Comparison of Histopathological Analysis, Culture, and Polymerase Chain Reaction Assays to Detect Invasive Mold Infections from Biopsy Specimens

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Background. With the advent of new antifungal agents, the identification of a causative pathogen is crucial to guide the antifungal treatment of invasive mold infection. However, tissue cultures often fail to grow a fungal pathogen in cases of suspected mold infection.

Methods. In a prospective multicenter study, we compared the results of histopathological analysis, culture, and 2 seminested polymerase chain reaction assays identifying Aspergillus species and Zygomycetes as causative agents of invasive mold infections using respiratory tract biopsy samples obtained from 56 immunocompromised patients who had suspected mold infection.

Results. Mold hyphae were detected histopathologically in 27 (48%) of the tissue specimens. Hyphae corresponded to either aspergillosis (n = 18) or zygomycosis (n = 6) or could not be further specified (n = 3). A mold was cultured from 14 of 18 samples with aspergillus hyphae, 2 of 6 samples with Zygomycetes hyphae, and 1 of 3 samples with unspecified hyphae. Polymerase chain reaction was superior to culture in detecting the infecting mold (26 of 27 samples vs. 17 of 27 samples, respectively; P = .006) from histopathologically positive samples. Genus or species identification by sequencing of the polymerase chain reaction products were in accordance with culture results in 16 of 18 culture-positive samples. Both polymerase chain reaction assays failed to detect fungal DNA in 1 sample that had unspecified hyphae and negative culture results.

Conclusion. The PCR assays offer a reliable etiologic diagnosis that is superior to culture in patients with proven invasive mold infection. This may improve patient management through tailored antifungal therapy when cultures fail to grow a pathogen.

Invasive mold infection of the respiratory tract is a significant complication in immunocompromised patients who have hematological malignancies or who have undergone solid-organ transplantation [1]. Although Aspergillus fumigatus is the most prevalent causative agent, other Aspergillus species and Zygomycetes have recently emerged as important opportunistic pathogens [2, 3]. Because of the different in vitro susceptibilities of these emerging pathogens to the increased number of antifungal agents, defining the exact microbial etiology of invasive mold infections is becoming increasingly important for the guidance of antifungal therapy. However, growth of a mold from specimens obtained using noninvasive techniques may represent colonization, rather than infection, and ~60% of organ specimens that have positive histopathological results have negative culture results [4]. As a result, antifungal therapy guided by the knowledge of the causative agent is possible only in a minority of patients. This may contribute to the failure of antifungal treatment in patients who have invasive fungal infections due to in vitro drug-resistant organisms.

We have previously shown that, through the use of 2 seminested PCR assays that target mitochondrial DNA of Aspergillus species and the 18S ribosomal DNA

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of Zygomycetes, mold DNA can be obtained from formalin fixed-tissue specimens, allowing the identification of fungal pathogens to the genus or species level [5, 6]. Here, we report the results of a prospective study that compared the 2 PCR assays for the identification of the causative agent of invasive mold infection with standard procedures (i.e., histopathological analysis and culture using unfixed biopsy specimens obtained from patients who have had suspected mold infection).

**PATIENTS, MATERIALS, AND METHODS**

**Study patients**

This study was conducted between February 2002 and December 2005. Tissue specimens from 58 consecutive immunocompromised patients with suspected invasive mold infection from whom a tissue biopsy sample was obtained were included.

A suspected invasive mold infection was defined as a new fever or increasing C-reactive protein levels and a new or progressive organ lesion suggestive of an invasive mold infection that developed or progressed during receipt of broad-spectrum antibacterial therapy. Suspicious lesions included focal lung lesions with or without surrounding halo, cavitating lung infiltrates, opacification of the sinuses as detected by high-resolution CT, or typical endobronchial lesions detected by bronchoscopy. The biopsy procedure depended on the location of the lesion and the patient’s status. In peripheral, pleurae-based lesions, a CT-guided biopsy using an 18-gauge needle and 1–2 passes through the pleura was performed. Bronchoscopy and video-assisted thoracoscopic were preferred in patients who had undergone lung transplantation or who were receiving mechanical ventilation. Contraindications for biopsy included a partial thromboplastin time >50 sec, a platelet count <50,000 platelets/μL, and a prothrombin time >19 sec.

Written, informed consent was obtained from all patients. Institutional review board approval was obtained, in accordance with local requirements, from the Universities of Frankfurt and Tübingen (Germany).

**Biopsy preparation.** Biopsy specimens were carefully divided by 1 of the investigators (V.R. or E.P.) by cutting with a knife without prior homogenization. Equal parts were used for histopathological analysis, culture, and molecular testing. PCR assays were performed centrally (by R.B. in Tübingen, Germany) within 2 months of sampling. Samples were stored frozen at −74°C until DNA extraction. Researchers performing the molecular testing were blinded to clinical, microbiological, and histopathological data. Histopathological analysis and culture were performed in Frankfurt, Germany, except for the 2 samples that were obtained from patients in Vienna, Austria.

**Histopathological analysis.** Periodic acid-Schiff and Grocott’s methenamine silver stain were used for the detection of hyphae in tissue. Hyphae were classified as being from *Aspergillus* species (hyaline septate hyphae with dichotomous 45°-angle branching) or Zygomycetes (wide, ribbonlike, paucisep- tate hyphae with right-angle branching), as previously described [7]. Mold hyphae not fitting the typical morphological characteristics of *Aspergillus* species or Zygomycetes were classified as unspecified. In samples without mold hyphae, a specific histopathological analysis was defined as evidence of a typical etiology, such as lymphoma. Histopathological analysis was considered to be nonspecific if findings were uncharacteristic of a specific pathology (e.g., necrotic tissue or fibrotic changes in the absence of a causative organism) [8].

**Culture.** Biopsy samples were immediately transported in saline to the mycology laboratory. Fungal pathogens were cultured on Sabouraud dextrose agar at 30°C in room air for 28 days. Bacterial cultures were performed on sheep’s blood and chocolate agar at 37°C in a 5% carbon dioxide atmosphere. All media were obtained commercially from Becton Dickinson (Heidelberg, Germany). Cultures for mycobacteria were performed in MB Redox (Heipha) for 8 weeks at 35°C. Identification of the cultured fungal and bacterial pathogens was performed using standard techniques.

**Molecular methods.** DNA extraction was performed as previously described [9]. Two seminested PCR assays that targeted the mitochondrial DNA of *Aspergillus* species (*Aspergillus* PCR) and the 18S ribosomal DNA of Zygomycetes (*Zygo- mycetes PCR*) were used. Negative, positive, and extraction controls were used in accordance with standard procedures. PCR products were detected by ethidium-bromide–stained gels after electrophoresis. Amplicons were sequenced as previously described [6]. PCR products that were <95% sequence homologous with the sequences of Zygomycetes or *Aspergillus* species deposited in GenBank were regarded as being nonspecific amplifications, and PCR results were reported as negative [5, 6].

**Definitions.** Neutropenia was defined as a neutrophil count <500 cells/μL. Immunosuppressive therapy was defined as the systemic administration of glucocorticosteroids, calcineurin inhibitors, or mycophenolate mofetil. Pretreatment with antifungals was defined as the systemic administration of antifungal agents that had in vitro activity against molds (e.g., itraconazole, voriconazole, posaconazole, caspofungin, or amphotericin B) during the 7 days prior to biopsy. The diagnostic accuracy for an invasive mold infection was classified according to the European Organization for Research and Treatment of Cancer/ Mycoses Study Group of the National Institute of Allergy and Infectious Diseases criteria [10].

**Statistical methods.** Data sampling and analysis were performed using a standardized case report form and Epi Info 2000 software (Centers for Disease Control and Prevention). Categorical variables were compared using the χ² test or Fisher’s exact test, as indicated. For continuous variables, the Wilcoxon
Table 1. Characteristics of 56 patients with suspected invasive mold infection.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, no. (%) of patients</td>
<td>37 (66)</td>
</tr>
<tr>
<td>Age, mean years (range)</td>
<td>53 (10–69)</td>
</tr>
<tr>
<td>Underlying condition</td>
<td></td>
</tr>
<tr>
<td>Acute leukemia</td>
<td>27</td>
</tr>
<tr>
<td>Other hematologic malignancies</td>
<td>14</td>
</tr>
<tr>
<td>Solid-organ transplantation</td>
<td>6</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>4</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>2</td>
</tr>
<tr>
<td>Othera</td>
<td>3</td>
</tr>
<tr>
<td>Organ biopsied</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>49</td>
</tr>
<tr>
<td>Paranasal sinus</td>
<td>6</td>
</tr>
<tr>
<td>Soft tissue</td>
<td>1</td>
</tr>
<tr>
<td>Risk factor for infection</td>
<td></td>
</tr>
<tr>
<td>Neutropenia</td>
<td>24</td>
</tr>
<tr>
<td>Immunosuppressive therapy</td>
<td>17</td>
</tr>
<tr>
<td>Both</td>
<td>13</td>
</tr>
<tr>
<td>Otherb</td>
<td>2</td>
</tr>
<tr>
<td>Received antifungal pretreatment</td>
<td></td>
</tr>
<tr>
<td>before undergoing biopsy</td>
<td></td>
</tr>
<tr>
<td>No. (%) of patients</td>
<td>33 (59)</td>
</tr>
<tr>
<td>Duration of therapy, mean days (range)</td>
<td>9.3 (1–63)</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of patients, unless otherwise indicated.

a Other underlying conditions include chronic sinusitis (1 patient), rheumatoid arthritis (1 patient), and severe psoriasis (1 patient).

b Other risk factors include therapy with iron chelator (1 patient) and chronic sinusitis (1 patient).

test was used. A P value <.05 in a 2-sided test was considered to be statistically significant.

**RESULTS**

We included 58 consecutive biopsy specimens. Two samples were excluded from the analysis: both patients had a clinical presentation that was suggestive of pulmonary aspergillosis and responded to voriconazole. In 1 sample, invasive aspergillosis was histopathologically confirmed but cultures were sterile. The negative PCR result was invalid because the result of the control PCR amplifying human DNA remained negative in 2 tests. This probably reflects insufficient amounts of tissue in the sample that was sent for PCR testing. In the second sample, only pleural tissue was observed in the histopathological analysis. Although sample cultures were sterile, PCR had results positive for *A. fumigatus*.

Underlying conditions, risk factors for invasive fungal infection, and the organs from which the analysed biopsy samples were obtained from the remaining 56 patients are listed in table 1. The mean platelet count at the time of biopsy was 76,000 platelets/µL (range, 55–191 platelets/µL), the mean prothrombin time was 16 sec (range, 13–19 sec), and the mean partial thromboplastin time was 42 sec (range, 36–45 sec).

Biopsies were performed through the guidance of CT (n = 25), bronchoscopy (n = 17), and video-assisted thoracoscopic surgery (n = 7). Specimens from the paranasal sinuses (n = 6) and soft tissue (n = 1) were obtained using surgical methods.

Complications of the CT-guided biopsies included minor hemorrhage without need for packed RBC support in 3 of 25 patients and pneumothoraces that resolved spontaneously in 6 of 25 patients.

**Histopathological results.** An invasive mold infection was proven, according to the European Organization for Research and Treatment of Cancer/Mycoses Study Group of the National Institute of Allergy and Infectious Diseases criteria [10], with the presence of hyphae in tissue samples in 27 (48%) of the 56 patients. Hyphae were considered to be indicative of aspergillosis in 18 samples (32%) and zygomycosis of in 6 samples (11%). Specification of hyphae was not possible in 3 samples (5%), either because of a low amount of fungal elements (n = 2) or an uncharacteristic morphology (figure 1). For 3 patients (5%) with specimens in which hyphae were not observed, an alternative etiology was detected through histopathological analysis (toxic changes of the lung in 2 patients, Hodgkin disease in 1 patient), whereas in 26 patients (46%), nonspecific histological results were obtained. In 8 (14%) of these samples, inflammatory changes consistent with fungal pneumonia without detection of hyphae were observed. According to the European Organization for Research and Treatment of Cancer/Mycoses Study Group of the National Institute of Allergy and Infectious Diseases criteria [10], 25 of these patients were classified as having a possible invasive mold in-
fection, whereas 1 patient was classified as having a probable invasive mold infection.

**Species identification by culture.** A fungal pathogen was cultured from 19 samples (34%), 17 of which had positive results by histopathological analysis. In 14 (78%) of 18 samples that had *Aspergillus* species hyphae, a mold was grown: *A. fumigatus* in 11 samples, *Aspergillus flavus* in 1 sample, *Aspergillus niger* in 1 sample, and *Trichoderma longibrachiatum* in 1 sample. Only 2 (33%) of 6 samples that had Zygomycetes hyphae grew a Zygomycete: *Rhizopus* species in 1 sample and *Rhizomucor pusillus* in 1 sample (*P* = .13). In 1 (33%) of 3 samples that had unspecified hyphae, *A. fumigatus* was grown.

In 1 sample, *A. flavus* was cultured from a sinus biopsy sample without detection of hyphae in the specimen by histopathological analysis. However, because the results of CT were consistent with an invasive infection and because the patient responded to antifungal therapy, the cultured fungus was considered to be a true pathogen. In contrast, in another sample that had negative results by histopathological analysis, *Penicillium citrinum* was cultured on 1 agar plate and was considered to be a contamination. The median duration of antifungal pre-treatment was 2 days (range, 0–32 days) in culture-positive cases versus 12.5 days (range, 0–46 days) in culture-negative cases (*P* = .14) with proven invasive fungal infection.

Bacteria were cultured in 13 (23%) of the 56 samples. In 6 samples, miscellaneous saprophytic gram-positive bacteria were grown. In 7 samples, facultative pathogenic bacteria were grown (*Pseudomonas aeruginosa* in 4 samples, *Stenotrophomonas maltophilia* in 2 samples, and *Staphylococcus aureus* in 1 sample). Although, hyphae were observed in 4 of these 7 samples during histopathological analysis, acute inflammation was detected in 3 cases by histopathological analysis without detection of mold hyphae. In these cases (2 samples with *P. aeruginosa* and 1 sample with *S. maltophilia*), the isolated bacteria may have caused the infection. These isolates were determined to be resistant to the applied antibacterials. Two of these patients improved after the addition of in vitro–susceptible antibacterials, and 1 patient died from refractory septic shock. All cultures for mycobacteria remained sterile.

**Species identification by PCR.** Using PCR, 30 molds were detected in 29 (52%) of the 56 samples (figure 1). The *Aspergillus* species PCR alone had positive results in 22 samples (39%), including all 18 samples in which typical *Aspergillus* species hyphae were detected. Sequencing revealed *A. fumigatus* sequences in 20 samples and *A. flavus* sequences in 2 cases. Two samples in which *A. fumigatus* DNA was detected grew *T. longibrachiatum* and *A. niger* (in 1 sample each). In 2 of the 3 samples that had unspecified hyphae, the *Aspergillus* species PCR revealed *A. fumigatus*–specific DNA. One of these samples grew *A. fumigatus*. In addition, 2 samples from which hyphae were not obtained had positive results by the *Aspergillus* species PCR. One sample was obtained from a patient who had sinusitis. *A. flavus* was grown from this sample and the results of sequencing of the PCR product were in accordance with culture results.

In another sample from a patient who had possible aspergillosis, PCR amplified *A. fumigatus* DNA, but fungal cultures remained sterile. Histological analysis revealed a neutrophil infiltration of a bronchus without the detection of hyphae. A retrospective review of the CT obtained during the biopsy showed the needle to be in the periphery of a typical lesion with surrounding halo. The patient responded to voriconazole, which was suggestive of invasive aspergillosis (as indicated by PCR).

The Zygomycete PCR alone had positive results in 6 (11%) of 56 samples, 5 of which were positive by histological analysis. Sequences were obtained from *R. pusillus* or *Rhizomucor miehei*, which share the same sequence and cannot be differentiated using this PCR (from 4 samples); from *Absidia corymbifera* (1 sample); and from *Rhizopus microsporus* (1 sample). The results of identification by PCR were in accordance with culture results in both culture-positive samples.

In 1 sample in which Zygomycete hyphae were detected by histopathological analysis and sterile cultures, the *Aspergillus* species PCR and the Zygomycetes PCR simultaneously had positive results, indicating a double infection with *A. fumigatus* and *R. microsporus* according to sequencing. In 1 sterile sample that revealed an organizing pneumonia using histopathological analysis, the Zygomycetes PCR was positive for *R. pusillus*. CT revealed a cavitating infiltrate consistent with invasive zygomycosis, whereas the clinical course, in which infiltrates slowly regressed in response to antibacterial treatment, supports a bacterial etiology.

Both PCR assays had negative results in 1 culture-negative sample that had unspecified hyphae, suggesting that infection might have been caused by a mold that was not able to be detected by these assays. In 25 samples that did not have hyphae in tissue samples and that had negative culture results, both PCR assays had negative results.

Identification of the infecting mold was possible using PCR in 26 (96%) of 27 histologically positive biopsy samples; in contrast, culture of biopsy samples revealed a mold in only 17 (63%) of 27 samples (*P* = .006). Species identification by PCR and sequencing results were in accordance with culture results in 16 (89%) of 18 culture-positive samples.

**DISCUSSION**

In this prospective study, we were able to increase the etiologic diagnosis from 63% using culture methods to 96% with the combined use of 2 PCR assays in patients who had a proven invasive mold infection. The PCR assays successfully distinguished between aspergillosis and zygomycosis and provided...
evidence of a double infection in 1 case. In addition, the PCR assays suggested a causative fungal agent in 2 patients who had probable or possible invasive mold infection.

Several factors may have an impact on the yield of cultures from biopsy specimens in patients who have invasive mold infection. First, standard culture conditions might be suboptimal for the recovery of Aspergillus species (and probably other molds as well) that are adapted to growth in necrotic tissue [11]; second, pretreatment of tissue samples by grinding may impair the growth of Zygomyetes because of disruption of the large, pauciseptate hyphae [12]; and third, prolonged antifungal pretreatment may lower culture yield, as is suggested by our data.

In animal models of fungal infections, PCR is superior to culture and even to microscopy for the detection of fungi in tissue specimens, probably because a smaller amount of vital fungus is needed to obtain a positive PCR result [13, 14]. In addition, PCR facilitates a species identification that cannot be achieved using microscopy.

Only a limited number of clinical samples have been analyzed by culture and different PCR methods for the detection of molds. Most reports show that the identification of a mold is possible by PCR in selected culture-negative samples [15–17]. However, to the best of our knowledge, this is the first prospective study comparing the yield of culture and PCR in consecutive tissue samples. Most PCR studies excluded Zygomyetes, whereas we were able to detect the most common Aspergillus species and Zygomyetes that are now emerging as important fungal pathogens. In addition, through the use of 2 separate PCR assays, we were able to detect double infection [5]. Mixed fungal infection with other molds has been described in 46% of patients with zygomycosis in a case series [18] and are probably difficult to diagnose using conventional culture and histopathological analysis.

Identification of the causative agent of a mold infection to the species or genus level is needed, because in vitro susceptibility varies among different Aspergillus species and different genera of Zygomycetes. By combining the 2 PCR assays, we were able to detect the most common etiologic agents of invasive mold infections—that is, A. fumigatus, A. flavus, and the agents of zygomycosis—at the participating centers. However, because infection due to Aspergillus terreus, Aspergillus ustus, or A. niger are more common in other centers, the use of these PCR assays must be further evaluated elsewhere [19, 20].

One PCR-negative sample in the present series that had mold hyphae that were uncharacteristic of aspergillosis or zygomycosis suggests that additional primers might be needed to detect the DNA of additional molds, such as Fusarium species or Pseudallescheria boydii. Although the results of species identification by sequencing were in accordance with culture results in 89% of the culture-positive samples in our study, they differed in 2 cases. Discordant results might be explained by contamination or by double infections not recognized by culture. In addition, rare fungal pathogens cannot be identified using our approach if the targeted sequences of rare organisms are not available in GenBank. Therefore, comparison of our method with other PCR-based strategies to detect mold in biopsy specimens should be performed to define the usefulness of the different approaches.

Although PCR suggested an etiologic agent in 2 histopathologically negative samples, the relevance of these results is difficult to establish, because there is no gold standard to distinguish between positive and false-positive results. However, the clinical course of infection in both cases was suggestive of invasive aspergillosis, and the number of false-positive samples obtained by the 2 PCR assays appears to be very limited in our study.

The etiology of organ lesions was not determined in 21 (38%) of the 56 biopsy samples in our study. In a retrospective case series that included patients who had hematologic diseases and suspected pulmonary aspergillosis, aspergillosis could not be confirmed after the resection of suspected lesions in 20% of the patients [21]. When CT-guided biopsies are used to confirm suspected invasive aspergillosis in neutropenic patients who have hematologic malignancies, nonspecific inflammatory findings have been described in 24% of the biopsy samples [22]. Although the number of biopsies is small, nonspecific inflammatory findings were more frequent in our study. Differences in the mode of biopsy and in patient characteristics might account for these differences. First, the diagnostic yield of CT-guided lung biopsies might be higher with an increased number of pleural passes, as has been used in previous series [22]. Obtaining more tissue samples may increase the likelihood of detecting a causative mold, but it increases the risk of bleeding and pneumothorax. Second, a large amount of hyphae and less inflammation has been described in tissue specimens from neutropenic patients and animal models, whereas the amount of fungal elements detected is lower and inflammation is more pronounced in nonneutropenic patients and animals treated with corticosteroids. Therefore, the relatively frequent detection of nonspecific inflammatory lesions in our study may reflect the changing risk factors for invasive mold infections—that is, the more-frequent use of glucocorticoids—compared with previous studies [4, 22, 23].

In evaluating the pros and cons of the identification of a mold from tissue specimens of the respiratory tract, this approach appears to be inappropriate for the early diagnosis of mold infection. Given the increasing use, as prophylaxis or empirical therapy, of the newer antifungals that have activity against Aspergillus species, and the emergence of molds, such as Zygomyetes or Aspergillus species, that are resistant to azoles [19], biopsy may become a useful tool for obtaining an etiologic.
diagnosis in pretreated patients who have progressive mold infection. These patients will benefit from mold identification by PCR in addition to the standard histopathological analysis and culture, because this allows for guided antifungal therapy in a greater proportion of patients.

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