Case Report

Cerebral aspergillosis diagnosed by detection of *Aspergillus flavus*-specific DNA, galactomannan and (1→3)- β -D-glucan in clinical specimens

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A case of cerebral aspergillosis was diagnosed by the detection of *Aspergillus flavus*-specific DNA in brain biopsy and serum specimens. The diagnosis was also supported by detection of elevated levels of galactomannan and (1→3)- β -D-glucan in serum specimens. Despite the presence of dichotomously branched septate hyphae in brain biopsy, the culture remained negative. The inability to isolate the organism in culture suggested that combined therapy of AmBisome and caspofungin was fungicidal for the fungus in the brain abscess.

Introduction

Invasive aspergillosis (IA) is an increasingly common mycosis among patients with haematological malignancy and those who have undergone organ transplantation (Singh & Paterson, 2005). Extension of IA to the central nervous system (CNS) is associated with high mortality that approaches nearly 100 % despite antifungal therapy (Schwartz & Thiel, 2004; Singh & Paterson, 2005). Because conventional diagnostic methods can be insensitive and the performance of invasive diagnostic procedures may not be feasible in thrombocytopenic or critically ill patients, the confirmatory diagnosis is often made post-mortem (Ascioglu *et al.*, 2002). Although demonstration of characteristic radiological findings, such as 'halo' or 'air crescent' signs, in a high-resolution computed tomography (CT) scan at various stages of pulmonary disease may suggest IA, they are not 100 % specific (Coley *et al.*, 1999). In view of these limitations, efforts have been focused on the development and evaluation of non-invasive markers for the specific and early diagnosis of IA (Hope *et al.*, 2005; Ostrosky-Zeichner *et al.*, 2005; Pickering *et al.*, 2005). In this communication, we describe a fatal case of cerebral aspergillosis that was diagnosed while the patient was alive by detection of *Aspergillus flavus*-specific DNA in brain biopsy and serum specimens. In the absence of positive culture, the diagnosis was also supported by detection of galactomannan and (1→3)- β -D-glucan in serum specimens.

Case report

A 62-year-old Kuwaiti male, a patient having insulin-dependent diabetes mellitus of 30-year duration, had complications of diabetes that included retinopathy, neuropathy, nephropathy and hypertension. He developed chronic renal failure that led to end-stage renal disease. He had triple-vessel coronary artery disease with a history of acute infarction. In March 2002, he presented with severe uraemic symptoms necessitating haemodialysis thrice a week. In October 2003, he underwent live unrelated kidney transplantation and he received a full induction course of antithymocyte-globulin immunosuppression besides prednisolone, CellCept (1 g twice daily) and Prograf (1 mg twice daily). Although the patient had respiratory complaints for the last two months, he was admitted on November 9 2004 with fever accompanied with chest infection and leucopenia. His white blood cell count was $2.26 \times 10^9 \text{ l}^{-1}$ (neutrophils $0.99 \times 10^9 \text{ l}^{-1}$). The patient was put on meropenem and AmBisome (3 mg kg^{-1}) empirically. A bronchoalveolar lavage (BAL) specimen obtained one week post-admission showed branched, septate, hyaline fungal hyphae suggestive of *Aspergillus*. Unfortunately, *Aspergillus* was not isolated. Cultures yielded *Candida tropicalis* and *Pseudomonas aeruginosa*. At this time, a CT scan of the chest showed bilateral apical lesions that appeared much larger than those seen one month earlier. In addition, many small air cavities and a large ill-defined hyper-dense area with multiple nodular shadows were observed. Since the patient had also developed neurological symptoms accompanied with drowsiness and hallucination, a CT scan of the head was performed that showed multiple bilateral low-density areas (Fig. 1). At this point, AmBisome

Abbreviations: BAL, bronchoalveolar lavage; CNS, central nervous system; CT, computed tomography; IA, invasive aspergillosis.

Fig. 1. CT brain showing ring enhancing lesions in the right occipital and left temporal lobe.

was combined with caspofungin treatment and total doses of 11.6 g and 820 mg were administered over a period of 38 and 30 days, respectively. Because the patient continued to deteriorate, a brain biopsy consisting of pus from the abscess was obtained that contained septate, branched hyphae when examined with KOH-Calcofluor and Gomori methenamine silver stain. The hyphae were suggestive of *Aspergillus* species (Fig. 2a, b). However, the culture of the biopsy on several Petri dishes containing either Sabouraud dextrose agar (without cycloheximide) or blood agar, which were incubated at 30 and 37 °C, failed to grow any organisms. Further attempts to isolate the fungus from the specimen on Sabouraud dextrose broth and brain heart infusion broth were unsuccessful. This led to the application of a PCR-based method for the diagnosis of his cerebral infection. The patient died 6 days after the biopsy was performed. The salient laboratory findings of the case are summarized in Table 1.

The amplification of the extracted DNA from brain biopsy and serum samples was carried out targeting rDNA using panfungal primers (Ahmad *et al.*, 2002), as well as species-specific primers of three *Aspergillus* species (*Aspergillus fumigatus*, *A. flavus* and *Aspergillus terreus*). The DNA sequences of the primers for *A. flavus* were: outer forward, 5'-TACCGAGTGTAGGGTTCCTAGCGA-3'; outer reverse, 5'-AAAAGATTGATTTGCGTTCGGCAA-3'; inner forward, 5'-CTAGTGAAGTCTGAGTTGATTGTAT-3'; inner reverse, 5'-CCGGAGAGGGGACGACGA-3'. The PCR amplification was carried out as described previously except that forward and reverse panfungal or outer or inner species-specific primers were used (Ahmad *et al.*, 2002, 2005). The amplified product obtained with *A. flavus*-specific outer primers was also reamplified with the *A.*

Fig. 2. Gomori methenamine silver-stained smear of the brain biopsy showing branched, septate hyphae suggestive of *Aspergillus*: (a) $\times 20$ objective, (b) enlarged view, $\times 100$ objective.

flavus-specific inner primer pair. The amplicons were detected by agarose gel electrophoresis (Fig. 3). The amplicon obtained with panfungal primers was also sequenced (Ahmad *et al.*, 2005).

Discussion

Our patient had respiratory symptoms for nearly three months prior to his death, but definitive proof of IA was considerably delayed until we used a PCR-based method for the detection of *A. flavus*-specific DNA. A BAL specimen collected on November 17th showed branched fungal hyphae, but cultures yielded *C. tropicalis* and *P. aeruginosa*. In retrospect, it appears that the isolation of *Aspergillus* did not occur perhaps due to the concomitant presence of *C. tropicalis* and *P. aeruginosa* in the BAL specimen that could have suppressed its growth (Randhawa *et al.*, 2005). A more likely possibility was that the fungus was non-viable due to antifungal therapy. Despite 8 days of initial treatment with AmBisome, the patient developed neurological manifestation. This necessitated initiation of combination therapy with AmBisome and caspofungin. While the pus obtained from one of the brain abscesses showed typical dichotomously branched septate hyphae, the culture was negative suggesting that the fungus was non-viable. The diagnosis of CNS aspergillosis was later confirmed by detecting *A. flavus*-specific DNA in serum and brain tissue. The nested PCR results were also supported retrospectively by demonstration of galactomannan (Pastorex *Aspergillus* and Platelia

Table 1. Summary of salient laboratory findings

Date	Specimen	<i>Aspergillus</i> antigen		(1-3)- β -D-glucan* (Fungitell) (pg ml ⁻¹)	<i>A. flavus</i> DNA by PCR
		Pastorex*	Platelia ELISA* (ng ml ⁻¹)		
November 16 2004	Serum	1:8	3.4	192.4	+
November 17 2004	Serum	1:16	3.2	156	+
December 11 2004	Brain biopsy†	ND	ND	ND	+
December 13 2004	Serum	1:4	2.8	87.8	+

ND, Not done.

*Performed retrospectively after patient's death.

†Direct microscopic examination showed branched, septate hyphae, but the culture was negative.

Aspergillus kits; Bio-Rad) and (1 $\rightarrow$ 3)- β -D-glucan in serum specimens (Fungitell kit; Associates of Cape Cod) (Table 1).

So far, only scant information is available on the intracerebral distribution of AmBisome or caspofungin and their access to brain abscesses in humans. Our inability to culture *A. flavus* from the brain biopsy suggests that possibly fungicidal concentrations of AmBisome and caspofungin were achievable at the site of infection.

Owing to the fact that these two antifungal agents have different target sites, and that caspofungin has been shown to be synergistic when used with some triazole antifungal agents, synergism may have been achieved. While we have not measured the concentration of these drugs in the aspirate of brain abscess, one available serum sample when tested against a clinical isolate of *A. flavus* by agar diffusion assay showed a clear zone of inhibition (6 mm in diameter).

Caspofungin is licensed in the United States and most European countries for use in refractory cases of aspergillosis, but clinical experience with this drug is still limited, either alone or in combination with azoles or polyenes (Marr *et al.*, 2004). Maertens *et al.* (2004) conducted an open, non-comparative, multicentre study in 90 patients who had failed to respond to treatment with amphotericin B, lipid preparations of amphotericin B or azoles; only 20% with disseminated aspergillosis had complete or partial response to caspofungin. Only two of the six (33%) patients in this series with cerebral involvement showed a favourable response to treatment with caspofungin. In a retrospective evaluation of the efficacy of caspofungin plus liposomal amphotericin B in 48 patients with documented or possible aspergillosis, Kontoyiannis *et al.* (2003) reported an inadequate response to liposomal amphotericin B monotherapy. The overall response rate was 42%; the combination was more successful as a primary therapy (53%) than as salvage therapy (35%). The response rate in patients with progressive documented IA was disappointingly low (18%). Aliff *et al.* (2003) treated 30 leukemic patients who showed inadequate response to amphotericin B alone, 60% gave a favourable response with combination therapy with caspofungin. Since caspofungin in *in vitro* tests alone is not fungicidal against *Aspergillus* species (Oakley *et al.*, 1998), it is possible that its combination with AmBisome exhibited synergistic effect resulting in the death of the fungus in our patient. This inference is consistent with *in vitro* studies where caspofungin and amphotericin B combination has yielded synergistic to additive results for at least half of the *Aspergillus* isolates with no antagonistic effect (Arikan *et al.*, 2002).

This case highlights the importance of the application of molecular methods in the specific diagnosis of fungal

Fig. 3. Agarose gel electrophoresis of PCR amplicons using outer (a) or inner (b) *A. fumigatus*-specific (lanes AF), *A. terreus*-specific (lanes AT) or *A. flavus*-specific (lanes AL) primer pairs and DNA isolated from brain biopsy or serum. Lane M, 100 bp DNA ladder, the 100 and 600 bp fragments are marked.

infections in the absence of positive cultures. Detection of (1→3)- β -D-glucan and galactomannan in serum specimens provided additional evidence in favour of *Aspergillus* infection, thus validating the efficacy of these markers in the diagnosis of cerebral aspergillosis. Detection of *Aspergillus* DNA and galactomannan in cerebrospinal fluid has been reported in some previous studies with distinct advantages over culture in the early diagnosis of CNS aspergillosis (Kami *et al.*, 1999; Verweij *et al.*, 1999). Because *A. terreus* and some other moulds exhibit resistance against amphotericin B (Sutton *et al.*, 1999), species-specific diagnosis by a PCR-based method could be helpful in instituting the most appropriate antifungal therapy. Additionally, while demonstration of dichotomously branched, septate hyphae in the brain biopsy may suggest *Aspergillus* infection, several other moulds also present similar tissue morphology leading to misdiagnosis (Liu *et al.*, 1998). Although our patient showed decreasing levels of (1→3)- β -D-glucan (192.4 to 87.8 pg ml⁻¹) and galactomannan (3.4 to 2.8 ng ml⁻¹) following therapy, he succumbed to infection suggesting that despite antifungal therapy cerebral aspergillosis is associated with dismal prognosis.

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