Detection of *Aspergillus* DNA in Cerebrospinal Fluid from Patients with Cerebral Aspergillosis by a Nested PCR Assay[▽]

M. Hummel,^{1*} B. Spiess,¹ K. Kentouche,² S. Niggemann,³ C. Böhm,⁴ S. Reuter,⁵
M. Kiehl,⁶ H. Mörz,¹ R. Hehlmann,¹ and D. Buchheidt¹

III. Medizinische Universitätsklinik, Fakultät für Klinische Medizin Mannheim, Universität Heidelberg, D-68305 Mannheim,¹
Klinik für Kinder- und Jugendmedizin, Friedrich-Schiller-Universität Jena, 07743 Jena,² Klinik für Kinderheilkunde,
Klinikum Mannheim, Universität Heidelberg, D-68167 Mannheim,³ Institut für Klinische Radiologie,
Klinikum Mannheim, Universität Heidelberg, D-68167 Mannheim,⁴ Medizinische Klinik III,
Universitätsklinikum Ulm, Ulm,⁵ and Klinikum Frankfurt/Oder, Frankfurt/Oder,⁶ Germany

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Invasive aspergillosis (IA), a complication with high mortality rates, especially in disseminated IA with cerebral involvement, is difficult to diagnose. Biopsy of cerebral lesions is often not feasible, and culture of *Aspergillus* spp. from cerebrospinal fluid (CSF) is frequently negative. New molecular methods have emerged for diagnosing IA. So far, there are only few reports of *Aspergillus* DNA detection in CSF. After modifying the DNA extraction protocol, we detected *Aspergillus* DNA in CSF samples by a previously described nested PCR assay. In six patients with hematologic malignancy and cerebral aspergillosis, CSF samples were investigated for *Aspergillus* DNA. IA was classified according to the EORTC/MSG 2002 criteria. Two patients each had proven, probable, and possible IA. Thirty-five CSF samples were investigated for *Aspergillus* DNA by nested PCR. Samples with positive results in the nested PCR assay were quantified by LightCycler PCR assay. Fourteen CSF samples showed positive results in the nested PCR assay. Of these, six samples gave positive results in real-time PCR. The range of CFU per ml was 2,154 to 63,100,000. The highest number of CFU per ml was found in a CSF sample of a patient with acute lymphocytic leukemia and probable cerebral aspergillosis. Detection of *Aspergillus* DNA in CSF samples is thus possible and has the potential to improve diagnosis of cerebral aspergillosis. Further prospective studies with larger numbers of patients must be performed to evaluate the clinical significance of *Aspergillus* PCR with CSF samples.

Cerebral aspergillosis is a rare but often fatal complication in patients with immunosuppression and has a worse prognosis than other localizations of aspergillosis. The case fatality rate of cerebral aspergillosis has been reported to be above 95%, compared to a 58% overall fatality rate for invasive aspergillosis (IA) (7, 15).

Cerebral aspergillosis mostly presents as a cerebral mass lesion or as cerebral infarction but rarely as meningitis. Differential diagnoses of cerebral mass lesions in the compromised host are various and range from infectious diseases caused by fungal pathogens other than *Aspergillus* spp. to toxoplasmosis, nocardiosis, and noninfectious disorders such as intracerebral bleeding, primary or secondary malignancies of the brain, and embolic or thrombotic strokes (6).

A cerebral mass lesion usually requires a tissue specimen for microbiologic and histologic evaluation (6). However, invasive procedures such as biopsy of the brain are often not feasible in hematologic patients, especially when thrombocytopenia is present.

With the exception of *Cryptococcus neoformans*, fungi are rarely detected in cultures of cerebrospinal fluid (CSF) obtained from patients having or suspected of having fungal ce-

rebral infection (18). This makes diagnosis of cerebral aspergillosis difficult.

In order to facilitate the diagnosis of invasive aspergillosis, molecular methods have been developed in recent years. Several PCR assays have been established to detect *Aspergillus* spp. in blood and in bronchoalveolar lavage (BAL) fluid samples, but experience with *Aspergillus* PCR with CSF samples is limited (4, 10, 11, 19, 25).

We investigated whether *Aspergillus* DNA can be detected in CSF samples from patients with cerebral aspergillosis by our nested PCR assay, which has been clinically and experimentally validated for blood and BAL fluid samples (2, 3, 5, 8, 22), and whether quantification of DNA by a previously described LightCycler assay (23) is possible.

MATERIALS AND METHODS

Patients and procedures. CSF samples from six patients with hematologic malignancy and possible, probable, or proven cerebral *Aspergillus* infection were investigated by a nested PCR assay and a LightCycler assay for the presence of *Aspergillus* DNA. Both assays have been previously described and validated for blood and BAL samples. CSF was obtained under sterile conditions either by lumbar puncture or, in one patient, from implanted ventricle catheters. Samples were obtained at diagnosis of cerebral disease and during follow-up. CSF samples were processed for routine microbiological studies such as Gram staining, microscopy, and bacterial and fungal culturing. Vials of 1 to 2 ml of CSF were shipped to our laboratory in sterile tubes for performance of *Aspergillus* PCR and were processed immediately.

Additional blood and BAL samples from five and two patients, respectively, were investigated for *Aspergillus* DNA by PCR. Patient characteristics are shown in Table 1. IA was classified according to the EORTC/MSG 2002 criteria (1). As

* Corresponding author. Mailing address: III. Medizinische Universitätsklinik, Fakultät für Klinische Medizin Mannheim, Universität Heidelberg, Wiesbadenerstraße 7-11, D-68305 Mannheim, Germany. Phone: 49-621-3834115. Fax: 49-621-3834201. E-mail: margit.hummel@med3.ma.uni-heidelberg.de.

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TABLE 1. Patient characteristics, clinical data, and PCR results with different samples

Patient no.	Age/ sex ^a	Underlying disease ^b	IA (by EORTC/ MSG 2002 criteria)	CNS lesion	Other site(s) of IA	Antifungal treatment	Outcome	Microbiological findings	No. of samples investigated by PCR	CSF PCR ^c		Blood PCR (no. of samples)	BAL fluid PCR (no. of samples)	LightCycler PCR (CFU/ml)			
										No. of samples	Δ days	+	-		+	-	
																	+
1	12/f	ALL	Proven	Solitary cerebral abscess	Lung, spleen, liver, kidney	Caspofungin, voriconazole	Improvement	Histologic proof from lung specimen; <i>A. fumigatus</i> and <i>A. flavus</i> in sputum	2 (CSF), 6 (blood), 3 (BAL)	1	1	113	0	6	0	3	Negative
2	62/m	NHL	Proven	Solitary cerebral abscess	None	Operation, voriconazole	Improvement	Histologic proof from brain biopsy	1 (CSF), 14 (blood), 0 (BAL)	1	0	NA	1*	12	0	0	Negative
3	63/f	AML, alloSCT	Probable	Multiple intracerebral abscesses	<i>A. fumigatus</i> growth in sputum	None	Death	<i>A. fumigatus</i> in sputum	1 (CSF), 0 (blood), 0 (BAL)	1*	0	NA	0	0	0	0	5,244
4	4/f	ALL	Probable	Multiple intracerebral abscesses, ventriculitis	Lung	Intraventricular amphotericin B, caspofungin, voriconazole	Improvement	Galactomannan ELISA from CSF positive	26 (CSF), 10 (blood), 1 (BAL)	9	17	7 (1–103)	0	10	1	0	3,871–6,31 × 10 ⁷
5	13/m	AML, alloBMT, GvHD	Possible	Solitary cerebral abscess	None	Liposomal amphotericin B, voriconazole	Improvement	Negative	3 (CSF), 4 (blood), 0 (BAL)	1	2	83 (73–93)	0	4	0	0	Negative
6	65/f	NHL	Possible	Multiple intracerebral abscesses	None	Caspofungin, voriconazole	No change	Negative	2 (CSF), 2 (blood), 0 (BAL)	1	1	24	0	2	0	0	2,154
Totals										14	21		1	34	1	3	

^a Abbreviations: f, female; m, male.^b AML, acute myeloid leukemia; alloBMT, allogeneic bone marrow transplantation; GvHD, graft versus host disease; alloSCT, allogeneic stem cell transplantation; NHL, non-Hodgkin's lymphoma.^c Δ days, number of days that separate consecutive CSF specimens from each other (if more than two samples were obtained, the median and range are given); NA, not applicable; *, sample not obtained during antifungal treatment.

treatment	voriconazole →															
	casposfungin →															
	intraventricular amphotericin B → →															
radio-imaging/ clinical data	cMRI +	ChCT +				ChCT +		Ommaya*								
<i>Aspergillus</i> antigen	CSF +															
BAL PCR						+										
blood PCR		-	-							-						
CSF PCR		+	+	+	+		-	+	+	+	-	-	-	-	-	-
			(2 samples)													
Light Cycler CFU/ml		neg	6.31x10 ⁷ and 3871	neg	5270 0			13002	neg	neg						
date (all 2003)	07/25	08/01	08/05	08/08	08/11	08/16	09/04	09/05	09/16	09/23	09/29	10/06	10/09	10/13	10/20	

FIG. 1. Timeline of patient 4 showing PCR results, results of radioimaging, and results of galactomannan testing during the initial phase of cerebral aspergillosis (a ninth specimen was PCR positive 11 months after the initial presentation of symptoms). Abbreviations: cMRI, cerebral magnetic resonance imaging; ChCT, chest computed tomography; neg, negative; Ommaya*, implantation of an Ommaya reservoir.

a control, CSF samples from 10 patients without cerebral aspergillosis were investigated by PCR.

PCR assays. (i) Nested PCR. *Aspergillus* DNA was detected by a nested PCR assay, as described previously (22). To minimize the risk of contamination, all samples were handled under sterile conditions in a laminar flow cabinet. The protocol for DNA extraction was modified: DNA was extracted from the whole CSF sample (1.5 to 2 ml) instead of from the cell pellet. Purification of DNA was performed by conventional phenol-chloroform extraction. The DNA concentration was assessed by spectrophotometry at 260 and 280 nm. Per 25 µl of PCR mixture, approximately 50 to 150 ng of total DNA was used as a template. The DNA solution used as the template also contained an unknown amount of *Aspergillus* DNA. In the nested two-step PCR technique, two pairs of oligonucleotide primers (AFU 7S and AFU 7AS for the first step and AFU 5S and AFU 5AS for the second step), derived from sequences of the *A. fumigatus* 18S rRNA gene (GenBank accession no. AB008401) and specific for *Aspergillus* species, were used. A 138-bp PCR fragment encoded by the human glucose-6-phosphate dehydrogenase gene (GenBank accession no. X55448) was amplified by primers G6PD1S and 1AS in each clinical sample as an internal control. The sensitivity of the nested PCR assay is 1 to 5 CFU per ml of blood.

(ii) Quantitative PCR. The quantitative PCR assay, using LightCycler technology previously established in our laboratory, proved to be less sensitive than the nested PCR assay. Therefore, only CSF samples with positive results in the nested PCR were investigated with the LightCycler assay (23). The DNA extraction method and the amount of total DNA used as the template were the same as for the nested PCR assay. Primers were derived from the sequence of the mitochondrial cytochrome B gene of *A. fumigatus* (GenBank accession no. AB 025434). The amplified fragment was 194 bp in length. Only DNA from *A. fumigatus* and *Aspergillus clavatus* is detectable by the LightCycler assay. The detection threshold of the quantitative PCR assay is 15 CFU/ml of blood.

RESULTS

Thirty-five CSF samples from six patients with hematologic malignancy were examined by the nested PCR assay. The

median number of CSF samples examined per patient was 2 (range, 1 to 26). Fourteen CSF samples were positive by nested PCR, and 21 samples were negative. Each patient had at least one positive PCR result in a CSF sample. For two patients, one CSF sample each was obtained at initial presentation with central nervous system (CNS) disease; for four patients, more than one sample (2 to 26 samples/patient) was investigated by PCR. Serial sampling of CSF showed a positive correlation of PCR results and clinical improvement for three patients. Clinical characteristics of the patients and PCR results are shown in Table 1.

In patient 4, an Ommaya reservoir was implanted because of increased intracranial pressure, and serial CSF samples ($n = 26$) were investigated by PCR. Initially, eight of nine serial CSF samples were positive for *Aspergillus* DNA. Consecutively, samples became negative during antifungal treatment, accompanied by clinical improvement (Fig. 1).

Both patients who underwent bronchoscopy and BAL fluid sampling had pulmonary infiltrates, but for only one patient was *Aspergillus* PCR positive with BAL fluid.

Thirty-five blood samples from five patients—two with proven, one with probable, and two with possible IA—were investigated for *Aspergillus* DNA by PCR; 34 samples were negative, and 1 sample was positive. The one blood sample that tested positive for *Aspergillus* DNA was obtained from patient 2 before antifungal treatment was begun; the remaining 34 blood samples were obtained during antifungal treatment.

All CSF samples that gave positive results in the nested PCR

were investigated by LightCycler PCR. Nine of 14 samples were negative by LightCycler PCR, and 6 of 14 samples were quantified; the results of quantification are shown in Table 1. The highest number of gene copies was found in a patient with acute lymphocytic leukemia (ALL), multiple intracerebral abscesses, and ventriculitis.

DISCUSSION

Cerebral aspergillosis is a severe and often fatal disease that is difficult to diagnose. Several PCR assays have been established in recent years; however, most of them have been validated for use with blood and BAL fluid samples but not with CSF samples (4, 9).

Aspergillus DNA was detected in samples from six of six patients with cerebral aspergillosis by our nested PCR assay. In all patients, samples that were obtained at the initial manifestation of cerebral aspergillosis showed a positive PCR result. In patients with serial sampling of CSF, follow-up samples obtained during antifungal treatment and after clinical improvement were negative.

Negative PCR results during effective antifungal treatment may reflect improvement of the infection, with reduction of the fungal load to below the detection threshold of the PCR. Negative results after treatment initiation with newer antifungal agents have been described for *Aspergillus* PCR with blood and BAL fluid samples and for galactomannan enzyme-linked immunosorbent assay (ELISA) with serum and CSF samples (13, 17, 25). It is therefore important for confirmation of the diagnosis of IA to obtain CSF samples for diagnostic purposes before the institution of antifungal treatment. Assuming that effective antifungal treatment lowers the fungal load, resulting in PCR negativity, persistently positive PCR results during antifungal treatment may indicate treatment failure (13).

Only 1 out of 2 BAL fluid samples and 1 out of 35 blood samples gave positive results in the nested PCR assay. This seems to be in contrast with our previous data, which showed a higher sensitivity rate for *Aspergillus* PCR with blood samples (3). Discrepancies between the data from previous studies and current data may be due to different antifungal treatment strategies: data from our previous studies were obtained in an era before highly effective and well tolerated antifungal agents were available. More-effective antifungal treatments with novel agents may lead to the clearance of fungal DNA from blood. In our patient cohort, all blood samples that tested negative in the nested PCR assay had been obtained during antifungal treatment. The only PCR-positive blood sample was obtained before antifungal treatment started. Therefore, the benefits of PCR diagnosis and screening of blood samples is limited if sampling takes place once treatment has started (12, 14, 24).

The finding that *Aspergillus* DNA could be detected in CSF samples but rarely in blood samples supports the hypothesis that PCR is more likely to detect fungal elements at the site of infection than in blood samples, because of the lack of fungemia during antifungal treatment (4).

Only 5 of 14 nested PCR-positive samples gave positive results by LightCycler PCR. This can be attributed to the following. The amount of *Aspergillus* DNA detectable in a sample by the nested PCR assay may be below the less-sensitive detection threshold of the LightCycler PCR assay. Fur-

thermore, additional *Aspergillus* species besides *A. fumigatus* are detectable by the nested PCR assay (22) but are missed by the *A. fumigatus*-specific LightCycler PCR assay performed here (23). Data published by Marr et al. confirm an increasing incidence of non-*fumigatus Aspergillus* species in hematopoietic stem cell transplant recipients (16).

In five patients, detection of *Aspergillus* DNA from CSF samples was the only positive microbiologic finding in the CSF. Culture and direct microscopy, as well as galactomannan ELISA, were negative for any pathogen. With the exception of *Cryptococcus neoformans*, fungi are rarely detected in CSF obtained from patients having or suspected of having fungal meningitis (18).

So far, reports of *Aspergillus* PCR with CSF samples are few (10, 11, 19, 25) and consist mostly of single-case reports. Kami et al. investigated one CSF sample each in five patients with cerebral aspergillosis and found positive *Aspergillus* PCR results (10). Verweij et al. investigated 26 serial CSF samples from a patient with proven cerebral aspergillosis and detected *Aspergillus* DNA in 4 of 26 samples (25).

In our series of patients with cerebral aspergillosis, four of six patients showed improvement in clinical symptoms and in radioimaging findings. With regard to the high mortality rates of cerebral aspergillosis, up to 95% (7), this response rate to antifungal treatment is favorable and can be attributed to newer and more effective antifungal agents (20, 21). With the availability of effective antifungal agents for the treatment of cerebral aspergillosis, early and sensitive detection of the fungal pathogen in CSF samples becomes even more important.

Detection of *Aspergillus* DNA from CSF samples is possible and helpful in diagnosing cerebral aspergillosis and can assist in distinguishing this disease from other cerebral conditions with space-occupying lesions in immunocompromised patients. Nevertheless, further prospective studies with larger numbers of patients must be performed in order to evaluate the clinical significance of *Aspergillus* PCR with CSF samples.

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