Invasive pulmonary aspergillosis due to *Aspergillus terreus*: value of DNA, galactomannan and (1→3)-β-d-glucan detection in serum samples as an adjunct to diagnosis

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A case of invasive pulmonary aspergillosis caused by *Aspergillus terreus* is described. The diagnosis was based on demonstration of branched septate hyphae in a sputum specimen and isolation of the fungus in culture. The diagnosis was further supported by detection of *A. terreus*-specific DNA, galactomannan (GM) and (1→3)-β-d-glucan (BDG) in consecutive serum specimens. The patient was treated for about 10 weeks with voriconazole. The decreasing levels of GM and BDG in serum samples were accompanied by symptomatic and radiological improvement. The report highlights the value of surrogate markers in the diagnosis and for monitoring the course of invasive aspergillosis during therapy.

Case report

A 9-year-old child with acute lymphocytic leukaemia presented in March 2008 with complaints of bony pain, pallor, fatigue, weight loss, nose bleeding and fever of 3-week duration. His haemogram revealed a white blood cell count of 68 × 10^9 l^{-1} (blasts 90 %) and platelets 77 × 10^9 l^{-1}, leading to suspicion of acute lymphoblastic leukaemia (ALL). The diagnosis of ALL was subsequently confirmed by bone marrow examination. Induction chemotherapy as per the UK-MRC-ALL-2003 protocol was started, followed by consolidation and maintenance chemotherapy regimes. While on maintenance therapy, he developed his first haematological relapse in January 2009 with complex cytogenetics along with positive Philadelphia chromosome. After necessary supportive therapy, he was started on induction chemotherapy with vincristine, epirubicin, dexamethasone, asparaginase, intrathecal Cytosar (cytarabine) and intrathecal methotrexate as per the UK-MRC bone marrow relapse protocol along with Gleevec (imatinib mesylate). About 10 days later, he developed *Klebsiella pneumoniae* septicaemia, pneumonia and signs of disseminated intravascular coagulopathy. With intensive supportive therapy, his condition improved. However, he continued to remain febrile with respiratory manifestations. X-ray and CT scan of the chest revealed multiple cavitary lesions of varying sizes in both the lung fields. No radiological abnormalities were detected in the CT scan of the brain. The examination of sputum specimens by 10 % KOH/calcofluor (0.1 %) mount showed branched septate hyphae (Fig. 1), and cultures on Sabouraud dextrose agar supplemented with chloramphenicol (50 mg l^{-1}) yielded *A. terreus*. The identity of *A. terreus* was established by...
typical colonial and microscopic morphology including demonstration of aleurioconidia on substrate hyphae. A provisional diagnosis of IPA due to *A. terreus* was made.

The antifungal susceptibility profile of the isolate was determined by E-test according to the procedure recommended by the manufacturer of the Etest (AB Biodisk). Since the isolate was considered resistant to amphotericin B (4 μg ml⁻¹) and susceptible to voriconazole (0.094 μg ml⁻¹) and posaconazole (0.047 μg ml⁻¹), the patient was started on voriconazole (6 mg kg⁻¹ first day, followed by 4 mg kg⁻¹ for 2 weeks and then continued on oral administration for about 8 weeks) along with broad-spectrum antibacterial antibiotics, acyclovir and intravenous fluid.

As an adjunct to diagnosis of IPA, BDG, GM and *A. terreus*-specific DNA were determined in sequentially collected serum samples. BDG and GM were determined by using the Fungitell kit (Associates of Cape Cod) and the Platelia *Aspergillus* kit (Bio-Rad), respectively. The tests were performed and interpreted for positivity according to the instructions supplied with the kit. The DNA from the serum samples was extracted as described previously (Ahmad et al., 2007). Nested amplification of rDNA was carried out by using outer as well as nested species-specific primers of three *Aspergillus* species (*A. terreus, A. fumigatus* and *A. flavus*) as described previously (Ahmad et al., 2007; Khan et al., 2007, 2008). In addition to the nested PCR amplification, a single-step PCR amplification of rDNA was also developed for detection of *A. terreus* DNA to avoid the risk of amplicon carryover. A new *A. terreus*-specific primer pair (ATEF, 5’-GAGTGCGGGTCTTTATGGCC-CA-3’; and ATER, 5’-CAAAGAATCACACTCAGACTGC-AAG-3’) was designed. The single-step PCR amplification was carried out as described previously for the first round of nested PCR except that *A. terreus*-species-specific ATEF and ATER primers were used, amplification was carried out for a total of 50 cycles and the amplicons were detected by agarose gel electrophoresis (Ahmad et al., 2007). Only *A. terreus* DNA was detected in the first two serum samples, but not in subsequent serum samples, by both the nested PCR and the single-step PCR amplification of rDNA as described above. No amplicons were detected when *A. fumigatus*-specific or *A. flavus*-specific primer pairs were used for nested PCR (data not shown). The identity of the *A. terreus* isolate was confirmed by DNA sequencing of the complete ITS region (ITS-1–5.8S rRNA–ITS-2), which was obtained as described previously (Khan et al., 2007, 2008). The DNA sequence of the isolate matched completely the sequence of reference strains of *A. terreus*.

Following the start of voriconazole therapy, the levels of both GM (from 6.4 ng ml⁻¹ to 1.2 ng ml⁻¹) and BDG (from 360 pg ml⁻¹ to 177 pg ml⁻¹) declined (Fig. 2). This was also accompanied by disappearance of *A. terreus* DNA in serum samples tested later. After 3 weeks of voriconazole therapy, sputum cultures became negative and no fungal elements were seen by direct microscopic examination. However, the patient’s condition deteriorated subsequently due to relapse of ALL. Induction therapy was restarted, which caused thrombocytopenia requiring platelets transfusion and cessation of all anti-infective treatment. The patient continued to deteriorate, developed septicaemia due to *Aeromonas hydrophila* infection and succumbed to septic shock.

**Discussion**

In this case report, we have illustrated the utility of biomarkers in establishing the diagnosis and assessing the prognosis of IPA. Although *A. terreus* was isolated from sputum on a solitary occasion, the diagnosis of IPA was supported by detection of *A. terreus*-specific DNA, GM and BDG in sequential serum samples. Following the initiation of voriconazole therapy, the patient was followed up for a duration of 3 months. His symptomatic and radiological improvement corresponded with decreasing levels of GM and BDG in serum samples assayed at different time points (Fig. 2). Also, *A. terreus*-specific DNA remained positive in two serum samples collected on 11 March and 16 March 2009, but was not detectable in four serum samples collected 2 weeks after the start of voriconazole therapy. This observation, although based on a solitary patient, is consistent with previous reports that rising or persistent levels of these markers may suggest poor prognosis, whereas falling levels may indicate favourable response to treatment and hence improved prognosis (Pazos et al., 2005; Senn et al., 2008; Koo et al., 2010). Unfortunately, the treatment was terminated prematurely due to relapse of ALL, the patient developed severe septicaemia due to bacterial infection and died due to septic shock.

The detection of high levels of GM in our patient suggests that *A. terreus* is a good producer of this cell wall component. This conforms with previous reports that GM concentrations may vary according to infecting *Aspergillus* species and that *A. terreus* produces significantly higher levels.
concentrations of GM than A. fumigatus in in vitro cultures (Mennink-Kersten et al., 2006; Hachem et al., 2009). It is possible that similar differences exist with respect to BDG.

In our patient, the diagnosis of invasive aspergillosis was supported by concomitant detection of three biomarkers: A. terreus DNA (species-specific), GM (genus-specific) and BDG (fungal-specific). Combined detection of more than one biomarker is helpful in enhancing the specificity of the diagnosis by ruling out false-positive or false-negative results (Sanguinetti et al., 2003; Kedzierska et al., 2007). The diagnostic marker BDG has been shown to have a higher sensitivity than GM in detecting invasive aspergillosis in haematological malignancy patients (Hachem et al., 2009), and thus may be more susceptible to false-positivity. However, the BDG test appears to have a greater value in excluding the diagnosis of invasive fungal infections rather than establishing the diagnosis (Pickering et al., 2005; Marty & Koo, 2009). It has been suggested that a negative BDG test in two consecutive serum samples may serve as a good indicator to rule out the presence of invasive mycosis (Marty & Koo, 2009). However, the cut-off values for a positive BDG test vary according to the brand of the diagnostic kit used (Kedzierska et al., 2007; Obayashi et al., 2008; Marty & Koo, 2009). This is likely to influence the sensitivity and specificity of the assays reported in different studies. In our patient, the maximum level of BDG estimated was 360 pg ml\(^{-1}\) (positive cut-off value of 80 pg ml\(^{-1}\)) against a GM index of 6.4 ng ml\(^{-1}\) (positive cut-off value of 0.5 ng ml\(^{-1}\)). The serum concentration of these biomarkers tends to vary considerably depending upon the location of the lesion as well as the extent of tissue invasion.

Aspergilli are ubiquitous in the environment and nosocomial outbreaks of invasive aspergillosis have often occurred in association with construction activities in and around haematological wards (Vonberg & Gastmeier, 2006). In Kuwait, A. terreus has been reported to be 6.2 % of the total aspergilli in outdoor air and <2 % in indoor air (Khan et al., 1999a), and it has also been implicated in two cases of pulmonary aspergillosis (Khan et al., 1999b, 2000). The relative aerial prevalence of A. terreus in comparison to other Aspergillus species is rather low in most of the surveys (Curtis et al., 2005; Guinea et al., 2006). Despite its low aerial prevalence, A. terreus has emerged as an important aetiological agent of invasive aspergillosis in some tertiary care hospitals (Blum et al., 2008; Hachem et al., 2004; Steinbach et al., 2004). In view of its low population density in the environment, the isolation of A. terreus from respiratory specimens may carry a greater clinical significance than for other Aspergillus species. Also, in patients receiving amphotericin B prophylaxis, the infection/colonization of the respiratory tract with A. terreus may have a higher predictive value for an invasive disease, since the species exhibits reduced susceptibility to this drug (Castón et al., 2007). It has been shown that accessory conidia (aleurioconidia) produced by this species are less susceptible to amphotericin B due to low ergosterol content in the cell membrane. Other attributes of A. terreus (such as adherence, excellent viability and rapid germination potential) may also contribute to its virulence (Deak et al., 2009). In one study, the percentage of A. terreus out of total Aspergillus isolates increased from 1.5 % in 1996 to 15.4 % in 2001 (P<0.001) (Baddley et al., 2003). The species has given rise to serious concern because of its inherent reduced susceptibility to amphotericin B (Hachem et al., 2004; Sutton et al., 1999). Steinbach et al. (2004) performed multicentre retrospective cohort analysis of 83 cases of proven and probable (47 % vs 53 %, respectively) invasive aspergillosis caused by A. terreus. The study revealed that patients who received voriconazole showed much higher survival (47 %, 16 of 34) than those who received other antifungal agents (24 %, 14 of 49). In several studies, higher mortality rates have been reported in patients who were infected with A. terreus than in those infected with A. fumigatus (Hachem et al., 2004; Lass-Flörfl et al., 2005). It is not known whether A. terreus is more virulent than A. fumigatus or A. flavus, or whether mortality differences observed in these reports were actually due to amphotericin B therapy, to which this species exhibits reduced susceptibility (Lass-Flörfl et al., 1998; Sutton et al., 1999). In general, regardless of the infecting Aspergillus species, improved therapeutic response has been achieved in patients who received voriconazole instead of amphotericin B (Herbrecht et al.,
2002; Steinbach et al., 2004). For this reason, voriconazole has been recommended as first-line therapy for invasive aspergillosis (Walsh et al., 2008). Our patient also showed a notable improvement with voriconazole therapy but died due to septic shock due to Aeromonas hydrophila infection.

In conclusion, this case demonstrates that decreasing levels of GM and BDG in serum samples are accompanied by symptomatic and radiological improvement in the patient’s condition. The report also highlights the value of surrogate markers in the diagnosis and for monitoring the course of invasive aspergillosis during therapy.

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