significantly greater than that in controls for all assays and combinations assessed.
[b] *P* value calculated for difference in positivity between cases and controls.
[c] As an example, the combination GM/GM means that a first positive test had to be confirmed by a second positive test within 11 days.

Fifteen BAL samples were tested by GM ELISA. All 12 positive tests were due to BAL samples from IA cases. *Aspergillus* cultures from clinical samples of IA cases (BAL, sputum, sinus aspirates, or brain tissue specimens) were positive in 11 samples (*A. fumigatus*, $n = 10$; *A. niger*, $n = 1$).

To investigate further the differences in biomarker positivity values, we analyzed clinical data, including blood counts, donor type, and the use of alemtuzumab and antimold effective drugs (assay positivity under treatment) in these patients. No correlation between these clinical data and the differences in diagnostic performance could be demonstrated (data not shown).

In a further analysis, the first positive test had to be confirmed by a second positive assay within 11 days. This could have been by the same assay or by any other test, resulting in 6 different combinations of GM/GM, WB/WB, serum/serum, GM/WB, GM/serum, and WB/serum. The time interval of 11 days was determined

TABLE 2 Biomarker sample positivity rates

Assay	No. of positive results/total (%)		% difference in positivity: case vs. control (*P* value)	% difference in positivity between assays (*P* value)		
	Cases	Controls		Assays	Cases	Controls
GM ELISA	145/495 (29.3)	1/308 (0.3)	29 (0.0001)[a]	GM vs WB[b]	4.3 (0.1515)	−5.3 (0.0001)[a]
WB PCR	128/490 (26.1)	17/300 (5.7)	20.4 (0.0001)[a]	WB vs serum[c]	0.7 (0.8794)	4.3 (0.0149)[a]
Serum PCR	118/467 (25.3)	5/287 (1.7)	23.6 (0.0001)[a]	Serum vs GM[d]	−4.1 (0.1859)	1.4 (0.2123)

[a] Statistically significant difference.
[b] A total of 486 samples from cases and 300 samples from controls were tested by WB PCR and GM ELISA.
[c] A total of 458 samples from cases and 281 samples from controls were tested by WB PCR and serum PCR.
[d] A total of 466 samples from cases and 287 samples from controls were tested by serum PCR and GM ELISA.

TABLE 4 The individual and combined diagnostic performance of GM ELISA, WB, and serum PCR

Assay	Performance value (% [95% CI])[a]						
	Sensitivity	Specificity	PPV	NPV	LR^{+}[b]	LR^{-}	DOR[c]
GM	80.9 (67.5–89.6)	96.8 (83.8–99.4)	97.4 (86.8–99.6)	76.9 (61.7–87.4)	25.3	0.20	126.5
WB PCR	85.1 (72.3–92.6)	64.5 (47.0–78.9)	78.4 (65.4–87.5)	74.1 (65.4–87.5)	2.4	0.23	10.4
Serum PCR	78.7 (65.1–88.0)	83.9 (67.4–92.9)	88.1 (75.0–94.8)	72.2 (56.0–84.2)	4.9	0.25	19.6
Combination testing[d]							
GM/GM	48.9 (35.3–62.8)	100 (89.0–100)	100 (85.7–100)	56.4 (43.3–68.7)	>489	0.51	>958.9
GM/WB	68.1 (53.8–79.6)	100 (89.0–100)	100 (89.3–100)	67.4 (53.0–79.1)	>681	0.32	>2,128.1
GM/serum	59.6 (45.3–72.4)	100 (89.0–100)	100 (87.9–100)	62.0 (48.2–74.1)	>596	0.40	>1,490
WB/WB	46.8 (33.3–60.8)	93.5 (79.3–98.2)	91.7 (74.2–97.7)	53.7 (40.6–66.3)	7.2	0.57	12.6
WB/serum	57.4 (43.3–70.5)	96.8 (83.8–99.4)	96.4 (82.3–99.4)	60.0 (46.2–72.4)	17.9	0.44	40.7
Serum/serum	53.2 (39.2–66.7)	100 (89.0–100)	100 (86.7–100)	58.5 (45.1–70.7)	>532	0.47	1,131.9

[a] PPV, positive predictive value; NPV, negative predictive value.
[b] LR, likelihood ratio.
[c] DOR, diagnostic odds ratio.
[d] As an example, the combination GM/GM means that a first positive test had to be confirmed by a second positive test within 11 days.

as optimal by receiver operating characteristic (ROC) curve analysis (data not shown).

This second analysis markedly reduced the number of false-positive patients in the control group for all combinations; only the combinations WB/WB and WB/serum detected control patients (2 and 1 patients, respectively; see Table 3). In the case group ($n = 47$), the various combinations performed differently, with the combination GM/WB yielding the highest positivity (68.1%; Table 3). Use of the same biomarkers to confirm positivity resulted in lower positivity rates.

Diagnostic parameters were calculated for single or confirmed positive assays. For single positive assays, WB had the highest sensitivity, whereas specificity and positive predictive value (PPV) were highest for GM, and specificity was significantly greater than in WB PCR (difference, 32.3% [95% CI, 11.6 to 50.4%; $P = 0.0026$]) (Table 4). However, as GM was used to define probable disease according to the EORTC/MSG criteria, its performance reflects inclusion bias and should not be used for comparative purposes. The negative predictive value (NPV) was similar for all three assays, ranging from 72.2% to 76.9%, but it must be noted that PPV and NPV are significantly affected by disease prevalence, which is artificially high in case-control studies (Table 4).

When a second assay was used to confirm positivity, the highest sensitivity was achieved by the combination of GM/WB followed by GM/serum. Specificity and PPV were excellent for all combinations (>91%) (Table 4).

Timing of assay positivity relative to CT scan or biopsy. To analyze the temporal relationship between the different molecular assays, the first positive results in each assay of a proven or probable case were compared to the definitive diagnosis based on computed tomography (CT) scan, biopsy, or tissue culture (time point "zero"). Patients who had a CT scan prior to the beginning of PCR and ELISA screening ($n = 10$) were excluded from this specific analysis. GM positivity preempted CT in 25 cases, WB in 27, and serum in 24 cases.

Prior to the definitive diagnosis based on CT scan, biopsy, or tissue culture, WB preceded the CT scan by a mean of 36 days (range, 1 to 118), GM by 24 days (range, 1 to 118), and serum by 15 days (range, 1 to 79). In 33 patients (70%) with proven or probable IA, the diagnosis might have been established earlier if it had been based on a positive PCR test instead of the CT scan, biopsy, or tissue culture. Twenty-eight cases had a confirmation of the first positive assay by a second assay (PCR or GM) prior to the CT scan. In 2 cases, the confirmation was concordant with the CT scan, and in 1 case only, a positive CT scan confirmed the GM result.

The diagnostic means (molecular assays and CT scan or biopsy) were compared to show the earliest signs that suggested IA in each patient. In 22 patients, WB PCR became positive prior to the other tests (mean, 37 days; range, 1 to 118 days), in 17 patients, GM was the first assay to be positive (mean, 32 days; range, 1 to 118 days), and in 14 patients, serum PCR showed the earliest signs of IA (mean, 14 days; range, 1 to 45 days) prior to diagnosis based on CT scan or biopsy).

DISCUSSION

Sensitive molecular assays leading to an earlier diagnosis of IA are needed for good patient prognosis. Several clinical studies have already shown the potential of PCR in this area (23), but the optimal clinical material to be used for diagnosis has yet to be determined. Our aim was to compare WB and serum directly in a cohort of patients at high risk for IA. All extraction methods used in this study were compliant with published EAPCRI guidelines (9, 10).

Besides its efficient DNA extraction and highly sensitive detection, performing PCR in duplicate contributed to increased sensitivity (24). Since the fungal load in blood is low (7, 8), the detection of pathogen DNA might be very close to the limit of PCR detection (LoD). Reproducibility within this quantification cycle (C_q) range is not consistent (25) and can lead to false-negative results. Duplicate testing increases the chance of detecting low concentrations of DNA but might give nonreproducible results concerning not only PCR technology itself, but also the different material used for DNA extraction. To overcome this problem, the enrichment of fungal target DNA by a higher extraction efficacy, amount of starting material, and frequency of screening might be beneficial. In this study, single positive results were considered positive, as recommended previously (18, 25).

We analyzed our data in two different ways, first by individual assay positivity and then by using a second assay within 11 days as

confirmation. In the first analysis, WB reached the best sensitivity but the lowest specificity. The combination WB/WB performed the worst, but in combination with another assay (GM), WB showed the best sensitivity, specificity, and diagnostic odds ratio (DOR) values. The second highest DOR was achieved by the combination of GM with serum. All combinations using different confirmatory assays (GM/WB, GM/serum, and WB/serum) were superior to confirmation using the same assay as the first (GM/GM, WB/WB, and serum/serum). No cases were missed when using all three assays, and if all were negative, IA could be confidently excluded. In this study, NPVs were lower than in previous studies, reflecting the artificial disease prevalence in case-control studies; the incidence for this study was 60.2%. If the sensitivity and specificity values determined in this study are applied to the same size population, but with a more representative incidence of 10%, the NPV for all assays is >97%, supporting a screening role to exclude diseases in routine clinical practice.

As reported previously (7), the use of one assay was insufficient for the diagnosis of IA, but a second complementary assay markedly improved specificity and PPV. Based on the results of a large prospective study in which we evaluated the utility of combining real-time PCR with galactomannan surveillance in high-risk patients (15), together with the findings of the present study, we recommend the use of a second confirmatory assay that is different from GM for the detection of IA. This could be PCR from either serum or WB. Furthermore, a second confirmatory test reduced false positivity within individual assays. More than 35% of the control patients were positive by a single positive WB assay; by using an additional WB confirmatory assay (within 11 days), false positivity was reduced by 29.0% (95% CI, 8.8 to 47.2%; $P = 0.0106$).

This reduction might be interpreted in different ways. First, control patients that were PCR positive were accurately defined by EORTC/MSG criteria, and false-positive results were generated by an oversensitive molecular assay resulting in low specificity. Second, PCR-based methods potentially have higher sensitivity to detect more cases, including subclinical manifestations of IA or potential exposure to the organism, an important factor considering that all patients were at high risk of developing IA. The accuracy of biomarker diagnosis is increased in patients who are found to be positive by more than one biomarker. The revised EORTC/MSG criteria were defined to determine accurate diagnosis of disease, principally for clinical trials, whereas biomarker detection tries to preempt disease by targeting the early infective process. This allows early initiation of empirical therapy, resulting in a better prognosis for the patient. However, 19 patients classified as having proven or probable IA were not detected by one, or even two, molecular methods. No PCR inhibition was detected, but the release of fungal DNA and antigens is poorly understood and is influenced by antifungal treatment (26–28). Almost all proven or probable cases received antimold effective drugs at some point, and diagnostic performance might be negatively influenced by this treatment.

Other clinical studies have attempted to determine what blood fraction is optimal for PCR-based detection of IA. Loeffler et al. (29) compared WB and plasma and found WB to be more sensitive. Plasma was also determined to be less sensitive than serum or WB using different blood fractions spiked with free *Aspergillus* DNA (30).

More recently, Bernal-Martinez et al. (31) compared serum and WB samples obtained from 26 patients. In their study, they did not observe any significant differences in performance between the samples and they concluded by recommending serum for *Aspergillus* detection, due to its convenience.

Early detection of IA is necessary for achieving a good patient prognosis. Therefore, in addition to assay sensitivity, the timing of assay positivity is important. With regard to the earliest positive assay, WB detected the most cases at the earliest time points, preceding GM and serum by 12 and 21 days, respectively, in comparison to CT scanning and biopsy or by 5 and 22 days, respectively. In the study of Meije et al. (32), *Aspergillus fumigatus* DNAemia preceded CT and GM by an average of 21 and 68 days, respectively. The same trend was reported by Challier et al. (7). In this study, WB preceded the CT scan by a mean of 36 days, GM by 26 days, and serum by 15 days.

In summary, there was no significant difference between the use of WB and serum samples for PCR-based diagnosis of IA. However, there was a trend for WB to be more sensitive (85% versus 79%) and show earlier positive results (36 days versus 15 days) compared to serum. Against this finding, false positivity is reduced using serum specimens, and they are also easier and faster to process than WB and can be used for simultaneous GM testing. A combination of biomarker testing offered the best diagnostic utility. Individual users should determine the sample type that meets the particular requirements of their individual center.

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