A Novel Extraction Method Combining Plasma with a Whole-Blood Fraction Shows Excellent Sensitivity and Reproducibility for Patients at High Risk for Invasive Aspergillosis

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Diagnosis of invasive aspergillosis (IA) is still a major problem in routine clinical practice. Early diagnosis is essential for a good patient prognosis. PCR is a highly sensitive method for the detection of nucleic acids and could play an important role in improving the diagnosis of fungal infections. Therefore, a novel DNA extraction method, ultraclean production (UCP), was developed allowing purification of both cellular and cell-free circulating fungal DNA. In this prospective study we evaluated the commercially available UCP extraction system and compared it to an in-house system. Sixty-three patients at high risk for IA were screened twice weekly, and DNA extracted by both methods was cross-analyzed, in triplicate, by two different real-time PCR assays. The negative predictive values were high for all methods (94.3 to 100%), qualifying them as screening methods, but the sensitivity and diagnostic odds ratios were higher using the UCP extraction method. Sensitivity ranged from 33.3 to 66.7% using the in-house extracts to 100% using the UCP extraction method. Most of the unclassified patients showed no positive PCR results; however, single-positive PCR replicates were observed in some cases. These can bear clinical relevance but should be interpreted with additional clinical and laboratory data. The PCR assays from the UCP extracts showed greater reproducibility than the in-house method for probable IA patients. The standardized UCP extraction method yielded superior results, with regard to sensitivity and reproducibility, than the in-house method. This was independent of the PCR assay used to detect fungal DNA in the sample extracts.

Despite intensive research over the last 2 decades, invasive aspergillosis (IA) is still a major complication in immunocompromised patients who have undergone allogeneic stem cell and solid organ transplantation. The mortality rate is very high (14) and is linked to difficulties in diagnosis of IA due to unspecific symptoms or the late appearance of clinical signs of disease; the insensitivity of conventional diagnostic techniques also contributes to delayed diagnosis of disease. Therefore, the development of sensitive molecular assays to enable early diagnosis of IA is highly warranted for good patient outcomes.

Detection of galactomannan (GM) is widely used as a molecular marker for IA and is already incorporated as a microbiological criterion into the criteria for invasive fungal disease (IFD) of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG). However, the reported sensitivity and specificity for this assay vary greatly between studies (12, 16, 22). PCR holds the potential to form the basis of a rapid and highly sensitive diagnostic assay for the detection of fungal DNA in blood samples. Blood is an easily accessible sample type that can be drawn regularly, even from severely ill patients, but it usually contains a low fungal load (9). Different blood fractions, even blood clots, have been evaluated as sample material for diagnostic tests, and fungal DNA was detected in each component of blood (13). The exact origin of *Aspergillus* DNA in blood is unclear since the fungus does not grow in blood. Recent studies in a murine model of IA and in *in vitro* growth studies have indicated that cell-free fungal DNA is released by actively growing mycelia (20). The suggested origin of cell-free fungal DNA in blood was as a by-product from the phagocytosis of fungal elements by immune cells. The absence of a standardized PCR assay for the diagnosis of IA had led

to its omission from the EORTC/MSG criteria for IFD. The first reports on the standardization of PCR-based assays for IA indicated that DNA extraction was the limiting step (28). This led to the publication of guidelines for the extraction of fungal DNA from whole blood and serum (26, 27). These guidelines will have to be incorporated into commercially available systems to ensure high sensitivity and specificity and provide a degree of methodological standardization.

The major drawback of PCR-based assays that target fungal DNA in blood is that they use either the cellular blood fraction or the plasma, with the other fraction being discarded (15). A combination of both available blood fractions should increase the sensitivity of the test system (19, 25).

In this study, we describe and evaluate a novel, commercially available diagnostic method to detect *Aspergillus* DNA in blood, including tools for DNA extraction and DNA detection. This is the first system to combine cell-associated and cell-free circulating DNA.

MATERIALS AND METHODS

Study design. Patients who had undergone allogeneic stem cell transplantation (alloSCT) and those receiving myeloablative chemotherapy with an

expected duration of neutropenia (leukocyte count of <1,000/μl) of at least 10 days were included in this study, after informed consent, for a maximum of 100 days after SCT or until termination of neutropenia. Between July and October 2010, blood and serum samples were prospectively taken twice weekly from patients with a high risk of invasive fungal disease. Signs and symptoms were collected together with microbiological data from patient charts to categorize the onset and type of fungal infections according to the EORTC/MSG criteria (4). Blood samples were used for each of the DNA extraction methods described below, and serum samples were used for galactomannan quantification. The study was approved by the ethics committee at the University Hospital in Wuerzburg.

DNA extraction from EDTA whole blood. Both extraction methods were compliant with the respective European *Aspergillus* PCR Initiative (EAPCRI) recommendations (27, 28). In each DNA extraction procedure, at least one negative control (water) was included. In addition, during the study period, blood from 20 healthy volunteers was analyzed by both extraction systems (detecting no *Aspergillus*-specific DNA in the donor blood, blood collection tubes, or laboratory work flow).

The in-house extraction (IHE) was performed as previously described (23). Briefly, the red and white cells from a 3-ml blood sample were lysed and centrifuged. The pellet was bead beaten, and DNA was isolated using the artus *Aspergillus* RG PCR kit (referred to herein as HHQ) (Qiagen, Hamburg, Germany). The elution volume was adjusted to 100 μl. IHE samples were analyzed in triplicate by two different quantitative PCR (qPCR) methods (an in-house assay [referred to as the PF assay] and a commercially available *Aspergillus* PCR assay [HHQ]).

A QIAamp UCP (for ultraclean production) PurePathogen Blood Kit ([UCP] (Qiagen, Germany) was used to isolate DNA from both blood fractions (cell pellet and plasma) according to the manufacturer's instructions. In brief, cellular and cell-free (plasma) fractions were separated at the beginning of the extraction process and combined after disruption of fungal cell walls. Human DNA within the cellular fraction was depleted by specific lysis of white blood cells, and fungal cells were lysed mechanically using pathogen lysis tubes which were part of the commercial kit. A minimum of 2 ml and maximum of 8 ml of EDTA blood were used for DNA extraction. The elution volume was adjusted to 100 μl. The UCP extract was analyzed in triplicate by two different qPCR methods (PF and HHQ). The reagents and consumables within the commercial kit underwent DNA decontamination processes to remove all traces of environmentally derived DNA contamination which occurs in some molecular biology products.

Detection of *Aspergillus* DNA. In all PCR assays, at least one negative control (PCR-grade water) and one positive control (cloned artificial template) were included.

The in-house assay (PF) was performed as previously described (23) with some modifications. Briefly, *Aspergillus*-specific qPCR assays were performed at a final volume of 21 μl, containing 10 μl of DNA extract, on the StepOnePlus platform (Applied Biosystems). Primer Asp fum F was replaced with the degenerate primer Asp-P1 degen (5′-GCAGTCTGAGT TGATTATYGYAATC-3′, where Y is C or T), which was used to increase sensitivity. Mean PCR efficiency was calculated by three independent experiments using 10-fold dilutions of *Aspergillus* plasmid standards using the following equation: efficiency = $10^{(-1/\text{slope})} - 1$. Six different dilutions close to the limit of detection were tested in duplicate. PCR efficiency was 92.5% for *Aspergillus fumigatus*, 90.3% for *Aspergillus terreus*, and 90.1% for *Aspergillus flavus*. No cross-reactivity with other fungi or human genomic DNA was observed. For each extract, triplicate assays were performed. The assay was termed positive if at least one replicate showed an *Aspergillus*-specific amplification signal (the threshold was set to 0.05).

The artus *Aspergillus* RG PCR kit (HHQ) is a qualitative multiplex PCR assay that is specific for *A. fumigatus*, *A. flavus*, and *A. terreus* and was designed for the RotorGeneQ platform (Qiagen). PCR was set up according to the manufacturer's instructions. Ten microliters of DNA extract was mixed with 15 μl of PCR master mix containing all PCR reagents including *Aspergillus*-specific oligonucleotides and probes. Each sample

TABLE 1 Performance of different methods

Method[a]	No. of samples			No. of positive PCRs by replicate		
	Negative	Positive	% Positive	1	2	3
IHE-PF	487	12	2.4	10	1	1
IHE-HHQ	483	16	3.2	14	1	1
UCP-PF	470	29	5.8	17	5	7
UCP-HHQ	470	27	5.4	14	4	9
GM	486	7	1.4			

[a] The eluates of two different extraction methods (IHE and UCP) were tested in crossover experiments by two different real-time PCR assays (PF and HHQ). GM represents the results for the galactomannan ELISA.

was tested in triplicate. PCR inhibition was monitored by an internal control (IC) featuring a cloned artificial PCR template. Samples were designated inhibitory if no amplification signal from the IC was detected. Samples showing no amplification signal but any *Aspergillus*-specific signal were considered not inhibitory. The specific assay was considered positive if at least one replicate showed an *Aspergillus*-specific amplification (fixed threshold of 0.05).

GM ELISA. Galactomannan (GM) was quantified in serum samples using a Platelia *Aspergillus* GM enzyme-linked immunosorbent assay (ELISA) (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's instructions, with a cutoff optical density of 0.5.

Data analysis. For data analysis, patients with probable IA under the recent EORTC criteria (4) were classed as true positives, and those diagnosed as unclassified were classed as true negatives. Patients having possible IA were removed from the analysis. Diagnostic odds ratios (DOR) were calculated by previously described methods (1). PCR results were compared by a *t* test.

RESULTS

Patient details and incidence of IA. In this prospective study 499 specimens were collected from 63 patients at high risk for invasive fungal infection. A mean of 7.9 specimens (range, 1 to 21) were taken from each patient. The cohort comprised 40 men (mean age of 53.4 years; range, 19 to 82 years) and 23 women (mean age of 56.4 years; range, 22 to 82 years). The main underlying disease was acute leukemia: 32 patients with acute myeloid leukemia (AML) and 8 patients with acute lymphoblastic leukemia (ALL). Eleven patients suffered from multiple myeloma, and 12 suffered from other diseases, including lymphoma, myelodysplastic syndrome, chronic myeloid leukemia (CML), and myelofibrosis. During the study period four cases of probable IA, 18 cases of possible IA, and 41 unclassified cases were identified according to EORTC/MSG criteria (4). As microbiological evidence for probable IA, three patients showed a positive GM from serum and one had a positive GM result from bronchiolar lavage (BAL) fluid; no positive culture or histopathological test was available. The incidence of proven/probable IA was 6.3%.

In contrast to all other patients analyzed within this study, one patient with probable IA was consistently serum GM positive; concurrently, the antibiotic drugs piperacillin-tazobactam were administered, which were reported to cause false-positive GM results (10, 24). This led us to the assumption that the patient was misclassified; thus, we retrospectively excluded this patient from further analyses.

Performance of molecular assays. The galactomannan (GM) ELISA from serum yielded seven positive results (1.4%) (Table 1); for six samples no serum was available (1.2%).

TABLE 2 Performance of different methods assorted due to EORTC/MSG classification

EORTC/MSG classification $(n)^a$	IHE-PF				IHE-HHQ				UCP-PF				UCP-HHQ				No. of serum GM positive-patients (%)
	No. of detected patients (%)	No. of positive PCR specimens by result type ^{b}			No. of detected patients (%)	No. of positive PCR specimens by result type			No. of detected patients (%)	No. of positive PCR specimens by result type			No. of detected patients (%)	No. of positive PCR specimens by result type			
		1	2	3		1	2	3		1	2	3		1	2	3	
Probable IA (3)	2 (66.7)	3	1		1 (33.3)	1	1		3 (100.0)			5	3 (100.0)	1		5	2 (66.7)
Possible IA (18)	3 (16.7)	2		1	4 (22.2)	4		1	8 (44.4)	5	2	2	8 (44.4)	6	2	4	0 (0)
Unclassified (41)	5 (12.2)	5			8 (19.5)	9			13 (31.7)	12	3		7 (17.1)	7	2		0 (0)

^{a} n, number of patients.

^{b} For triplicate PCRs, result types are indicated as follows: 1, single positive replicate; 2, two positive replicates; 3, three positive replicates.

DNA was extracted from 499 clinical specimens using two different extraction methods. The in-house extraction (IHE) used solely the cellular fraction of EDTA whole blood, whereas the QIAamp UCP PurePathogen Blood Kit (UCP) used the cellular and the cell-free (plasma) fractions. The UCP kit was designed to process large blood volumes of up to 8 ml; 10 extracts were produced from blood samples containing less than 3 ml, 242 extracts were from blood samples of 3 ml, and 247 were from blood samples of more than 3 ml. No significant increase in the number of positive samples was observed when more than 3 ml of blood was used for extraction (see Table S1 in the supplemental material). The number of large-volume blood samples was limited, so this question should be addressed in a future trial. Both DNA extracts were analyzed by crossover experiments to minimize the effect of the different qPCR assays (the PF in-house qPCR and the HHQ commercially available qPCR) and to validate the newly designed HHQ assay. Four different combinations of extraction and detection methods were possible: IHE-PF, IHE-HHQ, UCP-PF, and UCP-HHQ. The PCR inhibition rate as an indication for possible false-negative results in the HHQ assay was 2.4% in the IHE and 3.2% in the UCP extract.

Analysis of the IHE extracts showed that 12 (2.4%) and 16 (3.6%) specimens were positive for *Aspergillus* in the PF and HHQ assays, respectively (Table 1). Two specimens (0.4%) were not available for testing the UCP extract in the HHQ assay. Twenty-nine (5.8%) and 27 (5.4%) specimens tested positive using the UCP extract in the PF and HHQ assays, respectively (Table 1). This indicated an increased positivity rate associated with the UCP extract in comparison to the IHE extract that was independent of the qPCR assay used.

The qPCR assays were performed in triplicate, resulting in three different options for interpretation of test positivity: first, a single-positive result (and two negative); second, a double-positive result (and one negative); and third, a consistently positive result with three positive replicates. All three types of results were considered assay positive. The numbers of positive PCR replicates are indicated in Table 2. The majority of positive results using the IHE extract were single positives (10 and 14 specimens in the PF and HHQ assays, respectively). This was also the case for the UCP extract, with 17 and 14 single-positive results, but the number of positive specimens also increased in the double- and triple-positive assays. Comparing IHE versus UCP extract, the UCP-HHQ assay detected a maximum of nine triple-positive results, whereas IHE-HHQ detected just one triple-positive result. In addition to a generally higher positivity rate in the UCP extract, reproducibility was also increased, as indicated by the greater number of double- and triple-positive results. This was statistically significant ($P < 0.05$).

Correlation of diagnostic assays with EORTC/MSG classification. Analyzing the four different combinations of extraction and detection methods (IHE-PF, IHE-HHQ, UCP-PF, and UCP-HHQ) in correlation to the clinical data produced quite different results (Table 2). Out of three probable IA cases, only one patient was detected by the IHE-HHQ combination (33%), two were detected by IHE-PF (67%), and three patients were detected by UCP-PF and UCP-HHQ (100%). Serum GM testing also detected two cases (67%), whereas the third patient was categorized as probable due to a positive GM from a bronchoalveolar lavage (BAL) fluid sample (Table 3). A similar detection pattern was seen for possible IA; IHE-PF detected three patients (16.7%), IHE-

TABLE 3 Performance of different methods in episodes of probable IA patients

Patient code $(n)^e$	No. of positive results by method				EORTC/MSG factor and/or incidence for probable IA		
	IHE-PF	IHE-HHQ	UCP-PF	UCP-HHQ	Host^a	Clinical^b	Microbiological^c
M069 (11)	0	0	1	2	N	DL	1^d
M075 (5)	3	2	3	3	N	DL	2
T096 (1)	1	0	1	1	N, A, T	DL	1

^{a} Host risk factors for IA are as follows: A, allogeneic stem cell transplantation; N, neutropenia; T, T-cell-suppressive therapy.

^{b} DL, dense lesion.

^{c} Data represent the numbers of positive results of galactomannan ELISA (GM) performed from serum unless otherwise stated.

^{d} Positive result based on BAL fluid.

^{e} n, number of samples.

TABLE 4 Diagnostic parameters for each method[a]

Parameter[b]	IHE-PF	IHE-HHQ	UCP-PF	UCP-HHQ
Sensitivity (%)	66.7	33.3	100.0	100.0
Specificity (%)	87.8	80.5	68.3	82.9
PPV (%)	28.6	11.1	18.8	30.0
NPV (%)	97.3	94.3	100.0	100.0
DOR	13.2	2.0	169.0	205.0

[a] Patients with possible IA were excluded from analysis.
[b] PPV, positive predictive value; NPV, negative predictive value. DOR, diagnostic odds ratio. DOR was calculated by adding 0.5 to each cell as follows DOR = (TP × TN + 0.5)/(FN × FP + 0.5) to avoid dividing by zero, where TP, TN, FP, and FN are the numbers of patients identified as true positive, true negative, false positive, and false negative, respectively.

HHQ detected four patients (22.2%), and both methods using the UCP extracts detected eight patients (44.4%) (Table 2). The best performance was achieved using DNA extracted by the UCP method, which detected the highest number of probable and possible cases of IA.

In the 41 unclassified cases, serum GM tests were negative, but positive results for qPCR combinations ranged from 5 patients (12.2%) with IHE-PF to 13 patients (31.7%) with UCP-PF.

The highest PCR assay reproducibility was achieved using the UCP extract: five triple-positive results each within probable IA cases, and two and four triple-positive results within possible IA cases for the UCP-PF and UCP-HHQ, respectively (Table 2). Also the quantification cycle (Cq) values were lower in positive triplicates (the lowest in probable IA cases in comparison to possible IA), indicating a higher fungal load within reproducibly positive samples of the UCP extracts (data not shown). No triple-positive result was detected in unclassified patients in either the IHE or the UCP samples.

The number of single-positive PCR replicates was highest in unclassified patients, independent of the extraction and detection methods used, ranging from 5 cases (IHE-PF) to 12 cases (UCP-PF). None of these cases had a positive GM result.

A closer analysis of the three probable IA cases showed the different performances of the different extraction and detection methods used. This was indicated by the number of positive molecular assays (Table 3). Patient T096 had just one positive sample in each combination except IHE-HHQ. Patient M069 with 11 samples was classified as probable IA by a positive GM from BAL fluid and was detected only using the UCP extract. Patient M075 was detected by all combinations in several samples (range, two to three positive samples).

The unclassified patients fell into different subgroups according to their overall numbers of positive PCR results. Nineteen

patients showed no positive PCR at all, 12 had just a single positive event, and 9 patients had two or more positive signals (data not shown). In the last subgroup (see Table S2 in the supplemental material) more than one combination was affected, reducing the probability that a laboratory contamination issue was the reason for PCR positivity. All patients showed at least one host factor for EORTC/MSG classification, mainly neutropenia, and the majority were treated by an effective antimold drug during the study period.

Using the current EORTC/MSG classification, diagnostic parameters were calculated for each extraction and detection combination; patients with possible IA were excluded from analysis. Due to a very limited number of probable IA cases, a single undetected case could lead to large differences in sensitivity: 33% for IHE-HHQ, 67% for IHE-PF, and 100% for UCP-PF and UCP-HHQ combinations (Table 4). Specificity was good for all combinations (ranging from 80.5% to 87.8%) except for UCP-PF, with only 68%. The positive predictive values (PPV) were quite low, ranging from 11% to 30%, but the negative predictive values (NPV) were high (94% to 100%). This indicated that all PCR-based combinations were suitable as screening tests to rule out IA, but concerning sensitivity UCP-based combinations were favorable.

Patient classification independent of GM testing. GM testing is part of the EORTC/MSG classification system. A case of probable IA has to include *per definitionem* a positive microbiological criterion (here, a positive GM result from serum or BAL fluid). GM testing using serum samples was done twice weekly, whereas GM testing using BAL fluid was performed only in cases where a new infiltrate indicative of pulmonary aspergillosis was detected in a lung computerized tomography (CT) scan. BAL fluid samples were taken only from patients willing and able to undergo a bronchoscopy. The GM assay has been reported to produce false-positive and false-negative results due to numerous factors, including GM-contaminated food or antibiotics and use of antifungal agents (10, 12, 24). Patient data were analyzed independent of GM testing and the EORTC criteria using the results generated by the four different combinations of PCR-based detection: IHE-PF, IHE-HHQ, UCP-PF, and UCP-HHQ. Most of these episodes (n = 43) showed no or only one positive PCR result, including nine possible IA patients (Table 5). For comparison, patients with positive GM test results are also shown. All three were sorted into the groups showing two or more positive PCR-based methods.

To exclude false-positive PCR results due to, e.g., low-level contamination, a patient was further analyzed only if there was a positive result in at least two different PCR-based methods (n = 19). One patient was positive in all four PCR-based methods,

TABLE 5 Classification of patients independent of EORTC/MSG classification system sorted by different molecular PCR-based methods

No. of positive PCR-based methods	Total no. of patients	No. of positive patients by method (%)				EORTC/MSG classification (no. of patients)			No. of serum GM-positive patients (%)
		IHE-PF	IHE-HHQ	UCP-PF	UCP-HHQ	Probable	Possible	Unclassified	
4	1	1 (100)	1 (100)	1 (100)	1 (100)	1			1 (100)
3	7	2 (29)	6 (86)	6 (86)	7 (100)	1	3	3	1 (14)
2	11	3 (27)	3 (27)	9 (82)	7 (64)	1	5	5	1 (9)[a]
1	18	4 (22)	3 (17)	8 (44)	3 (17)		3	15	0 (0)
0	25	0 (0)	0 (0)	0 (0)	0 (0)		6	19	0 (0)

[a] This positive GM result was due to testing of a BAL fluid sample.

comprising a case of probable IA; seven patients were positive by three different assay combinations, comprising one probable, three possible, and three unclassified patients; and 11 patients were positive by two different assay combinations comprising one probable, five possible, and five unclassified patients (Table 5). This additional analysis identified all three probable (100%), eight possible (44%), and eight unclassified patients (20%). All analyzed unclassified patients (n = 8) are described in Table S2 in the supplemental material.

In this analysis most patients were detected using the UCP extracts, 16 (84%) were detected by UCP-PF, and 15 (79%) were detected by UCP-HHQ. For comparison, GM testing detected only 3 of 19 selected cases (16%).

DISCUSSION

Diagnosis of IA still remains difficult in routine clinical practice. Sensitive molecular assays leading to early diagnosis are needed for good patient outcomes. Several clinical studies have shown the potential of PCR (15), but an absence of standardized PCR assays has hindered a broader acceptance of this methodology for the diagnosis of IA. Our aim was to develop an efficient system which could be used as a standard diagnostic approach and to evaluate it against a second comparator assay on clinical samples. An optimal PCR-based diagnostic assay requires a sensitive detection system and an efficient extraction method, as proposed by published guidelines for detection of IA by PCR (28). Both extraction methods (IHE and UCP) used in this study were compliant with these EAPCRI guidelines (27, 28).

For a screening approach, serial sampling is mandatory, and therefore easily accessible material such as blood has to be used. In using both blood fractions for DNA extraction, it was possible to extract total fungal DNA from whole blood without the losses incurred by other methods. This extraction protocol (UCP) is unique to this study. In comparison to an in-house extraction method (IHE), the QIAamp UCP PurePathogen Blood Kit showed superior results in sensitivity and reproducibility.

In addition to efficient extraction and highly sensitive detection, sensitivity was also increased by performing the PCR in triplicates (3). Since the fungal load in blood is quite low, detection of pathogen DNA by PCR may be very close to the limit of detection (LoD) for PCR, further highlighting the importance of using triplicates. Reproducibility within this Cq range is not consistent (18), and many samples tested here ranked among this range. Testing such samples just once can lead to false-negative results. Testing in triplicate increases the chance of detecting low concentrations of DNA but results in mostly single-positive PCR replicates. Therefore, these single-positive results were also counted as assay positive in this study, but they have to be interpreted in the context of other clinical parameters and/or other non-culture-based methods (GM or beta-glucan assays). The greater extraction efficiency of the UCP method, due to the combined extraction of both blood fractions or a general higher extraction efficiency of the extraction method itself, led to improved reproducibility of the PCR assay and the detection of more positive replicates. A direct comparison of the extraction efficacies in both extraction systems was not performed because in the in-house assay the plasma fraction was not available for further analyses. The lower Cq values observed in PCR assays using the UCP extracts indicated a higher fungal burden in cases of probable IA cases. In contrast, the in-house method detected mainly single-positive PCR replicates, even in probable

IA cases, showing that a single-positive PCR replicate can be of clinical relevance, depending on the clinical and laboratory context.

To ensure consistent extraction efficacy, each patient sample should be monitored by an internal extraction control. This would also exclude operator-specific and inter- and intraexperimental differences and could be achieved by using an artificial DNA molecule as a spiked extraction calibrator, coextracted and analyzed for every single patient sample. Variances in the amount of the control DNA detected will reflect the efficacy of the extraction and possible PCR inhibition.

Use of the EORTC/MSG criteria led to the classification of a total of four cases of probable IA. Three of these were identified by PCR, but patient T150 was not detected as positive by any of the PCR-based methods. In contrast to all other probable cases, patient T150 suffered from an inherited severe immunodeficiency (ISID) and was consistently serum GM positive; in parallel, the antibiotic piperacillin-tazobactam was administered, which has been reported to cause false-positive GM results (10, 24). Assuming that this patient was misclassified, he was excluded from further analysis. A better performance of GM testing was also achieved by Steinbach et al. (24) excluding all patients receiving piperacillin-tazobactam. Confounding factors that interfere with the GM assay and infrequent CT scans may lead to misclassifications of IA when the EORTC/MSG criteria are used.

Patient M074 was another example of a potentially misclassified patient. This was an unclassified patient that gave positive results with two different PCR methods. Being only neutropenic, this patient did not formally fulfill the EORTC/MSG criteria. A high-resolution CT scan showed an unspecific infiltrate not sufficient for a clinical factor, and there was also a positive GM test result shortly before the beginning of the study period. Having no clearly defined chest infiltrate, this patient could not be classified as having probable IA. Using the UCP extract, PCR was positive within this patient episode but was stated as a false positive due to the unclassified EORTC/MSG status (21).

These two examples (patients T150 and M74) illustrate the problems inherent in developing a PCR-based diagnostic for IA. There is no real gold standard to guide clinical practice, and the current EORTC/MSG classification system is intended to be used only for clinical research. Testing new molecular markers within this system can cause bias, especially when criteria for defining IA such as the GM assay are false positive or false negative, leading to misclassifications of patients. We therefore classified the patients independently of the GM testing (and any clinical parameter). By analyzing two different DNA extracts by two different detection methods, four different combinations were possible. By showing a positive result in at least two different PCR-based methods, a patient episode was selected. This approach successfully identified all cases of probable IA and additionally some possible and unclassified patients. In the latter, the probability is quite high that these patients are in some way affected by the fungus *Aspergillus*. This could be due to a subclinical infection that did not result in any clinical manifestation or to effective antimold therapy, which most of these patients received and which can also influence the performance of GM and PCR testing (7, 12). These selected cases may therefore represent an infection that does not fit the definition or that exhibits weak signs of a partially treated disease. Also, in spite of extreme precautions, contaminations of different sources cannot be totally excluded and could cause these signals.

Despite successfully identifying patients with probable IA solely by means of positive PCR results and independently of the EORTC/MSG classification system, we are aware that this approach is of limited value in routine clinical practice. It is unlikely that two DNA extractions or two different PCR assays will be performed routinely in parallel. Nevertheless, we showed that PCR is able to assist in diagnosing IA and ruling out false-positive GM test results. Using PCR to complement GM testing can improve IA diagnosis. The cutoff for GM in serum is 0.5 GM index unit; in BAL fluid 1.0 GM index unit is recommended (11). Changes in cycle quantification threshold, number of positive replicates, and the confirmation by a second positive assay are possible for PCR-based methods and may be necessary to guide effective clinical management.

Using two positive PCR results was already reported to yield good results by Mengoli and colleagues in their meta-analysis (15). In that analysis, two consecutive positive PCR results were required to confirm IA diagnosis, but one negative result (on a regular screening basis) was sufficient to rule out IA at the tested time point. Such a diagnostics-driven approach can therefore reduce unnecessary antifungal treatment, costs, and toxic side effects (2). Halliday et al. included single and intermittent (within 14 days) positive PCR results for patient management (5). Another diagnostic approach used three markers instead of the usual two (6). A direct comparison of GM and beta-glucan testing and a real-time PCR method was prospectively performed and found comparable sensitivities and specificities for all noninvasive methods. Without standardization of all methods, a real comparison is not feasible, and a standardized animal model might be the right choice for assay validation by producing large numbers of samples from different sources. Such a preclinical evaluation study was recently reported (8). Unfortunately, no standardized PCR method was used.

In our comparison of PCR-based methods, we found that the DNA extraction method was the key for reproducible results in patients with probable IA. Whether some possible and unclassified patients were really affected by IA is difficult to prove. The detection of single-positive PCR replicates can improve the complex system used to diagnose IA by adding another independent parameter that can detect patients in an early phase of infection. In a diagnostics-driven approach it might be worthwhile to evaluate if these patients with a single positive PCR result benefit from *Aspergillus* active antifungal therapy.

False-negative PCR results can affect the reliability of a PCR assay and can be due to the presence of PCR-inhibiting factors. Therefore, PCR performance should be monitored by an adequate control system. The internal control (IC) detects variations in Cq values due to inhibitory substances in the eluate, and an IC is included in the artus *Aspergillus* RG PCR kit. High sensitivity can also cause false-positive results due to low-level contamination. Drawing of blood into tubes, laboratory processing of samples, and commercial reagents are potential sources of contamination. Even contaminated nutrition infused directly or transported through damaged intestinal barriers has been reported to cause false-positive PCR signals (17). Appropriate extraction controls monitor the whole laboratory work-flow and all reagents. Being aware of these risks, the UCP extraction system is especially adapted to the needs of *Aspergillus* diagnostics. All consumables and reagents in the kit underwent DNA decontamination processes (ultraclean production) to prevent putative false-positive

results. The in-house extraction and detection methods were monitored by negative extraction controls (human serum) and nontemplate controls (water), respectively. Therefore, the probability of low-level contamination was minimized.

To conclude, real-time PCR is a highly sensitive method for the detection of *Aspergillus* DNA, but the bottleneck to a sensitive assay has been DNA isolation from blood. This can now be achieved in a standardized fashion using the newly developed, commercially available UCP extraction kit and a multiplex *Aspergillus* PCR assay.

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