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Detection of *Aspergillus* Species DNA by PCR in Bronchoalveolar Lavage Fluid

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The usefulness of a nested PCR assay for detection of *Aspergillus* sp. DNA was evaluated in 177 bronchoalveolar lavage (BAL) fluid specimens. This test was accurate both to diagnose culture-negative BAL fluid specimens from patients with invasive pulmonary aspergillosis and to confirm culture-positive samples. However, it did not differentiate between infection and colonization.

Diagnosis in the early stages of invasive pulmonary aspergillosis (IPA) is very difficult, as clinical and radiological signs are nonspecific and the sensitivity of fungal cultures is low (40 to 60%), even when combined with direct microscopic examination (2, 5). Circulating-antigen detection may contribute to the diagnosis. However, up to 8% false positives are reported (11). Other antigens are under investigation as diagnostic tools (6), but attention has now turned to molecular methods. The role of PCR assay of bronchoalveolar lavage (BAL) fluid for diagnosing IPA does not clearly emerge in the literature (1, 3, 7, 9, 10, 14). Therefore, we have evaluated a nested-PCR-based amplification of *Aspergillus fumigatus* DNA that targets the genes encoding alkaline proteases of the fungus to determine the role of PCR in diagnosing IPA from BAL fluid under routine conditions.

Clinical and reference strains were tested to assess the specificity of the method: *A. fumigatus* (three strains), *Aspergillus flavus* (three strains), *Aspergillus niger* (two strains), *Aspergillus nidulans* (two strains), *Aspergillus terreus, Aspergillus glaucus, Fusarium oxysporum, Fusarium solani* (ATCC 10154), *Paecylomyces* spp., *Penicillium* spp., *Pseudallescheria boydii* (two strains), *Trichoderma harzianum* (two strains), *Rhizopus rhizopodiformis, Mucor* spp., *Candida albicans* (ATCC 10231), and *Candida glabrata* (ATCC 90030). The fungal strains were cultivated on Sabouraud dextrose agar and incubated at 37 or 28°C for up to 5 days, depending on the species. The clinical isolates were identified by macroscopic, microscopic, and culture characteristics (12).

All patients undergoing bronchoscopy at the University Hospital of Liège (Liège, Belgium) during a 12-month period (1997 to 1998) were included in the study. There were 74 immunosuppressed and 103 nonimmunosuppressed patients. Patients were referred to as immunosuppressed if they were under long-term corticotherapy for chronic obstructive pulmonary disease (COPD) ($n = 18$) or other diseases ($n = 13$) or if they had hematological malignancy ($n = 16$), organ transplantation ($n = 5$), AIDS ($n = 3$), or cancer ($n = 19$). Nonimmunosuppressed patients had bronchoscopy for investigation of severe pneumonia. Medical, radiological, histopathological, and microbiological records and autopsy findings were reviewed to assess IPA. Three groups were defined (A, proven or probable aspergillosis [$n = 10$]; B, colonization [$n = 5$]; and C, no evidence of aspergillosis [$n = 162$]) according to the following criteria: proven, histology with hyphal tissue invasion and *Aspergillus*-positive culture of one or more respiratory specimens or of a lung biopsy; probable, *Aspergillus*-positive culture from two or more respiratory samples and positive clinical or radiological findings (pulmonary infiltrates, nodular opacity, cavitation, or persistent fever under broad-spectrum antimicrobial chemotherapy) or positive histology without *Aspergillus*-positive culture; colonization, *Aspergillus*-positive cultures from one or more respiratory samples without clinical or radiological evidence (see above) of respiratory tract infection due to this pathogen; and no evidence of aspergillosis, *Aspergillus*-negative cultures and no clinical or radiological findings (see above) of aspergillosis.

After reception in the laboratory, each BAL fluid specimen was homogenized and separated into two parts: one part (1 to 5 ml) was stored at −20°C until it was analyzed by PCR, and the second part was included in the routine procedure and tested for the presence of bacteria, yeasts, fungi, parasites, and viruses. A PCR assay was performed a minimum of 1 week after sampling, and the clinicians were unaware of the results. The technique used for DNA extraction from fungal culture was adapted from that of Tang et al. (13), except that DNA was resuspended in 30 μl of water containing RNase A at a concentration of 50 μg/ml. The same technique was used for DNA extraction from yeasts except for the culture, which was made on Sabouraud dextrose agar and incubated for 48 h at 37°C. DNA extraction from BAL fluid was adapted from techniques already published by Tang and colleagues (14) except for two details: (i) 200 μg of proteinase K (Sigma, St. Louis, Mo.) was added to the 500 μl of BAL fluid and buffer and (ii) DNA was dried in a dry-heating block and resuspended in 30 μl of distilled H_2O containing RNase A (50 μg/ml). Positive-displacement pipettes were used throughout, and DNA extraction buffer was extracted in parallel in order to preclude contamination.

We developed a nested PCR using as external primers alp 11

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TABLE 1. Clinical data and aspergillosis detection for group A (proven or probable aspergillosis) and group B (colonization) patients

Case no.	Underlying disease[a]	IPA group (disease[b])	BAL fungal culture	Autopsy performed	BAL PCR results[c]	Outcome[d]
Group A (n = 10)						
1	Hepatic transplantation	Proven (DIS)	*A. fumigatus*	Yes	+	Died
2	COPD (corticosteroid dependent)	Proven (CNA)	*A. fumigatus*	Yes	+	Died (AmB)
3	Chronic lymphoid leukemia + COPD	Probable	*A. fumigatus*	No	+	Died
4	RA (corticosteroid dependent)	Proven	Negative	Yes	+	Died
5	COPD (corticosteroid dependent)	Proven (DIS)	*A. fumigatus*	Yes	+	Died
6	Gastric carcinoma	Proven	*A. fumigatus*	Yes	+	Died
7	COPD (corticosteroid dependent)	Proven (CNA)	*A. fumigatus*	Yes	+	Died
8	Chronic myeloid leukemia blast-crisis	Probable	*A. fumigatus*	No	+	Alive (Vor)
9	Chronic lymphoid leukemia blast-crisis	Proven	Negative	Yes	+	Died
10	COPD (corticosteroid dependent)	Probable	*A. fumigatus*	No	+	Died
Group B (n = 5)						
1	Mitral valvulopathy	None	*A. fumigatus*	No	+	Alive (AmB/It)
2	COPD (corticosteroid dependent)	None	*A. fumigatus*	No	+	Died
3	COPD (corticosteroid dependent)	None	*A. fumigatus*	Yes	+	Died
4	COPD (no corticoid)	None	*A. fumigatus*	No	+	Alive
5	Myelodysplasia blast-crisis	None	*A. flavus*	Yes	+	Died

[a] RA, rheumatoid arthritis.
[b] DIS, disseminated aspergillosis; CNA, chronic necrotizing aspergillosis.
[c] +, positive.
[d] Vor, treatment with voriconazole; AmB/It, treatment with amphotericin B and itraconazole; AmB, treatment with amphotericin B.

(5′-AGCACCGACTACATCTAC-3′) and alp 12 (5′-GAGAT GGTGTTGGTGGC-3′). These primers were derived from the sequence of cloned fragments of genes encoding the alkaline proteases (Alp) of *A. fumigatus* and *A. flavus* (14). As internal primers, we chose Alp13 (5′-CTGGCATACAACGCCGCTG-3′) and Alp14 (5′-TTGTTGATCGCAACC-3′), expected to amplify a fragment of 527 bp. The primers were synthesized by Eurogentec, Liège, Belgium. The PCR mixtures were identical for both steps except for $MgCl_2$. They were carried out in a 50-μl volume containing 10 mM Tris-Cl at pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$ (2.25 mM for the second step), with 20 pmol of both primers, 2.5 mM deoxynucleoside triphosphate (buffer and deoxynucleoside triphosphate were provided by Takara, Otsu, Japan), and 1.25 U of *Taq* polymerase (Takara). A 5-μl volume of DNA was added to the mixture. Positive and negative controls were amplified in parallel to assess the validity of the procedure. Thermal cycling conditions (GeneAmp PCR system 2400; Perkin-Elmer Cetus, Norwalk, Conn.) were identical for both PCRs: 5 min at 94°C; 30 cycles of 30 s at 94°C, 45 s at 63°C, and 2 min at 72°C; and a final extension step of 10 min at 72°C. For the nested PCR, 5 μl of the first amplified product was added to a new reaction mixture and amplified under the same conditions. The final amplified products were analyzed on 1.5% agarose gels stained with ethidium bromide and visualized by UV transillumination. Each sample was investigated for the presence of inhibitors by amplification of the β-globin gene (8).

No band of the expected size (527 bp) was detected with *A. niger, A. nidulans, A. glaucus, A. terreus*, or *A. flavus* or with the other fungal species. However, a band of 690 nm, corresponding to the sequence amplified by the first PCR, was observed for *A. flavus*. The sensitivity of the nested PCR was 25 fg of genomic DNA for *A. fumigatus* by ethidium bromide staining and 10 pg for *A. flavus*. Some BAL fluids (n = 5) were excluded because of lack of amplification of the β-globin gene. The PCR results are reported in Table 1. The sensitivity, specificity, and positive and negative predictive values of this PCR test for diagnosing IPA were 100, 96, 62, and 100%, respectively. All BAL fluids from patients with IPA were PCR positive. Those from the five cases of *Aspergillus* colonization were PCR and culture positive. In all, six PCR-positive cases were not associated with IPA among the 177 BAL fluid specimens, representing 3.4% false-positive results. Only one false-positive PCR result was induced in group C. Among the three patients with COPD and proven IPA (group A), two patients (cases 2 and 7) presented with chronic necrotizing aspergillosis and one (case 5) presented with disseminated aspergillosis. In two cases (4 and 9), the diagnosis of proven IPA was made at autopsy by histology and *A. fumigatus*-positive culture of a lung biopsy specimen.

In the present study, the PCR always confirmed the culture results, except for two culture-negative BAL fluid specimens, for which diagnosis of aspergillosis was missed and made at autopsy. In both cases, the PCR could have contributed to diagnosing IPA before death. Only one PCR-positive BAL result was observed for a patient with no evidence of aspergillosis: a burn patient who was intubated for 1 month. This false-positive result could be explained by contamination of the BAL fluid during sampling or the PCR process, or it could have been due to the colonization of the respiratory tract by *Aspergillus* spores during intubation. Some authors have reported up to 35% PCR-positive results for patients with no risk or low risk for aspergillosis and who did not develop IPA (1, 3, 9, 10, 14, 15). However, other studies performed in neutropenic patients (4) or in nonimmunosuppressed patients (7) reported no false-positive PCR results for BAL fluids. Among our population, only 3.4% positive PCR results not associated with IPA were observed. Half of the patients (3 out of 6) had COPD. These patients may have had a relatively high level of tracheobronchial colonization, which differs from patients with hematological malignancies, who may have had minimum involvement before developing severe invasive fungal infection.

In our study, the major risk factor associated with IPA was corticotherapy associated with COPD. However, the major risk factor for aspergillosis is known to be a prolonged neutropenia, accounting for its high frequency in patients with acute leukemia (2). In fact, in our institution, patients with hematological malignancies benefit from a good follow-up and are often treated early in cases where there is a suspicion of infectious disease.

In conclusion, nested PCR of BAL fluid is an accurate test to diagnose culture-negative patients with IPA, but it does not differentiate between infection and colonization. It is an appropriate method to exclude *Aspergillus* sp. infection in patients at risk of IPA and should be included in routine laboratory practice for this immunocompromised population.

REFERENCES

1. **Bart-Delabesse, E., A. Marmorat Khuong, J. M. Costa, M. L. Dubreuil-Lemaire, and S. Bretagne.** 1996. Detection of *Aspergillus* DNA in bronchoalveolar lavage fluid of AIDS patients by the polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. **15:**24–25.
2. **Bodey, G. P., and S. Vartarian.** 1989. Aspergillosis. Eur. J. Clin. Microbiol. Infect. Dis. **8:**413–437.
3. **Bretagne, S., J. M. Costa, A. Marmorat-Khuong, F. Poron, C. Cordonnier, M. Vidaud, and J. Fleury-Feith.** 1995. Detection of *Aspergillus* species DNA in bronchoalveolar lavage samples by competitive PCR. J. Clin. Microbiol. **33:**1164–1168.
4. **Jones, M. E., A. J. Barnes, B. A. Oppenheim, P. Balagopal, G. R. Morgenstern, and J. H. Scarffe.** 1998. PCR-ELISA for the early diagnosis of invasive pulmonary *Aspergillus* infection in neutropenic patients. J. Clin. Pathol. **51:** 652–656.
5. **Kahn, F. W., J. M. Jones, and D. England.** 1985. The role of bronchoalveolar lavage in the diagnosis of invasive aspergillosis. Am. J. Clin. Pathol. **86:**518–523.
6. **Latgé, J. P.** 1999. *Aspergillus fumigatus* and aspergillosis. Clin. Microbiol. Rev. **12:**310–350.
7. **Melchers, W. J. G., P. E. Verweij, P. van den Hurk, A. van Belkum, B. De Pauw, J. Hoogkamp-Korstanje, and J. F. G. M. Meis.** 1994. General primer-mediated PCR for detection of *Aspergillus* species. J. Clin. Microbiol. **32:** 1710–1717.
8. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, and H. A. Erlich.** 1985. Enzymatic amplification of β-globin sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350–1354.
9. **Skladny, H., D. Buchheidt, C. Baust, F. Krieg-Schneider, W. Seifarth, C. Leib-Mosch, and R. Hehlmann.** 1999. Specific detection of *Aspergillus* species in blood and bronchoalveolar lavage samples of immunocompromised patients by two-step PCR. J. Clin. Microbiol. **37:**3865–3871.
10. **Spreadbury, C., D. Holden, A. Aufauvre-Brown, B. Bainbridge, and J. Cohen.** 1993. Detection of *Aspergillus fumigatus* by polymerase chain reaction. J. Clin. Microbiol. **31:**615–621.
11. **Stynen, D., A. Goris, J. Sarfati, and J. P. Latgé.** 1995. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J. Clin. Microbiol. **33:**497–500.
12. **Sutton, D. A., A. W. Fothergill, and M. Rinaldi.** 1998. Guide to clinically significant fungi. The Williams & Wilkins Co., Baltimore, Md.
13. **Tang, C. M., J. Cohen, and D. W. Holden.** 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol. Microbiol. **6:**1663–1671.
14. **Tang, C. M., D. W. Holden, A. Aufauvre-Brown, and J. Cohen.** 1993. The detection of *Aspergillus* spp. by the polymerase chain reaction and its evaluation in bronchoalveolar lavage fluid. Am. Rev. Respir. Dis. **148:**1313–1317.
15. **Verweij, P. E., J. F. G. M. Meis, P. van den Hurk, B. E. De Pauw, J. A. A. Hoogkamp-Korstanje, and W. J. G. Melchers.** 1994. Polymerase chain reaction as a diagnostic tool for invasive aspergillosis: evaluation in bronchoalveolar lavage fluid from low risk patients. Serodiagn. Immunother. Infect. Dis. **6:**203–208.