Use of Real-Time PCR To Process the First Galactomannan-Positive Serum Sample in Diagnosing Invasive Aspergillosis

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Invasive aspergillosis (IA) is the main cause of mortality due to infection in leukemic patients and allogeneic stem cell transplant recipients (21). The average death rate is approximately 50% in leukemic patients and 90% in allogeneic bone marrow transplant recipients (14). This poor prognosis is due in part to a failure to diagnose early, which in turn results in delays in initiating antifungal therapy.

Definitions of proven, probable, and possible IA, based on a combination of host factors and radiological, clinical, and biological data, have been recently proposed so that homogeneous groups of patients can be formed for clinical research (3). In these consensual proposals, positive galactomannan (GM) antigenemia has been included as a microbiological criterion for probable or possible diagnosis. This has been mainly based on the reported 90% sensitivity of the Platelia Aspergillus test (Bio-Rad, Marnes-la-Coquette, France) (17). It is now a common practice in Europe, especially in hematological units, to screen patients at risk for IA twice a week (23). A positive GM result in a neutropenic patient frequently triggers antifungal therapy, as a positive GM result has been reported to appear before clinical signs (18, 25, 27).

However, false-positive GM results are frequent; GM has thus been used as a microbiological criterion only when two separate serum samples are positive (3). This procedure has led to an improvement in the predictive positive value, although the rate of false-positive results remains high, between 10 and 15% (7, 19). This problem of false positives has been recently exacerbated because of GM contamination of samples from patients treated with some piperacillin-tazobactam and amoxicillin-clavulanic acid batches (1, 22, 28–30). False-positive GM results could also be due to the digestive absorption of galactomannan in food, as well as some bacterial membrane-associated molecules (2, 23, 24).

This underlines the necessity for testing more than one sample. However, the wait for additional samples can delay the initiation of treatment. Instead of waiting for more samples, another test could be performed on the same sample to improve the specificity of the GM test.

Aspergillus DNA is another possible biological marker for the diagnosis of IA (6, 10–12). However, the international consensus defining invasive fungal infections (IFIs) in hematological patients does not include PCR as a diagnostic tool, because of divergent results due to the lack of standardization (3). Some of the shortcomings of the standard PCR assays are eliminated by real-time PCR assays (5). This new technology has been adapted for the detection of Aspergillus DNA (8, 9, 16, 26).

We wondered whether detecting circulating Aspergillus DNA in the first positive GM sample by real-time PCR could help in diagnosing IA in hematological patients. In an emergency diagnosis of IA, a real-time PCR performed on the same serum sample could shorten the time response and help in decision making. Therefore, we retrospectively screened the first GM-positive serum sample, collected as part of our routine practice and stored at −20°C, for Aspergillus DNA by real-time PCR.

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MATERIALS AND METHODS

Thirty-one consecutive patients from the hematology unit of Besançon University Hospital were studied between January 2001 and December 2002. The inclusion criteria were the presence of risk factors for IA, as they are defined by the National Institute of Allergy and Infectious Diseases Mycoses Study Group and the European Organisation for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (MSG/EORTC) consensus (neutropenia is defined as an absolute neutrophil count below $0.5 \times 10^9$/liter, and/or persistent fever under broad-spectrum antibiotic therapy, and/or recent or current use of immunosuppressive agents or corticosteroids) and one positive GM result.

The GM test was performed using the Platelia Aspergillus sandwich enzyme-linked immunosorbent assay (Bio-Rad). A GM test was positive when the index of purified DNA of $\textit{Aspergillus fumigatus}$ was detected by using real-time PCR with a Light Cycler instrument (Roche Molecular Biochemicals, Meylan, France) as previously described (9). Briefly, DNA extraction was performed using the High Pure PCR template kit (Roche Molecular Biochemicals). We used a starting volume of 200 nl of serum, eluted in 50 nl of elution buffer. PCR was performed with 5 nl of DNA extract. Mitochondrial DNA from $\textit{Aspergillus fumigatus}$ and $\textit{Aspergillus flavus}$ was detected by using previously described primers and probes (9). Fluorescence curves were analyzed with Light Cycler software, version 3.5. Quantitative results were expressed by determination of the threshold of detection, or crossing point (Cp), which marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. Thus, the higher the Cp value was, the smaller the amount of DNA in the sample was. Two independent DNA extractions were performed for each serum. Each DNA extract was submitted to PCR amplification. Thus, two Cp results, one for each replicate, were obtained for each serum sample tested. Two analyses of the results were performed: one analysis designated a sample positive when the Cp value was $\leq 40$ cycles in both replicates; the second analysis designated a sample positive when at least one replicate had a Cp value of $\leq 43$ cycles. One positive control, using $\textit{A. fumigatus}$ DNA, and two negative controls were added to each run. The negative controls were sterile water samples, one submitted to the extraction and amplification protocols and the other submitted only to the amplification protocol. This was done to verify that there was no contamination in any step of the technical procedure. Strict measures were taken to prevent DNA contamination. DNA extractions were performed inside a biological safety cabinet. Contamination with previously amplified products was prevented by the systematic use of uracil-N-glycosylase.

RESULTS

Classification of the 31 patients according to the consensual MSG/EORTC criteria was as follows: 1 patient had proven scedosporiosis, 1 patient had proven candidiasis, 1 patient had proven IA, 6 patients had probable IAs, 11 patients had possible IAs, and 11 patients had no IFI. Samples from the patient with candidiasis and the patient with scedosporosis were PCR negative and were excluded from further analysis because these infections were due to species that were not targeted by the primers or the probe used. Table 1 shows clinical and microbiological data from the 29 other patients.

These 29 patients had a first positive GM sample (one inclusion criterion). For four of these patients, no additional sera were tested for GM because of patient death or transfer to another unit. A second serum sample was available for GM testing for the 25 other patients. Fourteen of the patients had a second positive sample, which led to the inclusion of this result as a microbiological criterion for the final classification of IA. Thus, for 11 (44%) of the 25 patients who had a second test, the first GM result was considered a false positive; these 11 patients were non-IFI in the final classification. No PCR test was performed on these additional samples, as our study was not designed to compare the parallel kinetics of GM and DNA. Amplifications were performed in 15 different runs. Each run included a negative extraction control, a negative amplification control, a positive control, and 10 serum samples. No PCR-positive result was observed in the negative controls. A dilution of purified DNA of $\textit{A. fumigatus}$ (2.5 fg/μl) was used as a positive control and consistently gave a Cp value of 36.3 (± 0.5) cycles. The Cp values for the clinical samples were always $>36$ cycles, i.e., equivalent to a DNA concentration of $<2.5$ fg/μl of serum.

When a result was considered positive only when the Cp value was $\leq 40$ cycles in both replicates, samples from only four patients (patient numbers 1, 2, 4, and 6) (Table 1) were PCR positive. All four of the patients were classified as having proven, probable, or possible IA (Fig. 1A). With this analysis of PCR results, the association between probable-proven cases and positive PCR results was significant (Fisher’s test; $P = 0.033$).

When a result was considered positive when at least one replicate had a Cp value of $\leq 43$ cycles, 13 patient samples were PCR positive and 16 were PCR negative. No significant association between PCR results and final classification of IA was observed, especially because 4 of the 11 non-IA patients had samples that were PCR positive (Fig. 1B). However, a positive PCR was significantly associated with a fatal outcome, as all the 16 patients with PCR-negative samples had a favorable outcome, while 5 out of 13 PCR-positive patients died (Fisher’s test; $P = 0.01$). Radiological and/or clinical signs were recorded for these five patients in the final classification.

Given the reported high rate of false-positive GM results, some, but not all of the GM-positive patients were given anti-$\textit{Aspergillus}$ antifungals intravenously (i.v.) at the time of the first positive GM result. Seven of the 13 PCR-positive patients were given antifungals. Fourteen of the 16 PCR-negative patients were not given antifungals. These decisions were made with no knowledge of the PCR results, due to the retrospective design of the study. When PCR results were compared to the final analysis, if the 13 PCR-positive patients had been given antifungals, four additional treatments would have been prescribed to non-IFI patients. These patients were not neutropenic and had no clinical signs at the time of the first positive GM. However, two additional patients with possible IA would have been given antifungals. Both died having received no antifungal therapy. By contrast, negative PCR results would have lent further support to the decision not to administer antifungals, a decision that was made at the time of the first serum sampling for the 14 patients with positive GM. At final analysis, these 14 patients were classified as non-IFI ($n = 7$), possible IA ($n = 6$), and probable IA ($n = 1$). For all of them, the outcome was favorable with no anti-$\textit{Aspergillus}$ therapy.

DISCUSSION

In this pilot study, we were unable to show a clear correlation between a real-time PCR result at the time of the first GM-positive test and the final classification of IA using the consensus definitions (3). The lack of correlation could be linked to the high rate of false-GM-positive results. In our
study, the false-positive rate was 44% when only the first test was taken into account; this is much higher than what the literature reports. Therefore, the contamination of the reagents used with *Aspergillus* DNA is unlikely. In contrast, fungal DNA contamination from antibiotics could occur in the same way that GM contamination could (1, 22, 28, 29). To deal with this problem, the time of the blood draw should be recorded and compared to the administration of i.v. antibiotics, and GM and PCR testing of every batch of antibiotics should be performed (which would be difficult to implement on a routine basis).

An intriguing point in our study is the fact that the amount of DNA, as estimated from the Cp values, was very small in all the PCR-positive patients. This had already been reported when quantitative PCR assays were used (8, 9, 16). Consequently, several problems in interpreting real-time PCR were encountered. First, quantification of very small DNA amounts exceeded a few femtograms per microliter. According to Poisson's law, PCR results cannot be consistently positive at these concentrations calculated after the positive control never exceeded a few femtograms per microliter. Therefore, the contamination of the reagents used with *Aspergillus* DNA is unlikely. In contrast, fungal DNA contamination from antibiotics could occur in the same way that GM contamination could (1, 22, 28, 29). To deal with this problem, the time of the blood draw should be recorded and compared to the administration of i.v. antibiotics, and GM and PCR testing of every batch of antibiotics should be performed (which would be difficult to implement on a routine basis).
very low concentrations. Thus, one replicate can be positive and the other one can be negative, depending on whether the target DNA is present or not in the volume tested. For decision making, we suggest considering a sample as positive even if only one replicate is <43 cycles. In our retrospective study, two patients had only one positive PCR replicate and both died within 5 days; one of them did not receive i.v. antifungal therapy.

The small DNA amounts prevent any analysis of correlation between DNA and GM quantification. A larger DNA burden could indicate more advanced disease, as recently suggested, with the use of a different Aspergillus DNA quantification assay (31). In our study, some patients had very small DNA burdens, even in the presence of high GM levels and clinical and/or radiological signs and even just before death. The fact that PCR is less sensitive than GM in humans (6, 13) and animals (4) has already been reported. On the other hand, when the Cp value was ≥40 cycles in both replicates, the patients were all classified as IA. This suggests that the larger the DNA amount, the more probable the diagnosis of IA.

Indeed, one of the main points of our study is that a PCR-positive result, when obtained on the first GM-positive serum sample from patients at risk for IA, was associated with a poor prognosis. Clinical and radiological signs, also associated with a poor prognosis, have been reported to appear after (median, 6 days) the first GM result in more than half of the cases (18). Thus, in decision making, a PCR-positive result could reinforce the decision to initiate antifungal therapy, with the understanding that the therapy could be stopped if diagnosis of IA was not confirmed. In contrast, a negative PCR result could argue in favor of postponing costly treatment with potential side effects until additional arguments favoring therapy were presented. However, a PCR-negative result must be interpreted with caution, because our real-time PCR targets A. fumigatus and A. flavus and other molds could be involved. These preliminary data need to be confirmed by a prospective study with a larger cohort.

Our study suggests that performing real-time PCR as soon as the first serum sample is positive for GM can be of interest, not to improve diagnosis of IA but as a help in decision making with patients hospitalized in a hematology department and at risk for invasive fungal infections. Our results also underline the difficulties in interpreting the meaning of biological tests in the absence of definite standards. For prospective studies, our data suggest the need to record antibiotic therapy and possibly the time of the venous punctures and compare them to the results of GM and PCR testing.

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REFERENCES


