Comparison of Serum Galactomannan Antigen Detection and Competitive Polymerase Chain Reaction for Diagnosing Invasive Aspergillosis

Stéphane Bretagne, Jean-Marc Costa, Emmanuelle Bart-Delabesse, Nathalie Dhédin, Claire Rieux, and Catherine Cordonnier

To improve the diagnosis of invasive aspergillosis (IA), we retrospectively compared competitive polymerase chain reaction (PCR) and sandwich ELISA for detection of serum galactomannan (GM) antigen. We studied 281 serum samples collected weekly during the period at risk for IA from 41 selected hematology patients. Twenty-two patients had confirmed, probable, or suspected IA, according to clinical and mycologic data. Fifteen of them had positive GM titers (87 samples) and 12 had positive PCRs (20 samples). Nineteen of the 20 PCR-positive samples were also GM-positive. Of the 19 patients without IA (83 samples), one had 3 GM-negative samples. Neither test anticipated the initiation of antifungal therapy on the basis of clinical suspicion. Both tests were more likely to be positive before death. This study suggests that PCR on serum samples is not more sensitive than GM detection. However, PCR can improve the specificity of the GM test. Together, these noninvasive tests should improve the diagnosis of IA.

New methods of diagnosing invasive aspergillosis are currently being investigated to improve the ability to make the diagnosis noninvasively, particularly in high-risk patients. Problems in the diagnosis of invasive aspergillosis include the limited sensitivity of bronchoalveolar lavage (50%–60%) [1–3] and the difficulty in interpreting a positive culture which could reflect contamination or colonization as well as invasive infection. Both DNA detection by PCR [4] and antigen detection tests [5] have been investigated, but their usefulness has been limited to date.

In most studies of PCR, the method has been used on bronchoalveolar lavage (BAL) fluid samples and has been associated with a 10%–25% rate of false-positive results [4], mainly because PCR cannot distinguish between true infection, colonization, and contamination of BAL samples by conidia. A positive result of PCR on blood could be potentially more useful, as colonization of blood is unlikely and contamination with air-borne conidia should be avoidable when tubes are opened under a laminar-air-flow hood.

Among the techniques based on antigen detection, the one used most has been the sandwich ELISA for detection of Aspergillus galactomannan (GM) antigen [6]. This antigen is secreted in high amounts by the fungus. The detection threshold of the ELISA is 1 ng of GM per mL of serum, an amount 10 times lower than the threshold of the commercial Pastorex Aspergillus latex kit (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) [7, 8].

Probably because of this better sensitivity, the main limitation of routine GM testing is false-positivity, which has occurred at rates of 5.7% [9], 19% [7], and 29% [8]. When at least two consecutive positive tests have been required to consider a patient to be GM-positive, the false-positivity rate generally has decreased but only to 12% [10] or 16% [8]. False-positive reactions are probably due to molecules that mimic the epitope recognized by the monoclonal antibody to GM [11]. Another possibility may be that aspergillosis occurs but then resolves following recovery from neutropenia and treatment with amphotericin B, before confirmation of the diagnosis.

We postulated that using the competitive PCR test developed in our laboratory [12] on patient serum concurrently with GM testing could improve the specificity of GM detection. As the molecules detected by the two techniques are completely different, a GM-positive sample would be definitively positive for Aspergillus if it were concurrently PCR-positive. Moreover, we studied the timing of positive tests to determine whether one could anticipate the other in making early diagnosis possible.

Patients and Methods

Patients

A group of 41 hematology patients considered to have been at high-risk for developing invasive aspergillosis were retrospectively selected from 1,213 patients seen on a hematology service over a 2 1/2-year period. The risk factors for invasive aspergillosis were neutropenia (<500 neutrophils per μL of blood) and/or use of steroid therapy for >10 days and/or rehospitalization during the first 6 months following allogeneic bone marrow transplantation (in patients with graft-versus-host disease). These patients had at least three consecutive serum samples stored and had reliable clinical data available. Their medi-
probable: Aspergillus confirmed: invasive aspergillosis were categorized according to the following criteria: (1) confirmed: histologically proven disease and an Aspergillus-positive culture of a specimen obtained by percutaneous aspiration [13]; (2) probable: development of a new opacity in lung and isolation of an Aspergillus species or of septate branched hyphae on a wet mount examination of BAL fluid, transbronchial aspirate, or sputum (or histologically proven disease without any positive culture to confirm the species of filamentous fungus involved); and (3) suspected: temperature of >38°C for >5 days that was unresponsive to antibacterial agents, in a patient at risk for invasive aspergillosis who started receiving empirical antifungal treatment and had a new opacity on a chest radiograph (no evident etiology).

On the basis of these criteria, 22 patients were considered to have invasive aspergillosis (table 1), of whom 6 had confirmed invasive aspergillosis (patients 1–6), 12 (patients 7–18) had probable invasive aspergillosis, and 4 (patients 19–22) had suspected invasive aspergillosis. Nineteen patients did not develop invasive aspergillosis. Three of them had fungal disease other than aspergillosis, including one with invasive sinusitis due to Scopulariopsis brevicaulis and two with fungemia due to Candida tropicalis.

### Serological Tests

Serum were collected at admission and at least once weekly for each patient at risk for invasive aspergillosis, as long as the risk factors persisted. Serum samples were processed under a laminar-flow hood, heated at 56°C for 30 minutes, and split into two aliquots; 2 mL was kept at 4°C for <7 days until GM testing, and the remaining 2 mL was immediately stored at −70°C for PCR.

ELISA was performed as previously described with plates provided by Sanofi Diagnostics Pasteur (Platelia Aspergillus) [10]. Each run contained a negative control (Tris-buffer saline alone) and two positive controls, containing 1 and 10 ng of GM per mL. The optical density results were converted into GM concentrations expressed in ng/mL, as deduced from the calibration curve obtained for each run. Positive samples contained a concentration of GM of ≥1 ng/mL. Although the manufacturer recommends that the results should be expressed as an index (index <1, negative; index >1 and <1.5, doubtful; index >1.5, positive), we used quantitative results since the data are useful in evaluating the efficacy of treatment.

The frozen serum aliquots were coded and the amplification was done blindly. After thawing, 200 μL of serum was pro-

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### Table 1. Characteristics of 22 patients with confirmed, probable, or suspected invasive aspergillosis according to mycologic and clinical criteria and comparative results of galactomannan (GM) antigen tests and PCR on serum samples.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Patient’s sex/age (y)</th>
<th>Underlying disease</th>
<th>Allogeneic bone marrow transplant</th>
<th>Diagnostic specimen</th>
<th>Hyphae on direct examination</th>
<th>Fungal culture result</th>
<th>Classification of aspergillosis*</th>
<th>Total (n = 198)</th>
<th>GM-positive (n = 87)</th>
<th>PCR-positive (n = 20)</th>
<th>Specificity of PCR for Aspergillus</th>
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<td>M/24 MD</td>
<td>Yes</td>
<td>Skin biopsy</td>
<td>Yes</td>
<td>Aspergillus flavus</td>
<td>C</td>
<td>3</td>
<td>3</td>
<td>2</td>
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<tr>
<td>2</td>
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<td>Yes</td>
<td>Skin biopsy</td>
<td>Yes</td>
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<td>C</td>
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<td>No</td>
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<td>Yes</td>
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<td>C</td>
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<td>P</td>
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<td>M/35 Sarcoma</td>
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<td>Lung biopsy</td>
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<td>BAL</td>
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<td>8</td>
<td>7</td>
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<td>A. fumigatus</td>
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NOTE. ALL = acute lymphocytic leukemia; AML = acute myeloid leukemia; BAL = bronchoalveolar lavage (fluid); CML = chronic myelocytic leukemia; MD = myelodysplasia.

* C = confirmed; P = probable; S = suspected.
Each amplification run contained several negative controls. Sequencing of two PCR products from reference strains performed in a 48-well thermal cycler (Perkin Elmer Cetus). A sample was considered negative only if the signal with the internal control probe was positive. A clinical sample test was inconclusive if no signal was observed with both probes.

To differentiate the two main Aspergillus species responsible for aspergillosis (i.e., A. fumigatus and A. flavus), 1 μL of the PCR products of positive samples was run on a 24-cm-long acrylamide urea gel (6% acrylamide, 8.3 M urea, and 1 X TBE) for 4 hours under 1,500 V. PCR signals were read with the model 373 automated DNA sequencer (Applied Biosystems, Courtaboeuf, France) with an internal standard. The PCR product for A. fumigatus was 157 bp and that for A. flavus was 140 bp. Sequencing of two PCR products from reference strains (A. fumigatus IP 2279.94 and A. flavus IP 597.69) confirmed the difference between the two DNA sequences was a 17-bp deletion in the A. flavus sequence (data not shown). To confirm the specificity of the reaction, the DNA of collection strains was tested (Aspergillus niger IP 1431-83, Aspergillus terreus IP 1136, Aspergillus nidulans IP 17-60, Aspergillus ustus 1587-85, Penicillium purporogenum 2281-94, and S. brevicaulis IP 691-63); each of these reactions was negative.

Results
A total of 281 serum samples from 41 patients were available for analysis (mean number of samples per patient, 6.9; range, 3–20 samples). Thirty-two percent (90 of 281) were found to be GM-positive and 7% (20 of 281) were PCR-positive. All of the PCR-positive samples and all but three GM-positive samples were from patients who were classified as having invasive aspergillosis (figure 1). Of the 18 patients with positive mycologic data, 14 (78%) had at least 2 GM-positive sera and 9 (50%) had at least 1 PCR-positive serum.

Figure 1. Distribution of results for the 41 patients tested for Aspergillus galactomannan (GM) antigen in serum samples (GM+ = at least 2 positive serum samples) and for DNA by PCR (PCR+ = at least 1 positive sample). Classification as invasive aspergillosis (■) or no aspergillosis (●) was based on the basis of clinical and mycologic data.
At the time of the first GM-positive result. In those cases in which PCR and mycologic results were positive, the length of the PCR products was in congruence with the *Aspergillus* species identified.

Six patients met the criteria for confirmed invasive aspergillosis. All had multiple GM-positive samples, and two also had PCR-positive samples (patients 1 and 5). There were no positive mycologic data for patient 5 until shortly before death, but samples had been transiently GM- and PCR-positive 2 months earlier, in the setting of sinusitis treated with amphotericin B. The PCR-negative result was expected for patient 4, who had an *A. ustus* infection diagnosed following percutaneous aspiration of a pulmonary nodule. The GM antibody used in the sandwich ELISA is not specific for any *Aspergillus* species, and *A. ustus* DNA is not amplified in our PCR conditions.

Twelve patients were classified as having probable invasive aspergillosis. Eight (patients 7–14) had GM-positive samples, seven (patients 7–12 and 14) had PCR-positive samples, and four (patients 15–18) had GM- and PCR-negative samples. The data from patient 14 were informative, as the only mycologic findings were hyphae seen on wet-mount examination. The positive PCR result strongly suggested *A. fumigatus* as the fungus responsible. Data for two other patients (patients 15 and 16) were also informative, as they had repeatedly GM- and PCR-negative samples, despite a sputum culture positive for *A. fumigatus*.

Patient 15 had acute leukemia and had had pneumonia (successfully treated with antibiotics) 2 months earlier. He developed a cavitary pulmonary nodule and underwent serological testing only during this second hospitalization. Patient 16 had bronchiolitis obliterans related to graft-vs.-host disease. The positivity of these patients’ sputum for *A. fumigatus* could be explained by the colonization of previous pulmonary lesions. Unfortunately, both died and autopsies were not performed.

Four patients (patients 19–22) were classified as having suspected aspergillosis. All were given empirical amphotericin B therapy, but none had a definitive diagnosis. All had GM-positive samples; moreover, three of them also had PCR-positive samples. The PCR signals suggested that the species involved was *A. fumigatus* in patients 19 and 21 and *A. flavus* in patient 20. All of them survived and had a clinical course consistent with invasive aspergillosis.

All but one of the 19 patients with no evidence of aspergillosis had repeatedly GM- and PCR-negative samples. The one exception had three consecutive positive GM titers measured and died with a cerebral tumor in which no fungal organism was found at autopsy. These GM results were considered to be falsely positive. The three patients with fungal disease other than aspergillosis (one with invasive sinusitis caused by *S. brevicaulis* and two with fungemia due to *C. tropicalis*) had GM- and PCR-negative samples.

**Discussion**

In the present work we compared two new techniques, the double sandwich ELISA test for *Aspergillus* GM and a compet-
itive PCR on blood samples from patients at high risk for invasive aspergillosis. The findings of few studies of this kind have been published to date for comparison with our results. One study report described the simultaneous detection of GM and PCR products in BAL fluid samples [16]. From 35 nonneutropenic patients, 5 and 2 samples were PCR- and GM-positive, respectively. Although it would be interesting to know whether the patients were colonized with an Aspergillus species, the high rate of false-positive results suggests to us that BAL fluid is not the best target for PCR and GM tests.

Other authors recently published a comparison between the latex agglutination test, with use of the same monoclonal antibody as the GM ELISA, and a nested PCR of the 18S rRNA of A. fumigatus [17]. Twenty patients with histologically confirmed invasive aspergillosis were tested: 14 were PCR-positive and 12 were GM-positive, but only 10 were positive in both tests. Although the timing of positivity during the course of aspergillosis was not given, this first report was important in showing that serum could be used to detect Aspergillus DNA in patients with invasive aspergillosis.

Comparison with our results is difficult, as the sensitivity of the sandwich ELISA we used is 10 times higher than that of latex agglutination [7, 8]. Moreover, the PCR techniques, the DNA target, the sample processing, and the detection system used were completely different. In diagnostic PCR techniques, enzymatic prevention of contamination and detection of PCR inhibitors should be systematically included. Moreover, a nested PCR is too prone to contamination in a hospital laboratory when routine use is planned.

Recently, Einsele et al. reported on the use of a PCR technique on blood for the diagnosis of fungal disease [18]. They found 100% sensitivity for patients with invasive aspergillosis prior to treatment when two blood samples were considered. For the 8 patients who died, presumably of aspergillosis, the number of positive samples during the week before death was only 8 of 12 (67%). This is in contrast with our findings for the six patients who died with aspergillosis and were both PCR- and GM-positive. Samples were found to be positive before death for five of them, and all had negative tests at the initiation of antifungal treatment. These data suggest a correlation of positive results with expansion of the fungal burden despite therapy, which unfortunately is common in hematology patients.

While several studies have shown the usefulness of the GM antigenemia testing for the diagnosis and follow-up of invasive aspergillosis [6–10], the meaning of a positive PCR signal is less well established. Aspergillus fungemia is highly unusual [19] and was not observed in our patients. Therefore, the PCR-positive results may be surprising in view of the absence of live hyphae in the blood. The first explanation is that false-positive results occur because of contamination. However, we took numerous precautions to avoid false-positive results in the present study, including the use of UNG enzymatic treatment. The serum samples were coded and processed blindly, and no positive PCR result was obtained for any patient with either nonaspergillus infection or no fungal infection. Therefore, contamination is unlikely.

The circulating DNA necessary to initiate amplification could come from fungus that is unable to grow. This may explain the positive PCR obtained by investigators who extracted fungal DNA from the pellet obtained after cell lysis [18]; then, the pellet would contain fungal hyphae. After protoplast formation, the fungal DNA could be extracted and amplified. This seems to us highly unlikely in view of the ability of A. fumigatus to grow on various media and the number of negative blood cultures.

A more likely explanation for PCR-positive results is that Aspergillus DNA from dying hyphae is released into the blood, as occurs in human cancers where mutant DNA from cancer cells is found in blood plasma [20]. As the half-life of circulating DNA is short, probably less than 5 minutes [21], a positive PCR signal is observed only when the fungal burden is large enough to maintain at least 10 molecules of target DNA in 100 μL of serum, which is the sensitivity threshold of the PCR used. This threshold is estimated by the amplification of the internal control systematically added in every clinical sample [12].

Thus, a positive PCR result would have the same meaning as a positive GM titer that increases with fungal burden. Indeed, 12 of 20 positive PCR results were observed in association with GM titers of >5 ng/mL. The fact that the GM assay was more sensitive than PCR in our study could appear surprising to those who have considered PCR to be the ultimate sensitive tool. However, neither the amounts of GM and of DNA released into the blood nor their clearance from blood are known. If the clearance of DNA is faster than that of GM and if there is a huge quantity of GM released by the fungus, the better sensitivity of the GM test would not be surprising. However, whether optimal results of PCR are obtained with the use of plasma, serum, buffy coat, or whole blood remains to be determined.

The finding that neither test could predict the initiation of antifungal therapy was disappointing. This may be different for nonhematology patients who are not systematically given amphotericin B for fever unresponsive to antibiotics. The empirical treatment was probably responsible for the delay in the positivity of both tests. Nevertheless, although it is not possible to give any specificity, sensitivity, or predictive value of both tests since the patients were retrospectively selected, both tests appear to be potentially useful for analyzing cases.

The lack of false positives with PCR and the congruence of the PCR signal with the species obtained in mycologic cultures showed that the specificity of the PCR is high. As the basis of the two tests are completely different, with one targeted at a polysaccharide and the other at a specific DNA sequence, a GM-positive test is definitively positive for us if confirmed by PCR.

Thus, both tests appeared useful for the retrospective analysis of medical files. Three patients in our study had suspected aspergillosis with positive GM and PCR tests and survived...
after receiving amphotericin B. They should probably have been classified as having invasive aspergillosis, despite the lack of mycologic data. This retrospective analysis of medical files with the help of GM and PCR tests may be crucial to evaluate the efficacy of antifungal drugs when autopsies are not performed. In addition, PCR can indicate the fungal species involved, and this can have some epidemiological significance.

In patients at risk for invasive aspergillosis, the results of the sandwich ELISA with use of an antibody to GM and the competitive PCR correlate well with the diagnosis of invasive aspergillosis. The main value of the simultaneous use of these tests is to confirm the diagnosis. The GM test appears more sensitive and is more convenient than PCR for weekly screening in a hospital laboratory. However, the specificity of the GM test is not sufficient and can be improved by PCR. Therefore, when positive, these tests could provide an alternative to aggressive investigations such as lung biopsy. For clinical trials, these tests seem promising for objectively defining invasive aspergillosis.

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References