

Analysis of Performance of a PCR-Based Assay To Detect DNA of *Aspergillus fumigatus* in Whole Blood and Serum: a Comparative Study with Clinical Samples[∇]

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The performance of a real-time PCR-based assay was retrospectively analyzed (according to European Organization for Research and Treatment of Cancer/Mycosis Study Group criteria) in the samples of patients with invasive aspergillosis. A total of 711 serial samples (356 whole-blood and 355 serum samples) from 38 adult patients were analyzed. The *Aspergillus fumigatus* PCR assay results were positive for 89 of 356 (25%) whole-blood samples and 90 of 355 (25.35%) serum samples. Positive PCR results were seen in 29 of 31 (93.5%) patients for which serum was analyzed and in 31 of 33 (93.9%) cases with whole-blood specimens. Both blood and serum samples were available in 26 cases, and significant differences were not observed in this subgroup of cases. The average number of threshold cycles (C_T) for positive blood samples was 37.6, and the average C_T for serum was 37.4. The DNA concentration ranged between 2 and 50 fg per μ l of sample, with average DNA concentrations of 10.2 and 11.7 fg in positive blood and serum samples, respectively ($P > 0.01$). The performance of this PCR-based quantitative assay was similar for both serum and blood samples. We recommend serum samples as the most convenient hematological sample to use for *Aspergillus* DNA quantification when serial determinations are done.

The diagnosis of invasive aspergillosis (IA) remains a major challenge. This disease is still currently the most common fungal infection in patients with hematological malignancies (22). Despite new antifungal drugs that are available, mortality rates are as high as 50 to 70% (3, 26). *Aspergillus fumigatus* is the predominant etiological agent, followed by *A. flavus* and *A. terreus*. Early initiation of effective antifungal treatment is essential in order to improve the outcomes for infected patients. Microbiological cultures cannot be considered early diagnostic techniques, and their usefulness depends on the possibility of obtaining samples of deep tissues. The utility of standard blood cultures for *Aspergillus* spp. is limited because of a high percentage of false-negative results, even in patients with disseminated aspergillosis.

The diagnosis of IA has been improved in recent years with the generalization of the techniques for the quantification of galactomannan (GM) and β -D-glucan and techniques for high-resolution imaging (18, 28). The definitions of proven, probable, and possible invasive fungal disease developed by the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) are essentially based on these procedures. To define a case of probable aspergillosis, GM positivity in serum is required in conjunction with radiological evidence (8).

However, GM assay yields a number of false-positive results due to cross-reactivity between *Aspergillus* spp. and other fungi

(29) or to the detection of circulating GM resulting from contamination by certain antibiotics or parenteral nutrition preparation (1, 23, 25). Several studies have shown that previous administration of antifungals such as itraconazole prophylaxis reduces the sensitivity of this technique (19). Moreover, an article published in 2009 suggests that GM sensitivity is significantly lower in patients with IA caused by *A. fumigatus* than in infections caused by other species of *Aspergillus* (13% versus 49%; $P < 0.01$) (9). The β -D-glucan is a component of the fungal cell wall. It has a broader range of detection and, although it is useful in diagnosing IA, it has been less widely used than the quantification of GM (14).

Diagnostic techniques based on DNA detection have not been included as diagnostic criteria for fungal infection thus far. Given their advantages, such as sensitivity and speed, real-time quantitative PCR-based assays have been extensively studied and explored as a tool for use for the detection and identification of *A. fumigatus* and other pathogenic fungi in clinical samples (2, 4–6, 10–13, 16, 20, 24, 27).

The main concern about molecular detection of IA is the lack of standardization. Most of published studies do not include third-party validation. In addition, there are many PCR protocols, and consensus regarding the type of clinical sample (plasma, serum, whole blood, or bronchoalveolar lavage), extraction method, and type of PCR is needed. The European *Aspergillus* PCR Initiative (EAPCRI), a working party of the International Society of Human and Animal Mycosis (ISHAM), is in the process of standardizing PCR-based assays in order to determine the diagnostic role of the *Aspergillus* PCR and to evaluate its inclusion as a diagnostic option (30, 31). We studied retrospectively the performance of a PCR-based assay. A comparative study of sensitivity of blood and serum samples was also performed.

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TABLE 1. *A. fumigatus* PCR results categorized by sample and stratified according to EORTC/MSG definitions

| Sample type | PCR results for samples according to EORTC/MSG definitions ^a | | | | | | | |
|-------------|---|------------------|----------------|------------------|----------------|------------------|----------------|------------------|
| | Proven | | Probable | | Possible | | Not classified | |
| | No. of samples | No. positive (%) | No. of samples | No. positive (%) | No. of samples | No. positive (%) | No. of samples | No. positive (%) |
| Serum | 28 | 5 (18) | 265 | 65 (24.5) | 51 | 14 (27.4) | 11 | 6 (54.5) |
| Whole blood | 30 | 4 (13) | 267 | 63 (23.6) | 50 | 18 (36) | 9 | 4 (44) |

^a The total numbers of samples analyzed and the numbers and percentages of positive samples determined by the PCR technique are indicated.

MATERIALS AND METHODS

A. fumigatus PCR performance was retrospectively analyzed in samples of patients at high risk for IA and for which an infection was suspected. A total of 38 adult patients with positive PCR results were included in the study. Serial blood and serum samples were taken between 2005 and 2010 and sent from different Spanish hospitals to the Mycology Department of the Spanish National Center for Microbiology at the Instituto de Salud Carlos III for *Aspergillus* DNA detection by real-time PCR. A total of 356 whole blood and 355 serum samples were analyzed. Both blood and serum samples were available in 26 cases. Blood was only available for seven patients and serum only for five patients. Clinical data and microbiological and radiological results were reviewed to classify cases according to the EORTC/MSG 2008 criteria. Samples were stored at -20°C .

An *A. fumigatus* real-time PCR assay (RT-PCR) was performed as described previously by Cuenca-Estrella et al. (7) with a starting volume of 200 μl of serum or whole blood. A pair of primers and a molecular beacon probe specific to *A. fumigatus* were designed and directed at ribosomal DNA, specifically at the internal transcriber spacer 1 (ITS1). DNA was extracted using the QIAamp DNA minikit (Qiagen Izasa, Madrid, Spain) from a 200- μl sample, and it was finally eluted in 50 μl . Two microliters of DNA from each sample was used for each RT-PCR, which contained a final volume of 20 μl with 3 mM MgCl_2 , 0.5 μM concentrations of each primer, and a 0.4 μM concentration of the molecular beacon probe. Preincubation was performed at 95°C , followed by 45 amplification cycles and then denaturation (15 s at 95°C), annealing (30 s at 56°C), and extension (5 s at 72°C).

All samples were tested in duplicate. Controls were used throughout the process (DNA extraction and PCR amplification), including negative, positive, and PCR inhibition controls. For positive controls, DNA from the *A. fumigatus* CNM-CM-237 strain was used in both serum and blood samples (from the collection of filamentous fungi of the Mycology Department of the Spanish National Center for Microbiology). Commercial kits were screened for contamination.

Results were considered positive when an exponential increase in fluorescence was detected compared to negative controls, prior to 40 cycles of amplification (7). The detection limit was of 1 fg of DNA per μl of sample (cycle 42 of amplification). Amplified DNA was quantified by linear regression. A standard curve was made with five replicates of eight different genomic DNA concentrations of *A. fumigatus* CNM-CM-237 (range, 10 ng/ μl to 1 fg/ μl). Regression lines were made between the logarithms of DNA concentrations and the PCR cycle at which we began to detect fluorescence (crossing point and threshold cycle [C_T]).

Descriptive and comparative analyses were performed. Differences in the proportions of positive PCR results were determined by the Fisher exact test or by chi-square analysis. Differences in the concentration of DNA by samples and the C_T were determined by the analysis of variance (Bonferroni *post hoc* test). The association between the result of PCR assay and the type of clinical sample was assessed by the chi-square test. $P < 0.01$ was considered statistically significant. Statistical analysis was performed using Statgraphics Plus5.1 (Statpoint Technologies, Inc., Warrenton, VA).

RESULTS

Patient classification. Of 38 patients at risk for developing IA and with positive results for *A. fumigatus* PCR, 31 were classified as having IA according to the EORTC/MSG 2008 criteria (8). The most common underlying diseases of the patients included in the present study were oncohematological disorders, including 19 (50%) patients with acute myeloblastic leukemia, 5 (13.2%) with non-Hodgkin's lymphoma, 2 (5.2%)

with acute lymphoblastic leukemia, and 1 (2.6%) with myelodysplastic syndrome. Four patients (10.52%) had undergone organ solid transplant, and one was a patient treated with a high dose of corticoids. No data were available for 6 of 38 patients. Four patients were classified as having proven aspergillosis, fourteen as having probable aspergillosis, and thirteen as having possible invasive fungal disease. Seven patients were not stratified under this approach because they did not match the EORTC criteria and were categorized as not classified cases (GM enzyme-linked immunosorbent assay positive without radiological evidence).

PCR performance. We analyzed 711 samples retrospectively from a group of 38 adult patients (average of 9 samples per patient; range, 1 to 57) at high risk for IA. The *A. fumigatus* PCR assay results were positive for 89 of 356 (25%) whole-blood samples and 90 of 355 (25.35%) serum samples. Table 1 shows the results from PCR analyses categorized by sample type according to the EORTC/MSG classification criteria. No significant differences were found by sample and by EORTC/MSG criterion in the PCR performance. The rates for positive PCR results were very similar for both blood and serum specimens.

Comparable results were seen when the results of the analysis were categorized by patients. The PCR results evaluated according to IA classification criteria and patients are presented in Table 2. Briefly, 31 of 38 patients included in the exercise had serum samples available for analysis, and 33 had whole-blood samples. A PCR-positive result was seen in 29 of the 31 (93.5%) patients for which serum was analyzed and in 31 of the 33 (93.9%) patients with whole-blood specimens. Negative PCR results were observed more commonly in patients classified as having possible IA (Table 2).

Both serum and blood samples were available for study among a subgroup of patients (26/38 [68%]). This allowed a more comprehensive analysis, and a comparison by clinical specimen of the PCR performance could be made. In this subgroup, the PCR assay was positive in both serum and blood in 22 of 26 (84.5%) cases. In the remaining four patients, two had positive results only for serum samples, and the other two only had positive results for whole blood. Analyzing these results together, the PCR assay was positive in serum in 24 of 26 (92%) cases and showed the same rate of positivity in blood samples. While performing the same exercise using EORTC/MSG criteria (Table 2), we observed some nonsignificant differences between the samples. The serum samples were positive by PCR in 100% of the cases classified as probable IA, whereas the blood samples were positive in 92.8% of the cases classified as probable IA ($P = 0.326$). In possible fungal infec-

TABLE 2. *A. fumigatus* PCR results categorized by patient and stratified according to EORTC/MSG definitions

| Sample type | PCR results for patients according to EORTC/MSG definitions ^a | | | | | | | |
|-----------------------|--|------------------|-----------------|------------------|-----------------|------------------|-----------------|------------------|
| | Proven | | Probable | | Possible | | Not classified | |
| | No. of patients | No. positive (%) | No. of patients | No. positive (%) | No. of patients | No. positive (%) | No. of patients | No. positive (%) |
| Serum | 2 | 2 (100) | 13 | 13 (100) | 10 | 8 (80) | 6 | 6 (100) |
| Whole blood | 4 | 4 (100) | 14 | 13 (92.8) | 11 | 10 (90.1) | 4 | 4 (100) |
| Whole blood and serum | 2 | 2 (100) | 13 | 12 (92.3) | 8 | 5 (62.5) | 3 | 3 (100) |

^a The total numbers of patients with samples available for analysis and the numbers and percentages of patients with positive samples as determined by the PCR technique are indicated.

tion category, the serum was positive according to PCR in 80% of the patients and the whole blood was positive in 90.1% ($P = 0.475$).

Regarding the C_T and the concentration of DNA amplified, significant differences were not observed either. The average C_T for positive blood samples was 37.6, and the average C_T for serum was 37.4. DNA concentration ranged between 10 and 50 fg per μ l of sample, with average DNA concentrations of 10.2 and 11.7 fg in positive blood and serum samples, respectively ($P > 0.01$). Patients categorized as having proven, probable, and possible IA showed comparable C_T values and DNA concentrations since significance differences were not found when the positive PCR results were analyzed according to the EORTC/MSG definitions.

DISCUSSION

In recent years, molecular techniques based on nucleic acid detection of fungal pathogens, including *A. fumigatus*, have experienced a breakthrough. The optimal specimen for the molecular of IA is still under discussion. There are several papers using different types of samples such as respiratory samples, biopsy specimens, and hematological samples for diagnosing IA, but serum and blood samples have been more widely used since they are sterile, noninvasive samples and easy to obtain from patients (16).

Some reports have compared the performance of different blood fractions for the detection of *A. fumigatus* DNA in clinical specimens (17). Plasma PCR was found to be less sensitive than PCR performed on whole blood for the detection of *Aspergillus* DNA in neutropenic patients. Costa et al. (6) recommended the use of serum for detecting circulating *A. fumigatus* DNA for making a diagnosis. Some studies have reported, however, that the sensitivity of the PCR assay for IA diagnostics is lowest when serum is used as clinical sample (31).

We analyzed the performance of a specific RT-PCR technique for the detection of *A. fumigatus* DNA in a high number of blood and serum samples from patients at high risk for IA and for whom positive PCR results were noted (15). The results indicated that PCR performance is very similar in both blood and serum samples. The rate of positive samples, the percentage of patients diagnosed with IA by being PCR positive, the *A. fumigatus* DNA concentration, and the cycle in which DNA was detected (C_T) were comparable for both types of clinical sample.

A study published recently evaluated the diagnostic efficacy

of using a combination of two quantitative *Aspergillus* PCR assays targeting mitochondrial and ribosomal DNA in patients with risk factors for IA (21). PCR techniques were carried out on GM-positive serum samples. These authors demonstrated a good performance for the PCR assays and concluded that the use of serum for PCR determinations had several advantages, such as the use of the same sample for GM and DNA detection, the fact that serum is easy to store as frozen samples, and the fact that the standardization of DNA extraction is straightforward since automated commercial kits can be used (21).

We share that opinion and, from a practical point of view, we believe serum is the best type of hematological clinical sample to use for *Aspergillus* DNA detection. Serum processing is easier than processing whole blood. Blood samples for PCR determination require the use of an anticoagulant such as EDTA which could interfere with the PCR. The presence of anticoagulants leads to rather complicated purification procedures to avoid inhibition (1). Such procedures can get PCR-based assays away from clinical laboratories. In addition, sample contamination with airborne conidia or *Aspergillus* DNA can occur if complicated procedures of purification are needed, thus decreasing the performance and accuracy of the PCR techniques.

The use of a combination of serum and blood to increase the sensitivity of PCR determinations is also a possibility. However, our results showed that this combination does not significantly improve the PCR-based assay performance. The higher sensitivity of a combined analysis of blood and serum could be explained by the fact that performance of the DNA detection improved when large volumes of hematological samples are studied. Suarez et al. (24) proved that a PCR-based assay could be used for early detection of IA if large serum volumes were analyzed. An analysis of blood samples did not increase the accuracy of the assay. Another significant issue could be the number of different DNA targets available in whole blood and serum. Free circulating *Aspergillus* DNA in serum and fungal cell-associated DNA in whole blood can increase the likelihood of detection contributing to a higher sensitivity of combined serum and whole-blood analysis.

Our study did not include an analysis of large volumes since only 200 μ l per sample was processed. However, 711 samples from 38 adult patients (average, 9 samples per patient; range, 1 to 57 samples) were analyzed retrospectively showing that serial determinations of *A. fumigatus* DNA in small volumes of serum samples can be as reliable as detection in large volumes of hematological samples.

In conclusion, serum samples can be used for DNA detec-

tion of *A. fumigatus* in patients in risk for IA. The performance of this PCR-based quantitative assay was similar in both serum and whole blood samples. Therefore, we recommend serum samples as the most convenient hematological sample to use for *Aspergillus* DNA quantification.

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