Journal of Clinical Microbiology, Feb. 2004, p. 844–846 Vol. 42, No. 2
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.2.844–846.2004
Copyright © 2004, American Society for Microbiology. All Rights Reserved.

Development of a Serum-Based Taqman Real-Time PCR Assay for Diagnosis of Invasive Aspergillosis

Svetlana Challier,* Stéphanie Boyer, Eric Abachin, and Patrick Berche

Laboratoire de Microbiologie, Hôpital Necker-Enfants Malades, Paris, France

Received 28 July 2003/Returned for modification 31 August 2003/Accepted 27 October 2003

We compared an *Aspergillus fumigatus*-specific Taqman real-time PCR assay for the diagnosis of invasive aspergillosis (IA) with the enzyme-linked immunosorbent assay Platelia *Aspergillus* method. Patients with proven or probable IA had positive results with at least one of the two methods. The PCR method seems to be more specific, and a combination of the two should improve the diagnosis of IA.

Due to the absence of a highly sensitive and specific "gold standard" method for the early diagnosis of invasive aspergillosis (IA) (5, 6), we developed an *Aspergillus fumigatus*-specific Taqman real-time PCR assay. We retrospectively evaluated it on serum samples from 41 patients with suspected or confirmed IA and compared its performance with that of the enzyme-linked immunosorbent assay (ELISA) Platelia *Aspergillus* method, which detects *Aspergillus* galactomannan antigens.

Primers and probe were designed by comparison of the sequences of rRNA genes (GenBank database) from *A. fumigatus* (accession no. U28460), other *Aspergillus* spp. (*A. flavus*, *A. niger*, *A. nidulans*, *A. terreus*, and *A. versicolor*), other filamentous fungi (*Paecilomyces* spp., *Penicillium* spp., *Fusarium* spp., and *Alternaria* spp.), yeasts (*Candida* spp. and *Trichosporon* spp.), and bacteria (*Staphylococcus* spp., *Streptococcus* spp., *Enterobacteriaceae*, *Pseudomonas* spp., and *Actinomyces* spp.). The target, a 67-bp DNA fragment specific to the multicopy gene encoding the 28S rRNA of *A. fumigatus*, was chosen with the Primer Express, version 1.0, program for Taqman technology. The primers were 28S-466 (5′-CTC GGA ATG TAT CAC CTC TCG G-3′) and 28S-533 (5′-TCC TCG GTC CAG GCA GG-3′), and the Taqman probe was 28S-490 (5′-6-carboxyfluorescein-TGT CTT ATA GCC GAG GGT GCA ATG CG-3′-6-carboxy tetramethylrhodamine).

For the PCR assay, the QIAmp DNA minikit (Qiagen) was used to extract the DNA from *A. fumigatus* IHEM 6117 (Scientific Institute of Public Health-Louis Pasteur, Brussels, Belgium), other fungi and bacteria studied, and all serum samples. Purified DNA was eluted in 50 μl of elution buffer and stored at −20°C until use. The 50-μl PCR mixture contained 1× Taqman universal PCR Master Mix, 500 nM each primer, 100 nM probe, and 15 μl of DNA. DNA was amplified in a Thermocycler/ABI Prism 7700 sequence detector (Applied Biosystems) by incubation at 95°C for 10 min followed by 45 amplification cycles (15 s of denaturation at 95°C and 1 min of hybridization and elongation at 60°C). The fluorescence of the PCR products was detected by the same apparatus. A calibration curve was constructed for each assay to determine the amount of DNA present in serum samples (concentration versus number of copies). For this, the *A. fumigatus* target was ligated into pUC18 and used to transform a competent *Escherichia coli* strain. PCR inhibitors were systematically sought by amplification of the β-globin gene.

We tested 207 serum samples from 41 immunocompromised patients (21 adults and 20 children) hospitalized in the Hematology, Reanimation, or Infectiology department of Hôpital Necker-Enfants Malades, Paris, France (n = 39), Cochin Hospital, Paris, France (n = 1), or Amiens Hospital (n = 1) between January 1999 and July 2001. Patients were considered to have proven, probable, or possible IA according to the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group classification (2). At least two serum samples (range, 2 to 14 samples) were obtained from each patient over a maximum of 2 months before and/or after suspected infection (day 0 [D0]). Samples were collected in dry tubes and stored at −20°C. We also tested 39 serum samples from 29 control patients: 19 from 19 healthy subjects (11 adults and 8 children), 8 from 4 children with cystic fibrosis and *A. fumigatus* bronchial colonization, and 12 collected 15 and 30 days after bone marrow transplantation from 6 adults without suspected IA.

Aspergillus antigens were detected with the ELISA Platelia *Aspergillus* kit (Bio-Rad). Results were considered positive when they were equal to or higher than the cutoff value (1 ng of galactomannan/ml of serum). *A. fumigatus* DNA was detect-

TABLE 1. Results of the PCR tests and ELISA for the three IA groups

IA group	No. (%) of: PCR test-positive patients/no. tested	No. (%) of: ELISA t-positive patients/no. tested
Total	26/41 (63.4)	29/41 (71)
Proven	7/7 (100)	5/7 (71.4)
Adults	3/3 (100)	2/3 (66.7)
Children	4/4 (100)	3/4 (75)
Probable	15/19 (78.9)	15/19 (78.9)
Adults	11/11 (100)	7/11 (63.6)
Children	4/8 (50)	8/8 (100)
Possible	4/15 (26.7)	9/15 (60)
Adults	3/7 (42.9)	1/7 (14.3)
Children	1/8 (12.5)	8/8 (100)

* Corresponding author. Mailing address: Laboratoire de Microbiologie, Hôpital Necker-Enfants Malades, 149-161 rue de Sèvres, 75743 Paris cedex 15, France. Phone: 33-1-44-49-49-61. Fax: 33-1-44-49-49-60. E-mail: svetlana.challier@nck.ap-hop-paris.fr.

TABLE 2. Clinical, radiological, and biological features and test results for patients with proven, probable, and possible IA

IA group	Patient no.[a]	Sex/age (yr)	Feature(s)[b]			Mycology		No. of samples tested on D0/total no. of samples tested	No. of positive samples[e]/time of first positive test[f]	
			Clinical	Radiological		Site(s)[c]/result by ED[d] or histology	Culture		ELISA	PCR
				Nonspecific	Specific (scanner)					
Proven	1 (D, H)	M/28	P		P	Lung/+	−	1/19	3/D22	5*/D2
	2 (D, H)	M/76	P/C	P	/	Lung/+	*A. fumigatus*	0/2	0/NA	1/D24
	3 (R)	M/52	P	P	/	Trachea/+	*A. fumigatus*	0/2	2/D6	1/D6
	4 (D, T)	F/4	P/C	P/C	−	Disseminated/+	/	5/5	1/D0	1/D0
	5 (D, T)	F/16	P	P	/	Heart, thorax/+	*A. fumigatus*	1/9	0/NA	3/D12
	6 (D, T)	M/2	P/C	P	C	Lung/+	*A. fumigatus*	2/3	1/D14	1/D14
	7 (H)	M/2	P/C	P	C	Lung, brain/+	*A. fumigatus*	3/5	1/**D−9**	3/**D−9**
Probable	8 (D, H)	F/46	P/C		C	−	−	0/2	2/D19	2/D19
	9 (H, B)	M/51	P	P	/	+	*A. fumigatus*	0/7	2/D7	3/D7
	10 (H)	M/72	P		P	−	*A. fumigatus*	2/5	−	4/**D−29**
	11 (D, H, B)	F/45	P		P	−	−	1/14	−	5*/D22
	12 (D, H)	M/59	P/C	P	−	/	/	1/3	3/D0	1*/D0
	13 (D, H, B)	F/38	P		P	−	*A. fumigatus*	0/11	10/D2	11*/D1
	14 (D, H)	F/63	P	P	/	−	*A. flavus*	0/7	−	5/D2
	15 (D, H, B)	M/36	P/C		P	−	−	1/8	−	2/D13
	16 (T)	M/48	P	P	/	+	*A. fumigatus*	3/4	1/D4	2/**D−31**
	17 (H)	M/58	P		P	+	*A. fumigatus*	0/4	3/D3	2*/D3
	18 (D, H)	F/67	P		P	−	−	4/7	5/D0	5*/D0
	19 (T)	M/6	P	P	/	−	−	1/6	4/D0	−
	20 (D, H, B)	M/4	P/C	P	C	−	−	1/6	6/D0	−
	21 (D, H)	M/<1	P	P	/	−	*A. fumigatus*	0/2	2/D15	2/D15
	22 (D, H, B)	F/2	P	P	/	/	/	2/8	7/D0	−
	23 (D, H, B)	M/7	P	P	/	−	−	1/2	2/D0	−
	24 (H, B)	M/18	P	P	/	−	−	1/6	3/D11	3/D11
	25 (D, H, B)	M/15	P	P	/	−	*A. fumigatus*	2/4	3/D0	2/D6
	26 (D, H)	M/4	P	P	/	/	/	3/5	3/D21	3*/D21
Possible	27 (D, H)	M/65	P	P	/	−	−	0/8	−	6/D20
	28 (D, H)	F/71	P	P	/	−	*A. fumigatus*	1/2	−	−
	29 (H)	M/68	P	P	/	−	−	0/5	−	2/D10
	30 (H)	F/52	P	P	/	/	/	1/3	−	−
	31 (D, H)	M/71	P	P	/	−	−	0/2	−	−
	32 (D, H, B)	M/29	P/C	P	−	/	/	5/6	1/D7	3/**D−26**
	33 (D, H)	M/77	P	P	/	−	*A. fumigatus*	1/2	−	−
	34 (T)	M/2	−	−	/	/	/	1/2	2/D0	−
	35 (D, T)	M/4	P	P	/	−	*A. fumigatus*	0/4	1/D39	1/D30
	36 (D, I)	F/16	−	−	/	−	−	0/3	3/D2	−
	37 (H, B)	F/2	P	−	/	−	−	2/4	4/**D−22**	−
	38 (D, H, B)	F/2	−	−	/	/	/	0/4	4/D20	−
	39 (D, H, B)	F/3	−	−	/	−	−	3/9	7/D0	−
	40 (H, B)	F/4	−	P	/	/	/	3/4	3/D0	−
	41 (T)	M/4	−	P	/	/	/	1/3	3/**D−1**	−

[a] D, death; H, hematological disease; I, immunological disease; B, bone marrow transplantation; T, organ transplantation; R, human immunodeficiency virus infection.
[b] P, pulmonary signs; C, cerebral signs; P/C, pulmonary and cerebral signs; /, not tested; −, absent.
[c] Where no site is listed, the site is pulmonary.
[d] ED, cytological direct microscopic examination.
[e] *, PCR quantification of 1 to 10 copies.
[f] NA, not applicable. Boldface indicates days before D0.

ed with our specific PCR method. All samples were tested in duplicate. The sensitivity (related to the mean minimum number of cycles necessary to detect fungal DNA) was 34.3 cycles (1 copy). The reproducibility was good, with a linear function and a correlation coefficient of 0.983 for four DNA concentrations (1 to 10^3 copies) amplified on five different days.

PCR methods designed for fungi and the targets used (often located within the 18S rRNA gene) vary considerably among authors. These methods have been used to detect the *Aspergillus* DNA in serum or total blood, but the two types of sample have not been compared for the same patients (3, 4, 7–11, 14, 15, 18). Due to the retrospective nature of our study, the tests were carried out on serum samples. To avoid cross-reactions with microorganisms other than the target (e.g., *Penicillium* spp.), we chose a target within the 28S rRNA gene, which is specific to *A. fumigatus*, the main agent responsible for IA. We selected a multicopy target to maximize sensitivity. With the Taqman technology, there is little risk of false positives due to contamination with previously amplified products. The specific DNA could be detected and quantified within 2 h, and the method can easily be standardized. Thus, this method is suitable for routine biological diagnosis.

The sensitivity and specificity of the ELISA for the diagnosis of probable and proven IA are about 90% when clinical or radiological symptoms are present but about 50% in their absence (12, 16). The frequency of false-positive results for

at-risk pediatric populations is between 5.7 and 10% (13). Some studies compared this method and PCR. One study showed that nested PCR with a target within the 28S rRNA gene was more sensitive than the ELISA (18). Another found that the PCR method developed with a mitochondrial DNA target was less sensitive and should be used to confirm positive ELISA results (3).

In our study (Tables 1 and 2), all control serum samples were negative for the two tests. Of the 41 patients with IA, 26 were positive according to the PCR method: 20 had a mean of one copy, and 6 had between 1 and 10 copies. Twenty-six patients had proven or probable IA (14 adults and 12 children): 22 (7 of 7 proven, 15 of 19 probable), including all the adults, had a positive PCR test (84.6%), and 20 (5 of 7 proven, 15 of 19 probable) had a positive ELISA (75.2%). All gave a positive result with at least one of the two methods. When the two tests were positive for the same patient (5 proven, 11 probable), the PCR method was positive earlier than the ELISA for 3 patients with probable IA (patients 1, 13, and 16). In three (one proven [patient 7] and two probable [patients 10 and 16]) of the nine patients who had serological follow-up before developing fungal infection (three proven and six probable), the PCR test was positive before the radioclinical symptoms appeared (9 to 31 days before D0). For the two probable cases, the PCR test was positive before the ELISA. For four patients with probable IA, the PCR test was negative. Three were treated with antifungal agents on D0 and died from infectious respiratory distress syndrome. These results may be due to the lack of sensitivity of the method. Indeed, only very small amounts of *A. fumigatus* DNA circulate in the serum (between 100 fg/ml and 1 ng/ml). Similar levels were measured by Loeffler et al. in whole blood (11). Some authors observed that antifungal treatment rapidly results in negative PCR results (9). The detection of fungal DNA in the blood may be improved if DNA is extracted from a larger sample. It should also be possible to include a reverse transcription step.

In the possible IA group, the pulmonary symptoms of two (patients 27 and 29) of the three PCR-positive adults improved following treatment with antifungal agents but relapsed when specific treatment was stopped. The four PCR-negative patients were never treated with antifungal agents during the observed period and did not die. The discrepancy between the PCR and ELISA results for children with possible IA suggests that the PCR test is more specific in this population. ELISA was suspected to have given false-positive results for 10 patients (3 probable and 7 possible) with an associated infection (candidiasis or systemic bacterial infection) that may have resulted in cross-reaction (17) or with mucositis or host-versus-graft disease resulting in epithelial lesions that favored the digestive translocation of ingested galactomannans (1).

Thus, our PCR assay and the ELISA method are complementary for the diagnosis of IA. All the patients with proven or probable aspergillosis had a positive result with at least one of the two methods. However, the PCR method seems to be more specific than the ELISA method for patients with possible IA. Given these promising results, it is necessary to evaluate this method in a prospective study.

We thank F. Symoens and N. Nolard (IHEM, Mycology Section, Scientific Institute of Public Health-Louis Pasteur) for providing us collection strains, A. Paugam (Cochin Hospital) for data and samples concerning one patient, and C. Hennequin (Amiens Hospital) for data and samples concerning one patient and for critical reading of the manuscript.

REFERENCES

1. **Ansorg, R., R. van den Boom, and P. M. Rath.** 1997. Detection of *Aspergillus* galactomannan antigen in foods and antibiotics. Mycoses **40:**353–357.
2. **Ascioglu, S., J. H. Rex, B. de Pauw, J. E. Bennett, J. Bille, F. Crokaert, D. W. Denning, J. P. Donnelly, J. E. Edwards, Z. Erjavec, D. Fiere, O. Lortholary, J. Maertens, J. F. Meis, T. F. Patterson, J. Ritter, D. Selleslag, P. M. Shah, D. A. Stevens, and T. J. Walsh.** 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin. Infect. Dis. **34:**7–14.
3. **Bretagne, S., J. M. Costa, E. Bart-Delabesse, N. Dhedin, C. Rieux, and C. Cordonnier.** 1998. Comparison of serum galactomannan antigen detection and competitive polymerase chain reaction for diagnosing invasive aspergillosis. Clin. Infect. Dis. **26:**1407–1412.
4. **Costa C., D. Vidaud, M. Olivi, E. Bart-Delabesse, M. Vidaud, S. Bretagne.** 2001 Development of two real-time quantitative TaqMan PCR assays to detect circulating *Aspergillus fumigatus* DNA in serum. Microbiol. Methods **44:**263–269.
5. **Denning, D. W., A. Marinus, J. Cohen, D. Spence, R. Herbrecht, L. Pagano, C. Kibbler, V. Kcrmery, F. Offner, C. Cordonnier, U. Jehn, M. Ellis, L. Collette, and R. Sylvester.** 1998. An EORTC multicentre prospective survey of invasive aspergillosis in haematological patients: diagnosis and therapeutic outcome. J. Infect. **37:**173–180.
6. **Dupont, B., M. Richardson, P. E. Verweij, and J. F. Meis.** 2000. Invasive aspergillosis. Med. Mycol. **38**(Suppl. 1)**:**215–224.
7. **Einsele, H., H. Hebart, G. Roller, J. Loffler, I. Rothenhofer, C. A. Muller, R. A. Bowden, J. van Burik, D. Engelhard, L. Kanz, and U. Schumacher.** 1997. Detection and identification of fungal pathogens in blood by using molecular probes. J. Clin. Microbiol. **35:**1353–1360.
8. **Hebart, H., J. Loffler, C. Meisner, F. Serey, D. Schmidt, A. Bohme, H. Martin, A. Engel, D. Bunje, W. V. Kern, U. Schumacher, L. Kanz, and H. Einsele.** 2000. Early detection of *Aspergillus* infection after allogeneic stem cell transplantation by polymerase chain reaction screening. J. Infect. Dis. **181:**1713–1719.
9. **Lass-Florl, C., J. Aigner, E. Gunsilius, A. Petzer, D. Nachbaur, G. Gastl, H. Einsele, J. Loffler, M. P. Dierich, and R. Wurzner.** 2001. Screening for *Aspergillus* spp. using polymerase chain reaction of whole blood samples from patients with haematological malignancies. Br. J. Haematol. **113:**180–184.
10. **Loeffler, J., H. Hebart, U. Brauchle, U. Schumacher, and H. Einsele.** 2000. Comparison between plasma and whole blood specimens for detection of *Aspergillus* DNA by PCR. J. Clin. Microbiol. **38:**3830–3833.
11. **Loeffler, J., N. Henke, H. Hebart, D. Schmidt, L. Hagmeyer, U. Schumacher, and H. Einsele.** 2000. Quantification of fungal DNA by using fluorescence resonance energy transfer and the Light Cycler system. J. Clin. Microbiol. **38:**586–590.
12. **Maertens, J., J. Verhaegen, H. Demuynck, P. Brock, G. Verhoef, P. Vandenberghe, J. Van Eldere, L. Verbist, and M. Boogaerts.** 1999. Autopsy-controlled prospective evaluation of serial screening for circulating galactomannan by a sandwich enzyme-linked immunosorbent assay for hematological patients at risk for invasive aspergillosis. J. Clin. Microbiol. **37:**3223–3228.
13. **Rohrlich, P., J. Sarfati, P. Mariani, M. Duval, A. Carol, C. Saint-Martin, E. Bingen, J. P. Latge, and E. Vilmer.** 1996. Prospective sandwich enzyme-linked immunosorbent assay for serum galactomannan: early predictive value and clinical use in invasive aspergillosis. Pediatr. Infect. Dis. J. **15:**232–237.
14. **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.
15. **Spiess, B., D. Buchheidt, C. Baust, H. Skladny, W. Seifarth, U. Zeilfelder, C. Leib-Mösch, H. Mörz, and R. Hehlmann.** 2003. Development of a LightCycler PCR assay for detection and quantification of *Aspergillus fumigatus* DNA in clinical samples from neutropenic patients. J. Clin. Microbiol. **41:**1811–1818.
16. **Sulahian, A., F. Boutboul, P. Ribaud, T. Leblanc, C. Lacroix, and F. Derouin.** 2001. Value of antigen detection using an enzyme immunoassay in the diagnosis and prediction of invasive aspergillosis in two adult and pediatric hematology units during a 4-year prospective study. Cancer **91:**311–318.
17. **Swanink, C. M., J. F. Meis, A. J. Rijs, J. P. Donnelly, and P. E. Verweij.** 1997. Specificity of a sandwich enzyme-linked immunosorbent assay for detecting *Aspergillus* galactomannan. J. Clin. Microbiol. **35:**257–260.
18. **Williamson, E. C., J. P. Leeming, H. M. Palmer, C. G. Steward, D. Warnock, D. I. Marks, and M. R. Millar.** 2000. Diagnosis of invasive aspergillosis in bone marrow transplant recipients by polymerase chain reaction. Br. J. Haematol. **108:**132–139.