

Detection of *Aspergillus* DNA by a nested PCR assay is able to improve the diagnosis of invasive aspergillosis in paediatric patients

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Fungal infections are a leading cause of morbidity and mortality in severely immunocompromised patients and have been increasing in incidence in recent years. Invasive aspergillosis (IA) is the most common filamentous fungal infection and is, in adults as well as in children, difficult to diagnose. Several PCR assays to detect *Aspergillus* DNA have been established, but so far, studies on molecular tools for the diagnosis of IA in children are few. We evaluated the results of a nested PCR assay to detect *Aspergillus* DNA in clinical samples from paediatric and adolescent patients with suspected IA. Blood and non-blood samples from immunocompromised paediatric and adolescent patients with suspected invasive fungal infection were sent for processing *Aspergillus* PCR to our laboratory. PCR results from consecutive patients from three university children's hospitals investigated between November 2000 and January 2007 were evaluated. Fungal infections were classified according to the EORTC classification on the grounds of clinical findings, microbiology and radio-imaging results. Two hundred and ninety-one samples from 71 patients were investigated for the presence of *Aspergillus* DNA by our previously described nested PCR assay. Two, 3 and 34 patients had proven, probable and possible IA, respectively. Sensitivity (calculated from proven and probable patients, $n=5$) and specificity (calculated from patients without IA, $n=32$) rates of the PCR assay were 80 and 81 %, respectively. Our nested PCR assay was able to detect *Aspergillus* DNA in blood, cerebrospinal fluid and bronchoalveolar lavage samples from paediatric and adolescent patients with IA with high sensitivity and specificity rates.

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INTRODUCTION

Invasive fungal infections (IFIs) are a major clinical problem and a leading cause of morbidity and mortality in severely immunocompromised patients, especially in patients with haematological malignancies and intensive antineoplastic treatment.

Invasive aspergillosis (IA) is the most common filamentous fungal infection observed in these patients (El Mahallawy *et al.*, 2002; Hovi *et al.*, 2000). The incidence of IA has increased significantly in recent years in parallel

with the increasing number and improved survival of immunocompromised patients (Denning, 1998; Marr *et al.*, 2002). While IA in adults has been well characterized, there has been little investigation into IA in paediatric patients (Roilides, 2006; Steinbach, 2005a, b). In a large retrospective cohort study including 152 231 immunocompromised children in the United States, the annual incidence of IA was found to be 0.4 %. The highest incidence of IA was seen in children who had undergone allogeneic bone marrow transplantation (4.5 %) and those with acute myelogenous leukaemia (4 %) (Zaoutis *et al.*, 2006). Other predisposing host factors are acquired immunodeficiency syndromes, chronic granulomatous disease, severe combined immunodeficiency, organ transplantation, granulocytopenia, corticosteroid and other

Abbreviations: BAL, bronchoalveolar lavage; CNS, central nervous system; CSF, cerebrospinal fluid; GM, galactomannan; IA, invasive aspergillosis; IFI, invasive fungal infection.

immunosuppressive therapies and prematurity (Walsh *et al.*, 1996).

Morbidity and mortality of IFI are still high even though new antifungal drugs have brought some improvement (Herbrecht *et al.*, 2002a; Upton & Marr, 2006; Walsh *et al.*, 2004b). A recent Italian multicentre surveillance study including paediatric cancer patients reported a mortality rate of 34% in children with deep tissue infection (Castagnola *et al.*, 2006). To improve the outcome for patients with IA, early diagnosis and treatment initiation is important but establishing a reliable diagnosis remains difficult. In the majority of cases, the diagnosis of IA is made at autopsy (Chamilos *et al.*, 2006; Groll *et al.*, 1996; Lin *et al.*, 2001; Subira *et al.*, 2003). Immunocompromised children rarely display typical inflammatory responses. Radio-imaging is not specific and is difficult to interpret especially at the early stage of IA. Invasive diagnostic procedures can often not be performed because of the severity of the underlying disease and the risk of bleeding complications in thrombocytopenic patients. Numerous PCR assays to detect fungal DNA have been described, but most studies focus on adult patient cohorts (Baskova *et al.*, 2007; Florent *et al.*, 2006; Halliday *et al.*, 2006; Hummel & Buchheidt, 2007; Jordanides *et al.*, 2005; Schabereiter-Gurtner *et al.*, 2007; White *et al.*, 2006). Studies on the molecular diagnosis of IFIs in children are few (Roilides, 2006). Some studies have included children in larger cohorts, but results were not stratified for paediatric patients (Buchheidt *et al.*, 2001; Maertens *et al.*, 2001; Pinel *et al.*, 2003). From the data available, both galactomannan (GM) ELISA and PCR protocols appear to be less useful in children than in adults (Bochennek *et al.*, 2006).

To assess the role of molecular diagnosis of IA in children, we evaluated the results of a nested PCR to detect *Aspergillus* DNA in samples from paediatric and adolescent patients with IA.

METHODS

Patients and samples. Clinical samples from paediatric and adolescent patients with suspected IFI were sent for processing *Aspergillus* PCR to our laboratory. PCR results from all consecutive patients from three university children's hospitals investigated between November 2000 and January 2007 were evaluated in this study. The majority of patients had malignant haematological diseases. Neutropenia (absolute neutrophil count $<500 \mu\text{l}^{-1}$) was present in 38 (54%) of the patients. Patients' characteristics are shown in Table 1. The age of the youngest patient was 1 month. There were only three patients aged above 18 years included in the study. All of these patients were treated in a paediatric hospital according to paediatric chemotherapy protocols and were therefore included in the study. All samples were obtained during routine diagnostic procedures under sterile conditions and after informed consent of the patients or the parents. Samples were shipped to our laboratory in sterile tubes and processed immediately. Fungal infections were classified according to the EORTC classification (Ascioglu *et al.*, 2002) on the grounds of symptoms and clinical findings, microbiology and radio-imaging results.

Table 1. Patients' characteristics

Age, median	8 years (range 0–20)
Female/male	27/44
Underlying immunocompromising condition	<i>n</i> =71
Acute lymphoblastic leukaemia	22
Acute myeloid leukaemia	5
Allogeneic haematological stem cell transplantation*	17
Burkitt's lymphoma	3
Cystic fibrosis	4
Solid tumours	12
Congenital immunodeficiency	1
Chronic granulomatous disease	2
Others†	5

*Allogeneic haematological stem cell transplantation was performed for acute leukaemia (*n*=9), severe combined immunodeficiency (*n*=2), thalassaemia (*n*=2), chronic granulomatous disease (*n*=1), Hodgkin's lymphoma (*n*=1), secondary chronic myelomonocytic leukaemia (*n*=1) and Ewing's sarcoma (*n*=1).

†Others include Langerhans cell histiocytosis, juvenile chronic arthritis, haemophagocytic lymphohistiocytosis, respiratory distress syndrome and prolonged intensive care treatment.

PCR. *Aspergillus* DNA was detected in clinical samples by an experimentally and clinically validated nested PCR assay as described previously (Buchheidt *et al.*, 2001, 2002; Hummel *et al.*, 2004; Skladny *et al.*, 1999). To minimize the risk of contamination, all samples were handled under sterile conditions in a laminar flow cabinet. Between 0.5 ml [cerebrospinal fluid (CSF)] and 5 ml [blood, bronchoalveolar lavage (BAL)] was used for the PCR assay. For 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 *Aspergillus fumigatus* 18S rRNA gene (GenBank accession no. AB008401) and specific for *Aspergillus* species were used. The detection limit of the nested PCR assay is 1–5 c.f.u. (ml blood)⁻¹.

The definition of IA was based on the 2002 EORTC/MSG guidelines (Ascioglu *et al.*, 2002). Decisions about whether patients had proven, probable or possible IA were made strictly independently from PCR results. The PCR results were evaluated by correlating them with the clinical classification. Results of serological diagnostic techniques (GM assay, Platelia *Aspergillus* EIA; Bio-Rad) and post-mortem histological examination were included for clinical classifications.

Statistical analysis. Continuous variables such as age were summarized using descriptive summary statistics (*n*, median, mean, range). Categorical variables such as PCR results were tabulated and presented as a percentage. For calculation of sensitivity of the PCR assay, episodes with proven and probable IA were assumed to be true-positive episodes. Episodes with possible IA are non-conclusive and were therefore not considered in the calculation of sensitivity and specificity of the PCR assay. For calculation of specificity, episodes without evidence of IA were assumed to be true-negative episodes. Statistical analysis was performed with SPSS Advanced Statistics, version 10.0.6.

RESULTS AND DISCUSSION

Two hundred and ninety-one blood and non-blood samples from 71 patients were sent to our laboratory for

Table 2. Origin of samples, sample volumes and PCR results

Source of sample	Sample volume [median (range), ml]	Total no. of samples (n=291)	Samples with positive PCR results (n=34)	Samples with negative PCR results (n=251)	Samples not evaluable (n=6)
Blood	4 (1–5)	216	21	193	2
CSF	1 (0.5–2)	43	11	29	3
BAL	3 (2–5)	14	2	12	0
Bronchial aspirate	1.5 (1–2)	5	0	4	1
Sputum	0.5 (0.5–1)	3	0	3	0
Liver biopsy		4	0	4	0
Bone marrow aspirate	3 (3–4)	3	0	3	0
Sinus aspirate	0.65 (0.5–0.8)	2	0	2	0
Skin swab		1	0	1	0

PCR from three paediatric centres. Two hundred and eighty-five samples were evaluable. The median number of samples obtained per patient was two (range 1–41). Thirty-four samples gave positive PCR results and 251 samples gave negative PCR results.

Aspergillus DNA was detected in blood, CSF and BAL samples. Table 2 shows the source of the samples and PCR results.

Proven and probable IA, and sensitivity of the PCR assay

In our cohort of paediatric and adolescent patients, IA could be classified in only five patients as ‘proven’ ($n=2$) or ‘probable’ ($n=3$), reflecting the difficulties of verifying the diagnosis of IA (Table 3). All patients with proven or probable IA had acute leukaemia; one had undergone allogeneic haematological stem cell transplantation for acute myeloid leukaemia. Three patients had disseminated IA; in two of these there was involvement of the central nervous system (CNS). Table 4 shows the characteristics of patients with proven and probable IA. In patients with proven and probable IA, *Aspergillus* DNA was detected by PCR in blood, CSF and BAL samples. Four of five patients with probable or proven IA had at least one positive PCR result. Assuming that the patients with proven and probable IA were ‘true positives’, the sensitivity of PCR testing was 80 %. It is difficult to calculate sensitivity and specificity rates with small numbers of patients, but most studies report only few patients with proven or probable

IA, even if data are collected over a long time interval (Hovi *et al.*, 2007).

The patient with proven IA but negative PCR results had IA affecting the liver and the kidney. IA was proven from a liver biopsy specimen, which was positive for *A. fumigatus*. Only sputum and blood samples were investigated by PCR in this patient. An explanation for the PCR negativity is that the samples were not obtained from the site of infection and that there was no DNAemia at the time of blood sampling, so that *Aspergillus* DNA was not present in blood samples or the level of DNAemia was below the detection threshold of the PCR assay. There is still relatively poor understanding of DNA release and kinetics in fungal infections (Hope *et al.*, 2005).

In three cases with proven or probable disease, samples were obtained from the site of the fungal infection. In two patients with cerebral aspergillosis, CSF samples contained *Aspergillus* DNA and in another patient with pulmonary aspergillosis, a BAL sample was positive for *Aspergillus* DNA. This supports the hypothesis that investigating samples from the site of *Aspergillus* infection is more sensitive than blood sampling (Hummel & Buchheidt, 2007).

The majority of samples obtained from patients with proven, probable and possible IA were obtained during antifungal treatment. Mennink-Kersten *et al.* (2006) detected in their *in vitro* studies fungal DNA during autolysis caused by nutrient limitation or antifungal treatment. However, not all PCR samples were positive

Table 3. PCR results according to EORTC/MSG groups

	Proven	Probable	Possible*	No IA
No. of patients	2	3	34	32
No. of patients with positive PCR results	1	3	11	6
No. of patients with negative PCR results	1	0	22	26

*In the group of patients with possible IA, the samples of one patient were not evaluable.

Table 4. Characteristics of patients with proven or probable IA

Patient	Age (yrs)/sex	Underlying disease*	EORTC classification	PCR samples (n)	PCR positive	PCR negative	Localization of IA	Antifungal treatment	Other microbiological findings	Outcome
1	14/Female	ALL	Proven	Blood (2) Sputum (1)	0 0	2 1	Liver, kidney	Caspofungin, voriconazole	<i>A. fumigatus</i> in liver biopsy	Improved
2	12/Female	ALL	Proven	CSF (2) Blood (6) BAL (3)	1 0 0	1 6 3	Lung, spleen, liver, kidney, CNS	Caspofungin, voriconazole	Histological proof from lung specimen, <i>A. fumigatus</i> and <i>A. flavus</i> in sputum	Improved
3	1/Male	ALL	Probable	Blood (4)	1	3	Lung	Amphotericin B, voriconazole	Positive GM results	Died
4	4/Female	ALL	Probable	CSF (26) Blood (10) BAL (1)	9 0 1	17 10 0	CNS (multiple intracerebral abscesses, ventriculitis), lung	Intraventricular amphotericin B, caspofungin, voriconazole	Positive GM ELISA from CSF sample	Improved
5	12/Male	AlloHsCT, AML	Probable	BAL (2)	1	1	Lung	Voriconazole	<i>Aspergillus</i> hyphae in BAL	Survived

*ALL, acute lymphoblastic leukaemia; alloHsCT, allogeneic haematological stem cell transplantation; AML, acute myeloid leukaemia.

during the lytical phase. The detection of fungal DNA is certainly different in an *in vitro* setting as reported by Mennink-Kersten *et al.* (2006) as compared to the clinical setting where there is still relatively poor understanding of DNA release and kinetics. Several clinical studies report that the sensitivity of PCR testing for *Aspergillus* infections is limited during antifungal treatment due to a decrease of fungaemia (Kami *et al.*, 2001; Lass-Flörl *et al.*, 2004; O'Sullivan *et al.*, 2003). This may account for false-negative PCR results. This observation is supported by the course of PCR results in a patient of our study cohort where serial sampling for PCR testing was possible. In this patient (patient 4, Table 4) with disseminated IA with involvement of the CNS and the lungs, an Ommaya reservoir was implanted because of increased intracranial pressure. Serial CSF samples ($n=26$) were investigated by PCR for *Aspergillus* DNA. Initially, 8/9 serial CSF samples were positive for *Aspergillus* DNA. Samples became negative during antifungal treatment with intraventricular amphotericin, voriconazole and caspofungin, accompanied by clinical improvement.

So far, most studies on *Aspergillus* PCR testing have included only adult patients with only a few studies reporting on paediatric patients. Challier *et al.* (2004) report a sensitivity of an *A. fumigatus*-specific real-time PCR assay of 100 % and 50 % in children with proven and probable IA, respectively. In the study of El-Mahallawy *et al.* (2006) using a pan-fungal PCR assay in paediatric high-risk cancer patients for the diagnosis of yeast and mould infections, the sensitivity of PCR testing was 75 %.

Patients with possible IA

In 34 patients, IA was classified as 'possible'. Among patients with possible IA, 11/34 had at least one positive PCR result. Of these, four had CNS lesions and three had disseminated disease. In one patient with possible IA of the CNS, *Aspergillus* DNA was detected in a CSF sample, but other microbiological investigations were negative.

It remains unclear whether some of these patients actually had IA that could not be proven, as especially CNS aspergillosis is difficult to diagnose (Cunha, 2001; McGinnis, 1983). The EORTC/MSG definition of 'possible IFI' is the least specific: in this disease category it is uncertain whether IFI is present. In a series of 21 paediatric patients who underwent early surgical lung resection in pulmonary or disseminated IA, 12 patients were classified as having possible IA before surgery. Thereof, in 10 patients IFI was confirmed at surgery (Cesaro *et al.*, 2007). Subira *et al.* (2003) reported that 60 % of IA cases proven at autopsy were classified as 'possible IA' before patients' death.

Due to the uncertain disease status in the group of 'possible IFI', the statistical performance of PCR testing was calculated using different definitions of disease status (Table 5).

Table 5. Performance of *Aspergillus* PCR with use of different definitions of disease status

Data are percentage (95 % CI). Under definition A, proven/probable cases are true positives, at-risk cases are true negatives and possible cases are excluded from statistical analysis; under definition B, proven/probable/possible cases are true positives and at-risk cases are true negatives; under definition C, proven/probable cases are true positives and possible/at-risk cases are true negatives.

Value	Invasive aspergillosis definition		
	A	B	C
Sensitivity	80 (38–96)	38 (25–54)	80 (38–96)
Specificity	81 (65–91)	81 (65–91)	73 (61–82)
Positive predictive value	40 (17–69)	71 (50–86)	19 (8–40)
Negative predictive value	96 (82–99)	53 (39–66)	98 (89–100)

Specificity of the PCR assay

The initially considered differential diagnosis of IA could not be confirmed in 32 patients in the course of the disease and they were therefore classified as having no IA. Among patients without IA, 26/32 had negative PCR results, resulting in a specificity of 81 %. False-positive PCR results can be due to contamination. *Aspergillus* is a ubiquitously spread organism and therefore contamination is a considerable risk in spite of appropriate precautions in handling samples.

The specificity of PCR testing in our study was distinctly higher than the specificity of GM reported in other studies (Herbrecht *et al.*, 2002b; Mennink-Kersten *et al.*, 2004; Siemann & Koch-Dorfler, 2001). The problem with GM ELISA in children is the high rate of false-positive results. Specificity rates of as low as 47.6 % have been reported (Herbrecht *et al.*, 2002b). This has been attributed to the predominantly milk-based diet of children, as GM is present in milk and may translocate across the intestinal wall if chemotherapy-induced mucositis is present. Also, lipoglycan from *Bifidobacterium bifidum* as well as drugs of fungal origin such as piperacillin–tazobactam may cause false-positive GM results (Steinbach *et al.*, 2007; Sulahian *et al.*, 2003; Viscoli *et al.*, 2004; Walsh *et al.*, 2004a). In a prospective study on *Aspergillus* GM antigen testing in paediatric haematopoietic stem cell transplant recipients, Steinbach *et al.* (2007) reported a specificity for GM testing of 98.4 % after excluding samples from patients who were receiving piperacillin–tazobactam. With better specificity rates, *Aspergillus* PCR as a diagnostic test in invasive fungal disease is complementary to GM testing and other diagnostic tests.

Conclusions

PCR for *Aspergillus* DNA is a useful tool in establishing the diagnosis of IA with high sensitivity and specificity rates. Our nested PCR assay is able to detect *Aspergillus* DNA in

blood, CSF and BAL samples. However, PCR detection in blood samples is much less sensitive. Small sample volumes as low as 0.5 ml were sufficient for DNA detection. This is of advantage in small children where it is difficult to obtain appropriate specimens due to their small size (Roilides, 2006).

To increase the sensitivity of both PCR and serology testing, obtaining samples before the institution of antifungal treatment and serial sampling are desirable.

The combined use of culture, GM ELISA and PCR should result in an earlier and more definite diagnosis of IA in children. The diagnosis of IA remains a combination of the clinical presentation, findings on radio-imaging and results of conventional microbiological and molecular methods. PCR for *Aspergillus* DNA makes an essential contribution to the improvement of the diagnosis of IA.

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