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Detection of *Aspergillus* Species DNA in Bronchoalveolar Lavage Samples by Competitive PCR

STÉPHANE BRETAGNE,[1]* JEAN-MARC COSTA,[2] ANNE MARMORAT-KHUONG,[1] FRANÇOISE PORON,[3] CATHERINE CORDONNIER,[4] MICHEL VIDAUD,[2,5] AND JOCELYNE FLEURY-FEITH[3]

Laboratoire de Parasitologie-Mycologie,[1] Laboratoire d'Histologie,[3] and Service d'Hématologie Clinique,[4] Hôpital Henri Mondor, Créteil, France; Laboratoire de Biologie Moléculaire, Hôpital Américain de Paris, Neuilly, France[2]; and Laboratoire de Génétique Moléculaire, URA Centre National de la Recherche Scientifique 1484, Faculté de Pharmacie, Paris, France[5]

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A competitive PCR assay involving the use of bronchoalveolar lavage (BAL) samples for the diagnosis of invasive pulmonary aspergillosis (IPA) was developed. For this purpose, a 1-kb mitochondrial DNA fragment of *Aspergillus fumigatus* was sequenced. The primers used allowed amplification of *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. niger* DNAs but not DNAs of other fungi and yeasts. BAL samples from 55 consecutively enrolled patients were tested. Three samples were excluded because of failure of correct amplification of the internal competitive control. Of 28 immunocompromised patients, 6 were PCR positive; 3 died of IPA and their BAL cultures yielded *A. fumigatus*; and 3 were culture negative and did not develop IPA. Of 15 human immunodeficiency virus-positive patients and 9 immunocompetent patients, 5 and 4, respectively, were both PCR positive and culture negative, and none developed aspergillosis. Thus, PCR confirmed IPA in three patients but gave positive results for 25% (12 of 49) of the patients who did not develop aspergillosis. The predictive value of PCR-positive results seems low for patients at risk for aspergillosis. Moreover, the risk of contamination of reaction buffers or biological samples with *Aspergillus* conidia seems high and has to be weighed in regard to the potential diagnostic benefit of PCR testing as a routine procedure.

Invasive pulmonary aspergillosis (IPA), usually caused by *Aspergillus fumigatus* or *A. flavus*, is a major hazard for immunocompromised patients (2). Early diagnosis is one of the keys to the best outcome, but antemortem diagnosis is difficult. Some patients often have bleeding tendencies, which preclude invasive diagnostic approaches. Bronchoalveolar lavage (BAL) is usually a safe and simple procedure for immunocompromised patients and is often helpful in the diagnosis of diffuse pulmonary infiltrates (5). The presence of typical branching, septate hyphae obtained by aspiration, and/or the isolation of the organism from several cultures is generally sufficient for the diagnosis and the initiation of treatment (2, 10). Unfortunately, *Aspergillus* spp. are isolated from BAL fluid in only 50 to 57% of all cases (1, 11, 12). Similar to other investigators (14, 18, 20, 21), we have thus utilized PCR because of its high level of sensitivity to improve the diagnosis of IPA.

We have developed a competitive PCR technique similar to the one we used for the diagnosis of toxoplasmosis (3, 9). We used a competitive, internal control to minimize the risk of false-negative results due to amplification reaction inhibitors. Also, to prevent carryover with previously amplified DNA leading to false-positive results, sample processing and PCR were performed in two different laboratories, and all the PCRs were performed with dUTP instead of dTTP in the reaction mixture to allow the enzyme uracil-*N*-glycosylase to destroy any dUTP-containing amplified product that could have been carried over from previous PCRs. Moreover, for diagnostic purposes, it is essential to use repeated sequences, such as ribosomal or mitochondrial DNA, as targets to ensure good sensitivity of the amplification. We have thus chosen primers within a mitochondrial DNA fragment isolated from an *A. fumigatus* library.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Georgetown University Medical Center, 3900 Reservoir Rd., NW, Washington, DC 20007-2197. Phone: (202) 687-1801. Fax: (202) 687-1800. Address for reprints: Laboratoire de Parasitologie-Mycologie, Hôpital Henri Mondor, 51 avenue du Général DeLattre de Tassigny, 94010 Créteil Cedex, France.

MATERIALS AND METHODS

Cloning of the 1-kb fragment of *A. fumigatus* mitochondrial DNA. *A. fumigatus* (IP 2279.94; Institute Pasteur Collection, Paris, France) DNA was partially digested with *Sau*3A, and 500- to 1,500-bp fragments were inserted into the *Bam*HI site of pUC19 before transformation of *Escherichia coli* DH5α. Total *A. fumigatus* DNA was ^{32}P radiolabelled by random priming and was used as a probe for screening 200 colonies by hybridization to duplicate nitrocellulose filters. Four strongly hybridizing colonies were selected, from which inserts were amplified by PCR with two 21-mer primers which overlap the pUC-M13 direct and reverse sequencing primers. Only one of these inserts showed hybridization patterns identical with those of 30 *A. fumigatus* isolates tested by standard Southern blotting after *Eco*RI digestion (data not shown). This 1-kb insert was sonicated, and the 300-bp fragments obtained were cloned into M13mp18 phage and sequenced with a Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio). Both strands were read, and the sequence obtained was compared with DNA sequences in GenBank. The result showed a strong identity (87%) with *A. nidulans* (*Emericella nidulans*) mitochondrial DNA (accession no. J01390). The 1-kb sequence of *A. fumigatus* overlapped with the *A. nidulans* mitochondrial DNA from the end of the second exon in the rRNA gene (position 11160) to the end of the Met-tRNA gene (position 12180) (4, 16).

Clinical specimens. BAL samples were obtained from 55 consecutive patients undergoing fibroscopy at the Henri Mondor Hospital, Créteil, France, from December 1992 to June 1993 (one sample per patient). BAL was performed by fiber-optic bronchoscopy with slow infusion and aspiration of three 50-ml aliquots of sterile 0.9% saline solution at 37°C (8). For each BAL, 1.5 ml from the second aliquot was processed for DNA amplification. The remaining fluid was processed by the mycology and cytology units for culture and cellular counts. For cultures, about 3 ml of each wash was seeded on Sabouraud slants with gentamicin and chloramphenicol (Institut Pasteur Production, Lyon, France). All cultures were incubated at 37°C for 4 weeks and inspected for growth daily for the first week and then twice weekly. Fungi were identified on the basis of morphological and cultural characteristics. Direct examination of 500 μl of the three fresh washes was performed with the optical brightener Blankophor-P-Flüssig (Bayer, Cologne, Germany) (19). In the cytology unit, the three fractions

of fluid were transferred to low-adherence flasks (Nunc) and smears were prepared with the pellets and stained with a Papanicolaou stain and a methenamine-silver stain. Each lavage sample was coded, the amplification was performed blind, and the results were subsequently compared with clinical and microbiological data.

DNA amplification. Samples were processed as previously described (3). The contaminating erythrocytes of the 1.5-ml aliquots for DNA amplification were eliminated by a selective lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-100). After centrifugation, the pellets were stored at −20°C. They were subsequently resuspended in 100 μl of heat-detergent extraction buffer (10 mM NaOH, 0.5% Tween 20, 0.5% Nonidet P-40) and then heated at 100°C for 10 min and centrifuged at 15,000 × *g* for 10 min. PCR was performed with 10 μl of the supernatant. In order to avoid contamination by the PCR products, processing of the BAL fluids and DNA amplification were performed in two different laboratories and dUTP nucleotides were substituted for dTTP nucleotides in the amplification reaction mixture.

The target DNA was the mitochondrial DNA fragment previously sequenced. The upper primer (5′ GAA AGG TCA GGT GTT CGA GTC AC 3′) was a 23-mer (sense) at position 804 to 826 on the *A. fumigatus* sequence, and the lower primer (5′ CTT TGG TTG CGG GTT TAG GGA TT 3′) was a 23-mer (antisense) at position 914 to 938. To avoid false-negative results, we developed a positive internal control with M13mp18 phage DNA. First, two 39-mer composite primers (5′ GAA AGG TCA GGT GTT CGA GTC ACC ACC ATC AAA CAG GAT 3′ and 5′ CTT TGG TTG CGG GTT TAG GGA TTG GGC GCC AGG GTG GTT 3′) that contained the M13mp18 sequences flanked at their 5′ ends were synthesized by the *A. fumigatus* primer sequence used in the PCR amplification. The primary PCR with the composite primers generated an M13mp18 fragment with the *A. fumigatus* sequence incorporated at the ends. This fragment was then amplified with the *A. fumigatus* primers only to ensure a homogeneous product of 182 bp. After purification and dosage, this 182-bp fragment was diluted, and each dilution was tested for amplification. The highest dilution consistently giving positive results corresponded to approximately 5 to 10 molecules of the internal control per μl, and 1 μl was added to each amplification reaction mixture. Because this control contains the same primer template sequences, it competes with the *A. fumigatus* gene for primer binding and amplification. Thus, a result was considered negative only if the *A. fumigatus* gene did not amplify but the control sequence did.

The samples were amplified, in duplicate, in a 50-μl reaction mixture containing 2 mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 0.01% (wt/vol) gelatin; 0.2 mM (each) dATP, dGTP, and dCTP; 0.4 mM dUTP (Pharmacia, Orsay, France); 20 pmol each of *A. fumigatus* primers (Genset, Paris, France); 1 μl of internal control (approximately 5 to 10 molecules); 0.5 U of uracil-*N*-glycosylase (Gibco BRL, Cergy Pontoise, France); and 1.25 U of *Thermus aquaticus* DNA polymerase (Perkin Elmer, Roissy, France). The samples were initially incubated for 5 min at 50°C to allow the action of uracil-*N*-glycosylase. This step also eliminated PCR products due to nonspecific annealing of the primers occurring at temperatures below 50°C during the preparation of the samples for amplification and thus enhanced the specificity of the reaction. This incubation was followed by a 5-min denaturation at 95°C prior to temperature cycling (40 cycles at 94 and 60°C for 30 s each and at 72°C for 1 min) in a 48-well thermal cycler (Perkin-Elmer Cetus). After 40 cycles, primer extension was continued for 10 min at 72°C, and then an equal volume of chloroform was added to prevent renaturation of uracil-*N*-glycosylase. Each amplification run included several negative controls (heat-detergent extraction buffer) and positive controls (internal positive control in heat-detergent extraction buffer).

The PCR products were analyzed by ethidium bromide staining after electrophoresis in an 8% acrylamide gel. The specificity was confirmed by hybridization with an oligonucleotide labelled at the 5′ end with ^{32}P and complementary to the amplified region (5′ GCG GGT TGA TGT AAT AGT 3′). PCR products were electrotransferred (1 h at 80 V) onto nylon membranes (Hybond N; Amersham). Hybridization with the oligonucleotide (1 pmol/75 cm^2) was performed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt's solution–1% sodium dodecyl sulfate (SDS) for 4 h at 60°C, and two posthybridization washes were performed in 5× SSC–0.1% SDS at 65°C for 30 min. The nylon membranes were exposed to Amersham film between two intensifying screens at −70°C for 16 h.

To check the specificity of the reaction, human DNA, DNAs from 30 clinical *A. fumigatus* isolates, and DNAs of several yeast and fungal strains were extracted and 100 ng was used for amplification. PCR products were transferred and hybridized as described above. The strains used in this study included *A. flavus* (IP 597.69), *A. terreus* (IP 1136.76), *A. niger* (IP 1431.83), *A. nidulans* (IP 17.60), *Penicillium purpurogenum* (IP 2281.94), *Alternaria alternata* (IP 2280.94), *Candida albicans* (ATCC 32354), *Candida tropicalis* (ATCC 66029), *Candida parapsilosis* (ATCC 22019), *Candida glabrata* (IP 810.63), *Candida krusei* (IP 208.52), and *Cryptococcus neoformans* (IP 1209.79).

The accession number of the *A. fumigatus* sequence is GenBank L37095.

RESULTS

Specificity of PCR. Each of the 30 clinical isolates of *A. fumigatus* tested showed the same signal upon amplification, with a band of the expected size (135 bp). In contrast, this band was not observed with yeast or human DNA or DNAs from *P. purpurogenum*, *Alternaria alternata*, or *A. nidulans*. The result with *A. nidulans* DNA was expected, as the lower primer does not match the *A. nidulans* sequence. The *A. flavus*, *A. terreus*, and *A. niger* strains showed amplification signals of different sizes in an acrylamide gel after ethidium bromide staining (Fig. 1). Only the *A. fumigatus* and *A. niger* DNAs gave a strong hybridization signal after blotting and probing with an internal oligomer (Fig. 2). Thus, the primers and the parameters of the PCR allowed us to amplify the main *Aspergillus* spp. involved in human pathology.

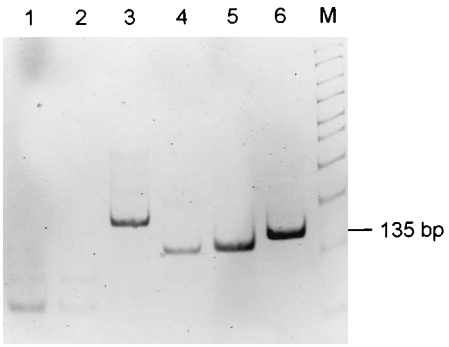

FIG. 1. Results of amplification of different fungus DNAs after 8% acrylamide gel electrophoresis and ethidium bromide staining (negative image). Only *A. fumigatus* yields the expected band at 135 bp. *A. nidulans* and *P. purpurogenum* DNAs are not amplified. Lane 1, *P. purpurogenum*; lane 2, *A. nidulans*; lane 3, *A. niger*; lane 4, *A. terreus*; lane 5, *A. flavus*; lane 6, *A. fumigatus*; lane M, size markers (AmpliSize; Bio-Rad).

Clinical sample testing. Of 55 consecutive patients undergoing BAL at Henri Mondor Hospital, 46 patients were immunocompromised. All the immunocompromised patients had symptoms of acute respiratory illness or unexplained fever. Thirty patients were human immunodeficiency virus (HIV)-negative, immunocompromised patients (19 males and 11 females; mean age, 48 years; range, 12 to 71 years); 7 had undergone organ grafts; 9 had undergone bone marrow transplantation; 11 were receiving chemotherapy for hematological malignancies; and 3 were receiving steroids for vasculitis. Sixteen patients were infected by HIV (15 males and 1 female; mean age, 39 years; range, 29 to 68 years). The remaining 9 patients (9 males; mean age, 49 years; range, 16 to 73 years) were considered immunocompetent and underwent fibroscopy for chronic or acute respiratory disorders.

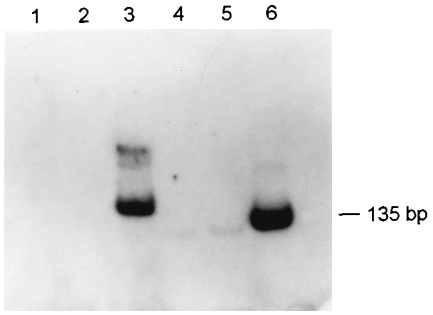

FIG. 2. Results of hybridization after Southern blotting of the amplified fragments with an oligonucleotide completely within the amplified fragment of *A. fumigatus*. A strong hybridization signal is observed only with *A. fumigatus* and *A. niger* DNAs. Lanes 1 to 6 are the same as those in Fig. 1.

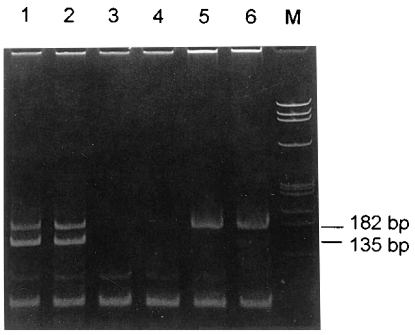

FIG. 3. Duplicated detection of amplified products after 8% acrylamide gel electrophoresis and ethidium bromide staining. The 135-bp fragment corresponds to the internal control of the amplification, and the 182-bp fragment corresponds to the *A. fumigatus* amplified DNA. Lanes 1 and 2, positive results; lanes 3 and 4, nonconclusive results; lanes 5 and 6, negative results; lane M, ϕX174 DNA cleaved with *Hae*III.

Figure 3 shows the three possible results expected for the competitive PCR: positive, negative, and nonconclusive. Of the 55 BAL samples, 3 (one from an HIV-positive patient and two from HIV-negative, immunocompromised patients) contained residual amplification inhibitors, as demonstrated by the lack of amplification of the internal control. The inhibitors were not eliminated by proteinase K digestion and phenol-chloroform extraction performed on these samples. Thus, only 52 BAL samples were subsequently analyzed (Table 1).

A. fumigatus DNA was amplified from 15 of these 52 samples: 6 from the 28 HIV-negative, immunocompromised patients; 5 from the 15 HIV-positive patients; and 4 from the 9 immunocompetent patients. In the HIV-negative group, *A. fumigatus* was cultured from four samples, *Rhizopus rhizopodiformis* was cultured from one, and yeasts were cultured from five (*C. albicans*, four; *C. glabrata*, one), and *Pneumocystis carinii* was identified in three samples. In the HIV-positive group, yeasts were cultured from nine samples (*C. albicans*, eight; *C. glabrata*, two; *C. tropicalis*, one; *C. krusei*, one), *P. carinii* was identified in four samples, and *Toxoplasma gondii* was identified once, with five patients harboring several pathogens. No pathogen was recovered from the nine immunocompetent patients. Cytology did not reveal any fungal hyphae for any patient. The percentages of cell types in the BAL samples were in the same range for the PCR-positive and the PCR-negative patients.

Among the 28 HIV-negative immunocompromised patients, 3 patients (bone marrow transplantation, one; renal transplantation, one; chemotherapy for hematological malignancy, one) were PCR positive and developed IPA. IPA was diagnosed on the basis of the presence of numerous colonies of *A. fumigatus* on Sabouraud medium seeded with BAL fluid and the clinical setting (fever, neutropenia with <100 neutrophils per mm^3 of blood, and pulmonary infiltrate) according to the definitions generally accepted (6, 7). The three patients died despite amphotericin B treatment. Autopsy was performed only on the recipient of the renal transplant, and the histological specimens showed characteristic *Aspergillus* hyphae.

Three other patients were PCR positive but did not develop IPA. The first one suffered from graft-versus-host disease after allogeneic bone marrow transplantation. The BAL was done because the patient suffered from acute interstitial pneumonitis, and the results showed *P. carinii*. This patient was not neutropenic at the time of or after BAL and has not received drugs active on *Aspergillus* spp. since that time. He is still alive with severe pulmonary fibrosis. The second patient of this group had resistant lymphoma. During an episode of neutropenia, he experienced multifocal pneumonia and underwent fibroscopy. The only pathogen isolated was *Streptococcus mitis*. The patient received 4 days of amphotericin B until he recovered from aplasia. He died 1 month later with gut bleeding, recurrence of pneumonia, and uncontrolled lymphoma. IPA cannot be ruled out in this case but seems unlikely because of the complete cure of the first episode of pneumonia after only 4 days of amphotericin B. No autopsy was performed. The third patient underwent BAL for cytomegalovirus primary infection 45 days after a renal transplant. She had fever but no clinical or radiological pulmonary symptoms and was not neutropenic. She was receiving steroids, cyclosporine, and azathioprine at the time of BAL. She never received any antifungal drug and is alive and well 2 years after the transplant.

The sample from one other patient yielded one colony of *A. fumigatus* but was PCR negative. This patient did not develop any pulmonary diseases, and the colony was considered a result of contamination of the Sabouraud medium.

None of the five PCR-positive samples from the 15 HIV-positive patients yielded any filamentous fungi. The CD4 lymphocyte titers were under 50 cells per μl for 4 of the 5 PCR-positive patients and for 7 of the 10 PCR-negative patients. Of the five PCR-positive samples, two yielded no pathogens, one yielded *C. tropicalis* and *T. gondii*, one yielded *C. glabrata*, and one yielded *C. albicans*.

Of the four PCR-positive patients in the immunocompetent group, consciousness impairment was present in two, one had pulmonary fibrosis, and one had Lyell's syndrome. One patient died from alcoholic encephalopathy. None developed aspergillosis.

TABLE 1. PCR and culture of BAL samples for *Aspergillus* spp.

Diagnosis	No. of patients	No. positive for *Aspergillus* spp. by PCR	No. positive for *Aspergillus* spp. by culture
HIV negative, immunocompromised			
With aspergillosis	3	3	3
Without aspergillosis	25	3	1[a]
HIV positive without aspergillosis	15	5	0
Immunocompetent	9	4	0
Total	52	15	4

[a] PCR-negative patient.

DISCUSSION

No commonly accepted criteria for defining IPA are available. Histology is recommended for confirmation of aspergillosis in clinical trials but is rarely performed. Therefore, and especially for patients with profound neutropenia, a positive culture from BAL fluid from a patient with a lung infiltrate is usually considered sufficient for the diagnosis of aspergillosis in this population (2, 10). On the other hand, it is commonly admitted that BAL is often falsely negative for IPA (1, 11, 12). The main advantage of PCR is that it detects low burdens of hyphae and thus allows the beginning of an early, specific treatment. That was the rationale for developing PCR for aspergillosis. As false-negative results are a risk in the development of a tool for the diagnosis of aspergillosis, our decision was to develop a competitive PCR which avoids false-negative results due to residual amplification reaction inhibitors. In-

deed, 3 of 55 BAL samples had to be excluded from the analysis, as they did not allow correct amplification of the internal competitive control and could have led to false-negative results. These inhibitors are unknown and are not eliminated by DNA isolation with proteinase K digestion and phenol-chloroform extraction.

The PCR used confirmed the diagnosis for three patients who died of IPA, but the clinical data and the sample cultures had easily established the diagnosis. This result was expected, and the PCR could have saved 2 days (the time taken for the culture in these cases) for the diagnosis. Nevertheless, antifungal treatment (intravenous amphotericin B) was given before the culture results were obtained since presumptive treatment is the rule in our wards of immunocompromised patients (7). PCR seems, therefore, to be of limited value in cases of full-blown, diffuse IPA since the diagnosis is easily established with routine culture.

For one PCR-negative patient, the culture yielded one *A. fumigatus* colony. This patient did not develop IPA. This result might be due to contamination by *A. fumigatus* conidia after collection of the BAL fluid or due to the lack of *A. fumigatus* DNA in the aliquot tested, whereas *A. fumigatus* was present in the aliquot seeded on Sabouraud slants. For the first hypothesis, negative PCR results could help to establish contamination. Nevertheless, the *Aspergillus* hyphae are often clustered and not equally spread in the BAL fluid, and the second hypothesis cannot be ruled out.

More questionable is the meaning of the 23% (12 of 52) PCR-positive results without IPA. These results could be due to a weak specificity of the amplification reaction. Indeed, amplification of DNAs from yeast and fungus strains tested did not occur except for those of other *Aspergillus* species. *A. flavus*, *A. terreus*, and *A. niger* DNAs are equally amplified, although the lengths of the amplified fragments are slightly different from those of *A. fumigatus*. However, these data allow us to include other *Aspergillus* spp. in the diagnosis of IPA. Moreover, specific PCR products were not systematically observed upon amplification when other pathogens were recovered from BAL samples. In addition, the specificity of the PCR products was controlled by hybridization with an internal oligonucleotide completely within the amplified sequence. A lack of specificity therefore seems unlikely.

One other explanation is the presence of *Aspergillus* species in the sample not related to IPA. Indeed, *Aspergillus* species have been cultivated from 5 to 73% of BAL samples, even when the patients did not develop IPA (12, 13). The positive PCR may result from colonization of the patients' respiratory tracts or contamination of the samples during processing. A very small number of *Aspergillus* conidia or hyphae could thus be detected upon amplification even if they do not grow on Sabouraud medium. Similar results have been reported by authors using PCR directed at other DNA targets: Spreadbury et al. reported two PCR-positive results for 7 immunocompetent patients (20), Tang et al. reported five PCR-positive results for 28 immunocompetent patients (21), and Melchers et al. reported three PCR-positive results for 8 neutropenic patients with negative cultures (14). Positive PCR results could be considered only if the patient had risk factors for aspergillosis. In view of our results, the patient would be given antifungal therapy on the basis of clinical and radiological data (2) rather than PCR results. A PCR-positive result would probably boost the decision in favor of therapy, but a PCR-negative result should not delay the initiation of treatment. The question of whether PCR would have a predictive value among patients at high risk for IPA remains to be answered. Studies which focus on larger cohorts of immunocompromised patients should be designed to answer this question. However, our preliminary results are not encouraging, as a PCR-positive result was not predictive of aspergillosis in the subsequent 6-month time interval for the immunocompromised patients, including HIV-positive patients with advanced AIDS.

Quantitation of the PCR signal could help in distinguishing between contamination of the sample and true aspergillosis. Competitive PCR allows semiquantitation, as has been done for amplification of *T. gondii* DNA (9). Only a large amount of *Aspergillus* DNA would direct the physician to the use of therapy. Unfortunately, IPA is not always diffuse, especially in AIDS patients (15). For these patients, the amount of *Aspergillus* hyphae recovered depends on the lobes washed during the BAL procedure. Moreover, the yield of the BAL fluid recovered is variable for each patient. The BAL can even be noncontributive for patients who have focal pulmonary lesions (13). For these patients, the reliability of a quantitation system would remain questionable.

The high proportion of positive results might also be due to contamination of samples or reaction buffers by previously amplified products or by *Aspergillus* conidia. The risk of contamination of samples by PCR products seems low. All the PCRs were performed according to the same technique as those used for the diagnosis of toxoplasmosis, and we have not yet observed any contamination by amplified *T. gondii* DNA in our laboratories (3, 9). On the contrary, the risk of contamination of reaction buffers by *A. fumigatus* conidia is high. Indeed, fewer than five conidia diluted in water could be detected (data not shown). In contrast with *T. gondii*, *A. fumigatus* is ubiquitous in the environment, and every step of the processing of the sample cannot take place under filtered air to prevent contamination. This constraint seems out of proportion in regard to the weak benefit that PCR could bring to the diagnosis of aspergillosis. The risk of false-positive results due to contamination of samples or reaction buffers seems too high to be managed in an everyday diagnostic procedure. Contamination could lead to pseudoepidemics as previously described (22). PCR could be performed with biological specimens less susceptible to aerocontamination. Blood or urine samples have been suggested (18), but *Aspergillus* spp. are rarely cultured from blood or urine specimens even from patients with disseminated infection (2). The probability of a positive PCR result is too low to perform prospective screening. On the other hand, when aspergillosis is disseminated and blood cultures are positive, the illness is almost always out of control. In contrast, PCR could be performed with pulmonary biopsy specimens and help establish diagnosis when the culture is negative because of previous antifungal treatment or when the fungus is unable to grow on Sabouraud medium.

Larger prospective studies focusing on immunocompromised patients with a high risk of IPA could improve knowledge on the clinical significance of positive PCR results, at least as a predictive factor for the subsequent development of IPA. However, we do not totally agree with previous reports (14, 20, 21) which indicate an important contribution of PCR performed with BAL fluid in the diagnosis of IPA. The risk of false positives will make interpretation difficult. The reliability of the PCR technique would then be questionable, as is already the case for the detection of *Mycobacterium tuberculosis* (17) and hepatitis C virus (23).

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