ASPERGILLUS ANTIGENEMIA SANDWICH-ENZYME IMMUNOASSAY TEST AS A SERODIAGNOSTIC METHOD FOR INVASIVE ASPERGILLOSIS IN LIVER TRANSPLANT RECIPIENTS

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Received 23 March 2000.
Accepted 4 May 2000.

Abstract

Background. Invasive aspergillosis (IA) is an important cause of mortality in liver transplant patients. Clinical and microbiological diagnosis is difficult, and it is frequently achieved only after autopsy. Early diagnosis and antifungal therapy could improve the survival of these patients.

Methods. A retrospective case-control study of IA in liver transplant recipients (OLT) was performed to determine the value of the detection of galactomannan Aspergillus antigen in serum using a sandwich-ELISA test (Platelia, Sanofi Diagnostic Pasteur). Stored frozen serum specimens obtained during the posttransplantation period were used.

Results. Fourteen cases of IA were diagnosed in 240 OLT recipients (IA incidence: 5.8%) during 5 years with 13 deaths (mortality: 93%). Nine case patients and 33 control patients met the criteria required for being considered “valid” for antigenemia analysis. In five of the nine case patients, a serum sample was positive for Aspergillus antigenemia detection. The median value was 5.7 ng/ml (range: 1.6–6.6). Sensitivity of the test was 55.6%, specificity was 93.9%, the positive predictive value was 71.4%, and the negative predictive value was 88.6%. The likelihood ratio of a positive test was 9.2.

Conclusions. Galactomannan detection in serum could be useful for an early diagnosis of IA in OLT recipients.

Invasive aspergillosis (IA) has been reported in 1.5–6% of liver transplant patients (OLT), with high mortality rates. Prompt diagnosis and specific therapy could improve the survival in these patients. Serological methods based on Aspergillus antigen detection could help in the diagnosis. Galactomannan (GA) is a cell-wall antigen of Aspergillus sp. that has been detected in patients with IA. The sandwich-enzyme immunoassay (EIA) is a highly sensitive method developed for detecting GA, and the serum is the best specimen for performing the assay. A retrospective analysis to determine the value of the detection of Aspergillus antigen (using an EIA-sandwich method) in serum for the diagnosis of IA in liver transplant recipients was conducted.

We used stored frozen serum specimens routinely obtained during the posttransplantation period of liver graft recipients. The samples had been stored at -20°C.

Definitions of Fungal Infections (1)

Proven IA infection defined as:

• Tissue histopathology showing septate, acute branching hyphae with or without a positive culture for Aspergillus spp. from the same site or

• In the absence of histopathology, a positive culture from tissue obtained by an invasive procedure (i.e., transbronchial biopsy or percutaneous needle aspiration).

• Histopathologic demonstration of IA at autopsy or surgical procedure.

Probable IA applied only to patients with pulmonary disease and defined as:

1.1.1.
Patients with chest radiographic appearance of new nodules or new cavities.

In addition all patients had to have:

- two sputum cultures or one bronchoalveolar lavage, washings or brushings culture for Aspergillus spp. or
- cytologic examination on bronchoalveolar lavage showing characteristic septate hyphae.

The patients were classified as controls, colonized or with Aspergillus disease by two of the authors (P.M., J.F.), after careful chart review and without knowledge of GM assay results.

Definitions of Case Patient (Cs-Pt) and Control Patient (Cnt-P)

Case patients (Cs-Pt).

A Cs-Pt was any OLT recipient with a proven or probable diagnosis of IA. For the antigenemia analysis, a Cs-Pt was considered valid if more than three serum samples, drawn in intervals, were available. One of the samples had to be obtained within a month before IA diagnosis.

Control patients (Cnt-P).

Three Cnt-P were chosen for each case. They were the nearest (in time) liver transplant recipients, considering Cs-Pt transplantation date. A survival of more than 2 months after transplantation was also required.

A Cnt-P was considered valid for the antigenemia analysis if three or more serum samples, separated in time and obtained during the first six months after transplantation, were available.

Colonized patients with Aspergillus sp were excluded because, based on IA definitions, we were unable to classify them either as cases or as controls for antigenemia specificity analysis.

Antigen detection.

The procedure was performed under sterile conditions, to avoid Aspergillus airborne contamination. The Aspergillus antigen detection test was performed in serum using a sandwich-ELISA technique (Platelia, Sanofi Diagnostic Pasteur) following the method described by the manufacturing laboratory. Briefly, 100 µL of test serum was added to 100 µL of the conjugate (4% EDTA solution) and boiled at 100°C for 3 min and centrifuged at 10000×g for 10 min. After centrifugation, 50 µL of a reaction mixture containing anti-GM monoclonal antibody EB-A2 was added to 50 µL of the supernatant to be tested. The 100-µL mixture was placed in wells of a microtitration plate previously coated with the same monoclonal antibody EB-A2 and incubated at 37°C. After 90 min of incubation, the plates were washed five times before 100 µL of buffer containing o-phenylenediamine dihydrochloride solution was added. Plates were incubated another 30 min in darkness at room temperature, followed by the addition acid to stop the reaction of 100 µL of 1.5 M sulfuric. The optical density was read. Positive and negative controls were included in each assay. An OD index of Aspergillus antigen detection with a value of 1 nanogram of GM per microliter or higher was considered positive.

Positive samples were retested in parallel in another assay. A positive sample was considered positive only after retest confirmation. A single positive specimen was required to classify a patient as "Positive" for the antigenemia assay.

Statistical analysis.

Sensitivity, specificity, and predictive values were obtained in comparison with the reference diagnosis, which was based on cases of definite or probable aspergillosis. The 95% confidence intervals for the proportions were calculated using the Fleiss quadratic method (binomial distribution). Analysis was performed with the Epinfo software package (CDC, Atlanta GA). Likelihood ratios and theirs confidence intervals were also calculated.

Fourteen cases of IA were diagnosed in 240 liver transplant recipients (IA incidence: 5.8%) from 1994 to 1999. Thirteen of them died (93%). Death was directly related to Aspergillus infection in all of them.

For our study, nine Cs-Pt and 33 Cnt-P met the criteria required for being considered valid for antigenemia analysis.

Cs-Pt.

Forty-five serum samples from Cs-Pt were analyzed. The mean number of serum samples per patient was 4 (range: 3–9). In 5 (55%) of 9 patients, a serum sample was positive for GA EIA-sandwich test. High positive antigenemia values (>5 ng/ml) were obtained in 60% of the cases. The mean value of titers was 4.5 ng/ml (range: 1.7–6.6 ng/ml).
The description and characteristics of the IA cases are shown in Table 1. In five cases, they were retransplanted patients, with a median time from transplantation to IA diagnosis of 125 days (range: 5 to 1117 days). In nine patients, stored serum samples were available for performing the antigenemia analysis.

### Table 1. Results of Aspergillus antigenemia analysis in 14 IA cases in OLT recipients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Season</th>
<th>OLT date</th>
<th>IA diagnosis time</th>
<th>Aspergillus isolation</th>
<th>Aspergillus antigenemia (Ag level mg/L)</th>
<th>Medical therapy (starting date, EDI)</th>
<th>Related outcome</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55M</td>
<td>Apr 1594</td>
<td>May 1594</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Postmortem findings</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>45M</td>
<td>Jun 1594</td>
<td>Feb 1594</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Negative confirmation</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>68M</td>
<td>Oct 1394</td>
<td>Feb 1596</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Negative confirmation</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>65M</td>
<td>May 1595</td>
<td>May 1596</td>
<td>Yes</td>
<td>0.45</td>
<td>Yes</td>
<td>Postmortem findings</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>60M</td>
<td>May 1595</td>
<td>May 1596</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Negative confirmation</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>73F</td>
<td>Aug 1596</td>
<td>Oct 1596</td>
<td>No</td>
<td>No</td>
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<td>Negative confirmation</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>55M</td>
<td>Oct 1596</td>
<td>Nov 1595</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Negative confirmation</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>65M</td>
<td>Dec 1596</td>
<td>Jan 1596</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Negative confirmation</td>
<td></td>
</tr>
<tr>
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<td>65M</td>
<td>Jun 1596</td>
<td>Jun 1597</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Postmortem findings</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>55M</td>
<td>Jun 1596</td>
<td>Jun 1597</td>
<td>Yes</td>
<td>0.45</td>
<td>Yes</td>
<td>Postmortem findings</td>
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<tr>
<td>11</td>
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<td>Aug 1596</td>
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<td>Dec 1596</td>
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<td>65M</td>
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<td>Oct 1596</td>
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<td>No</td>
<td>Yes</td>
<td>Negative confirmation</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>65M</td>
<td>Nov 1596</td>
<td>Jan 1596</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Negative confirmation</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: OLT: orthotopic liver transplantation; NA, not available; POS, positive results; NEG, negative result; CD, Cumulative dose.

Aspergillus antigenemia has been performed using sandwich ELISA assay (Platelia, Sanofi Diagnostic Pasteur) and the GM detection level is measured in ng/mL.

In two cases (patients 1 and 9), Aspergillus infection was diagnosed in the postmortem study. The positive sera for GM detection in these two patients had been collected 25 and 8 days before death, respectively. High Aspergillus antigen detection values were obtained (5.8 and 6.6 ng/mL).

In the other three positive cases (patients 4, 6, and 14), antigenemia was positive in serum obtained previous to IA diagnosis and antifungal therapy initialization.

In four IA patients (patients 2, 3, 5, and 14), the Aspergillus antigen detection was negative:

In patient 2 the assay was performed in serum drawn 7 days after IA diagnosis while the patient was already receiving amphotericin B.

In patients 3 and 5, the sera analyzed had been collected 20 and 37 days before IA diagnosis, respectively, without receiving concomitant antifungal therapy.

The serum from patient 14 was drawn 12 days before diagnosis. He did not receive any antifungal drug at that moment.

Control patients.

A total of 126 serum samples from Cnt-P were analyzed. The mean of sera per Cnt-Pt was 3 (range: 3–6). In this group there were two patients with a positive antigenemia. Medical charts were reviewed and no data of fungal infection or Aspergillus isolation was noted in these patients.

Table 2 shows the yield of antigenemia EIA-sandwich in this retrospective study. The sensitivity was 55.6% (CI: 22.7–84.7), with a specificity of 93.9% (CI: 78.4–98.9), and a positive and negative predictive value of 71.4% (CI: 50.3–94.9) and 88.6% (CI: 72.3–96.3), respectively. Using Taylor’s method, the likelihood ratio (LR) for a positive test was 9.17 (2.12–39.66) and LR for a negative test was 0.47 (0.22–1.01).
Table 2. Characteristics of the aspergillus antigen sandwich-EIA test in this study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>LR+</th>
<th>LR-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55.6%</td>
<td>93.9%</td>
<td>71.4%</td>
<td>86.6%</td>
<td>0.17</td>
<td>0.47</td>
</tr>
<tr>
<td>95% CI</td>
<td>22.7–84.7</td>
<td>78.4–98.9</td>
<td>30.3–94.9</td>
<td>72.3–96.3</td>
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<td></td>
</tr>
</tbody>
</table>

The results of the Aspergillus antigen detection using a sandwich-ELISA in serum in the group of case and control patients are shown in this figure. Confidence intervals for LR were calculated using Taylor’s method. Abbreviations used in table: LR+, likelihood ratio for a positive test; LR-, likelihood ratio for a negative test.

Confidence intervals for LR were calculated using Taylor’s method. Abbreviations used in table: LR+, likelihood ratio for a positive test; LR-, likelihood ratio for a negative test.

The diagnosis of IA is usually deferred and based on histological criteria demonstrating tissue invasion, that confers an ominous prognosis, mainly in immunocompromised patients. Prompt diagnosis is crucial to initiate specific therapy before vascular invasion appears. Humoral immune response in transplant patients is poor, and antibody-based assays are not helpful for diagnosis. The detection of circulating antigens or nucleic acids in serum or other fluids does not depend on the host immunity, and the development of either immunosassays or nucleic acid amplification methods may become in the near future the serological methods of choice for diagnosis of aspergillosis. GM is a cell-wall antigen of Aspergillus sp. and circulating levels can be detected during IA. The antigen levels are higher in patients with disseminated infection, and quantitative assays may be helpful in monitoring treatment response. Serum is the most appropriate specimen for GM detection, and different techniques have been developed.

Different techniques has been performed to detect the presence of GM. The sandwich-EIA is the most sensitive method developed, with a global sensitivity of 50–90% and a specificity of 81–93% (2–4). In comparison with the latex agglutination test, EIA-based assays are able to detect GM antigens at least 2 to 3 weeks earlier, usually before signs and symptoms of aspergillosis become clinically apparent (5, 6). Nucleic acid amplification diagnostic tests such as Aspergillus polymerase chain reaction are also under development, with high rates of sensitivity, although further evaluation of these assays are needed (3, 7).

Most studies that assess the value of Aspergillus antigenemia have been performed in high-risk hematological patients. A recent prospective study using a sandwich-EIA test in hematological patients at increased risk for aspergillosis showed sensitivity, specificity, and predictive values higher than 92%, and was detected before clinical suspicion in more than half of the patients with IA (8).

In transplantation, most studies assessing the efficacy of Aspergillus antigen detection have been performed in bone marrow recipients. No experience has been reported in OLT recipients. In this retrospective study, performed in liver transplant recipients, we found a sensitivity and a specificity of 55.6% and 93.9%, respectively. This lower sensitivity in comparison with other series can be explained partly because of the retrospective design and the absence of an established schedule to collect samples, circumstances that do not allow us to define the interval between antigen detection and presentation of the disease.

Different authors have confirmed a decrease of titers during antifungal therapy. In one of our four IA cases with a false-negative result, the serum was drawn when the patient had received 7 days of antifungal therapy. In another two patients the serum samples available had been obtained more than 20 days before IA diagnosis. The GM detection in serum is transitory and GM is rapidly cleared from blood because of immune complex formation and the mannose receptor of the Kupffer’s cells of the liver. In high-risk patients it is recommended that samples be obtained twice weekly (9, 10).

It is noteworthy that in two of the five patients with true positive results the Aspergillus infection diagnosis was done in the postmortem study. The retrospective analysis of their sera showed a high GM titer in the samples obtained several days previous to death. If this assay had been performed in real time (for example, weekly), they could have benefited from an early initiation of antifungal therapy.

In the control group two patients have a false-positive result. The frequency of false-positives is near 8% in most of studies using the EIA-sandwich test (4). Many causes have been hypothesized to explain the rate of persistent false-positive results, although it remains unclear. Subclinical aspergillosis, intestinal Aspergillus colonization, or cross-reactivity with an unidentified serum compound are some of the explanations proposed. Aspergillus airborne contamination of serum samples before performing the procedure is other possible cause of a persistent false-positive result. The ubiquitous nature of this mold warrants careful manipulation of the samples to be tested.

In some studies, GM value detected by sandwich-EIA has a good correlation with the grade of dissemination of Aspergillus infection. It has been observed that antigenemia level was higher in patients with an IA than in those with an infection limited to the lung (11, 12). In the present study we found high antigenemia levels (>5 ng/ml) in 60% of
IA has been reported in 1.5–6% of liver transplant patients. Mortality rates are higher than 85%. Poor graft function, prior cytomegalovirus infection, antirejection drug use (OKT3), prolonged antibiotic therapy, and renal failure have been identified in liver transplant recipients as the main risk factors associated with IA (13–15). With the results obtained in this retrospective study, a likelihood ratio of 9.2 for a positive test, we cannot recommend the systematic determination of antigenemia for all patients, because for this pretest probability (0.058), the posttest probability only increased to (0.53). However, in other scenarios with a higher pretest probability, as in patients with the previously described risk factors for IA, GM detection in serum could be a useful marker for beginning preemptive therapy before full-blown disease compromises patient's life.

REFERENCES


