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The prospective evaluation of a nested polymerase chain reaction assay for the early detection of *Aspergillus* infection in patients with leukaemia or undergoing allograft treatment

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Summary. Patients with acute leukaemia or undergoing allogenic bone marrow transplantation at University College London Hospital Trust were screened for the presence of aspergillosis by polymerase chain reaction (PCR). *Aspergillus* DNA, from whole blood samples, was amplified by nested PCR to detect a 135 bp fragment in the mitochondrial region of *Aspergillus fumigatus* or *A. flavus* (121 bp). One colony-forming unit (CFU) per 2 ml of blood or 1–10 fg DNA could be detected. Patients at risk of aspergillosis were classified as probable or possible based on the European Organization for Research and Treatment of Cancer definitions. Antifungal drugs given were recorded. In four of 17 patients studied, infection was not suspected and the PCR was negative. Four patients were considered to have possible aspergillosis infection and were PCR positive on at least one occasion. Of the three patients in the probable group, four of the nine samples tested PCR positive from one patient and in another patient only one of nine samples tested positive. The remaining six patients were not suspected of having fungal infection but each had one or two PCR-positive results. In summary, six of seven patients thought to have clinical evidence of infection were PCR positive on at least one occasion and treatment with antifungals may have reduced infection below detectable levels.

Keywords: *Aspergillus*, infection, PCR, diagnosis, allograft.

Invasive aspergillosis is one of the commonest causes of death due to infection in neutropenic cancer patients and recipients of allogeneic stem cell transplants (Bodey *et al*, 1992; Denning, 1996; Wald *et al*, 1997). The apparent failure of antifungal chemotherapy to reduce the overall mortality rate of this disease highlights the need for a more effective diagnostic test. However, establishing a clinical diagnosis is difficult as the immunocompromised host rarely presents with a characteristic clinical picture. In 1977, standards for the diagnosis of aspergillosis were described using histological examination of tissue sections and microbiological criteria (Ainser *et al*, 1977). Despite 25 years of medical advances, these criteria remain the gold standard for the diagnosis of aspergillosis today.

Early diagnosis of infection is important as early treatment with antifungal drugs may increase patient survival (Ainser *et al*, 1977). Many studies have developed and compared culture, serological and molecular techniques for detection of *aspergillus*. Polymerase chain reaction (PCR) techniques have been shown to be the most sensitive and specific (Einsele *et al*, 1997; Van Burik *et al*, 1998; Löffler *et al*, 2000a), but levels of sensitivity for *Aspergillus fumigatus* vary between 5 colony forming units (CFU) or 10 fg of DNA (Skladny *et al*, 1999; Löffler *et al*, 2000a), 4 CFU (Van Burik *et al*, 1998), 50 fg of DNA (Yamakami *et al*, 1996), 100 fg of DNA (Jaeger *et al*, 2000) and 100 pg of DNA (Fletcher *et al*, 1998). Some of these studies have been methodological with little evaluation of the test in a clinical setting. Two prospective reports of a panfungal PCR assay in an unselected cohort of patients, undergoing chemotherapy for acute leukaemia or high-dose therapy followed by stem cell transplantation, demonstrated the potential value of screening these patients for early signs of fungal infection (Hebart *et al*, 2000a,b).

In this study, we have used a nested PCR assay with primers that detect a sequence in the mitochondrial region of *A. fumigatus* to screen for the early diagnosis of

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aspergillosis in patients undergoing chemotherapy for acute leukaemia and those undergoing allogenic bone marrow transplantation (BMT) at University College London Hospital Trust (UCHT). The diagnosis of aspergillosis was classified into probable or possible on the basis of clinical history and radiological observation of shadows within the lung [computerized tomography (CT) scans; Williamson *et al*, 2000; Caillot *et al*, 2001], and the PCR results were retrospectively compared with clinical data and antifungal treatment. The benefits and limitations of such a study are discussed.

PATIENTS AND METHODS

Patients. Ninety-four blood samples from 17 patients, undergoing chemotherapy for acute leukaemia (10) or undergoing allogenic BMT (seven) on the haematology unit at the UCHT, were screened. These two groups of patients were chosen as they were thought to be at significant risk of developing aspergillosis. Those patients undergoing allogenic BMT usually received antifungal prophylaxis with itraconazole from the onset of conditioning therapy, whereas those patients undergoing chemotherapy for acute leukaemia did not receive prophylaxis. Blood samples were taken weekly and at least three samples were required for inclusion in the study. Those patients thought to have *Aspergillus* infection were classified according to the European Organization for Research and Treatment of Cancer (EORTC) guidelines as proven, probable or possible (Williamson *et al*, 2000). Any antifungal drugs given were noted from the patients' drug charts and any microbiological findings were recorded. The PCR results did not influence the management of the patients.

Fungal cultures. A. fumigatus spores (Nesqua QC1480/7/9, obtained from UCHT Microbiology Department) which had been cultured on Sabouraud-glucose agar for 72 h at 30°C were collected and counted, then inoculated into 2 ml of whole blood (10^{6}–10^{-1} spores) in duplicate and then incubated overnight at 37°C before being extracted. This extraction series was performed in duplicate, and used to determine the sensitivity of the extraction and PCR. DNA from laboratory cultured *A. flavus* (clinical isolate 8942/10/94, obtained from UCHT Microbiology Department) was also extracted and amplified by PCR.

DNA extraction. An extraction protocol supplied by Qiagen (Crawley, UK) was modified as follows. All extractions were carried out in a biosafety hood. Uncoagulated whole blood (2 ml) (EDTA treated) was mixed with 10 ml of cold-erythrocyte lysis buffer (0·155 mol/l NH_4 Cl, 0·01 mol/l NH_4HCO_3 and 0·1 mmol/l EDTA pH 7·4) for 10 min on ice. The tubes were then spun at 400 ***g*** for 10 min to precipitate lysed cells. The pellet was resuspended in 10 ml of lysis buffer, and incubated again on ice and centrifuged. The final pellet was transferred to a 1·5 ml tube and a modified Qiagen kit protocol followed for the extraction of the fungal DNA. The pellet was resuspended in 180 µl of QIAamp DNA Blood mini kit (Qiagen^{TM}) ATL buffer and 5 µl of lyticase (at 25 units/µl; Sigma, Poole, UK) and incubated overnight at 37°C (some samples were stored at −20°C at this point). Proteinase K (20 µl) was added to the samples or controls and then incubated at 56°C for 1 h then the DNA was extracted using the mini kit with a 75 µl elution volume.

Controls. Each extraction included, 200 µl of saline suspension of laboratory cultured *A. fumigatus* which was vortexed in the lysis buffer as well as a negative control containing only lysis buffer. Each PCR run also contained a 10^{-2} and 10^{-3} dilution of stored positive DNA and two negative water-only controls.

Nested PCR. PCR was performed on 20 µl of extracted DNA. Separate pipettes were used from those used for the extraction protocol. First-round primers, detecting a mitochondrial region of the *A. fumigatus*, were at 791–813 (5′-TCG YTT TAC ACR CGA AAG GTC AG-3′) for the forward primer and 964–982 for the reverse primer (5′-CTC AAA CCA TCA TGA GTG G) (GenBank accession number *A. fumigatus*: L37095). Internal primers, used in the second round, were as reported by Bretagne *et al* (1995) and at positions 804–826 (5′-GAA AGG TCA GGT GTT CGA GTC AC) and 914–936 (5′-CTT TGG TTG CGG GTT TAG GGA TT).

For the first round, 20 µl of extracted DNA from either patient or control samples were amplified in 50 µl reaction mix containing 2 U of AmpliTaq Gold^{TM} (Perkin-Elmer Cetus, Cambridge, UK) with 4 pmol of first-round primers in 1X GeneAmp PCR buffer and 6·25 nmol of deoxynucleoside triphosphates (dNTPs). The first-round product (1·5 µl) was transferred to the second-round mix in a room dedicated to handling PCR amplicons. Concentrations of *Taq* and dNTPs were the same but the second-round primer concentrations were increased fourfold to 16 pmol. After an initial denaturation step at 94°C for 10 min, the following cycling conditions were performed on a PHC3^{TM} Techne thermal cycler (Cambridge, UK) for 35 cycles: 92°C for 30 s, 45°C for 30 s and 72°C for 30 s. Only the primers and anneal temperature (now 57°C) were changed in the second round. After separation on a 4% agarose gel, the products were visualized with UV light. Any DNA sample that gave a positive result was retested at least once more. For a run to be considered viable both the control positive and negative extraction, and DNA samples had to give the correct result and the 10^{-2} and 10^{-3} DNA PCR samples had to be observed on the gel. The PCR product for *A. fumigatus* was 135 bp and that for *A. flavus* was 121 bp. As whole blood from the patients was not stored, repeat testing was performed on the −20°C stored DNA when a positive PCR result was obtained. Only results that were repeatedly positive on at least two occasions were considered positive. DNA extracted from three patients with probable infections was spiked with 2 fg of positive DNA to look for inhibition of PCR by the patient DNA. DNA from a laboratory reference strain of *A. fumigatus* and *A. flavus* were sequenced, using an ABI PRISM^{TM} 377 DNA sequencer (Perkin-Elmer) and their BigDye^{TM} terminator method.

Aspergillus enzyme-linked immunosorbent assay (ELISA). All samples, as serum, were also tested in the Platelia *Aspergillus* antigen ELISA (Sanofi Diagnostic Pasteur, France) in accordance with the manufacturer's instructions.

RESULTS

PCR sensitivity

A titration series of *A. fumigatus*-cultured spores from 10^6 spores/2 ml of whole blood (amount added to lysis/extraction buffer) to 0·1 spores/2 ml whole blood was used to determine the sensitivity of the nested PCR. Bands of the right size (135 bp) were seen in samples containing 10^6–0·1 spores. If one spore was equivalent to 1 CFU, the nested PCR was able to detect less than 1 CFU, that equates to 1–10 fg DNA (Fig 1). Whole blood was used as a negative control for extraction and PCR, and showed no signal.

PCR specificity

Sequencing data using the reverse inner primer (Fig 2) confirmed a 135 bp sequence as known for *A. fumigatus,* and *A. flavus* showed a shorter (121 bp) sequence not currently reported in GenBank. Only these two *Aspergillus* species were investigated as they were considered to be the main species likely to cause aspergillosis in the patients studied, although these primers detected other *Aspergillus* species of differing size, namely *A. niger* and *A. terrus* (results not shown).

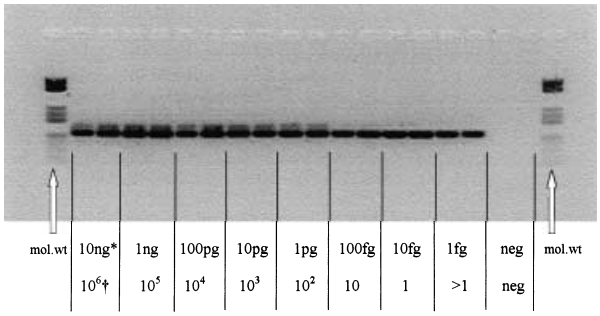

Fig 1. Titration of *Aspergillus* spores in duplicate showing sensitivity of PCR per 2 ml of whole blood. *DNA; †CFU; mol wt, molecular weight marker.

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A fla    1    CTT TCC AGT CCA CAA GCT CAG TGG : : : : : : : : :
A fum    1    CTT TCC AGT CCA CAA GCT CAG TGG ATA AAG AAT

A fla   34     : : : : : : : : GAT AAA  GGA ATT  AAT ATT TAC  GGC
A fum   34    AGC TCA ATG GAT AAA   : GA A : T  AA : ATA TAC  : GC

A fla   67     CTA ACT ACA TTA TCA TTG GTA TCT ACC GAG TAC
A fum   67     CCA ACT ACA TTA TCA TT:  GTA TCT ACC GAG TAC

A fla  100    TGG GTT  TTA TAA ATC CA :  : TT AGG GAT TTG GGC
A fum  100    T : G GTA TTA TAA ATC CAC  GTT AGG GAT TTG GGC

A fla  133    GTT GGT TTC
A fum  133    GTT GGT TTC
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Fig 2. Alignment of *A. flavus* and *A. fumigatus* sequences amplified by nested PCR.

Correlation of patients' results with clinical findings

None of the 94 samples tested from the 17 patients were above the cut-off value when tested as serum in the Platelia *Aspergillus* antigen ELISA. Four patients (A, B, F and P) who had no radiographical investigations and were considered fungal infection free were tested by PCR and found to be negative by PCR (Fig 3). All these patients were given antifungals at some time during the course of the study.

Specimens from four patients (J, K, L and M) were suspected of having a possible *Aspergillus* infection. The supportive evidence for a possible infection was obtained (in patients J, K and L) from the detailed CT scan reports where ground glass shadowing or attenuation or partial halos were seen. In patient M, a possible infection was determined on clinical grounds alone. A PCR-positive result from these patients was detected on at least one occasion and one of these patients (M) died with a *Candida* infection 2 weeks after the end of the study.

Three patients (C, E and O) were considered to have a probable *Aspergillus* infection. In all three patients, there was a more defined diagnosis of aspergillosis from the CT scan reports. Several air crescents and partial halos were seen in patient C. Patient E had multiple irregular nodules with partial halos to them and ground glass shadowing, while the CT scan from patient O showed a patchy nodular change and ground glass shadowing predominantly peribronchovascular in distribution that was new and typical of an active infection. Patient O showed positive-PCR signals on four of the nine samples tested. PCR positives were seen both in the presence and absence of antifungals (Fig 3). Seven of nine DNA samples from this patient were spiked with 2 fg of *A. fumigatus* DNA and no inhibition of the PCR signal by the sample was seen. This patient recovered from the infection. Only one sample of nine tested from one of the other two probable patients (patient E) was PCR positive. Antifungal drugs were given throughout the time of PCR screening. This may have reduced the infection to below detectable levels. No inhibition of the PCR by these samples was observed.

PCR-positive signals were also found in six other patients (on more than one occasion in four patients). These patients not did have CT scans or radiographs as they were not considered to have symptoms of infection but in four cases antifungals were given. Whether these results should be classified as false-positives is questionable but, as defined in the methods, these results were confirmed at least once from the stored DNA samples. Overall, eight different extraction runs were carried out and none had contaminated negative lysis buffer samples, and the positive samples were as expected.

DISCUSSION

This small study, confirmed some of the difficulties when screening for *Aspergillus* infection by PCR. Care was taken at all steps in the procedure to avoid and test for false results. For diagnostic purposes, it is essential to target a region of the fungal genome where repeated sequences are present, i.e. ribosomal or mitochondrial region as a target, to ensure

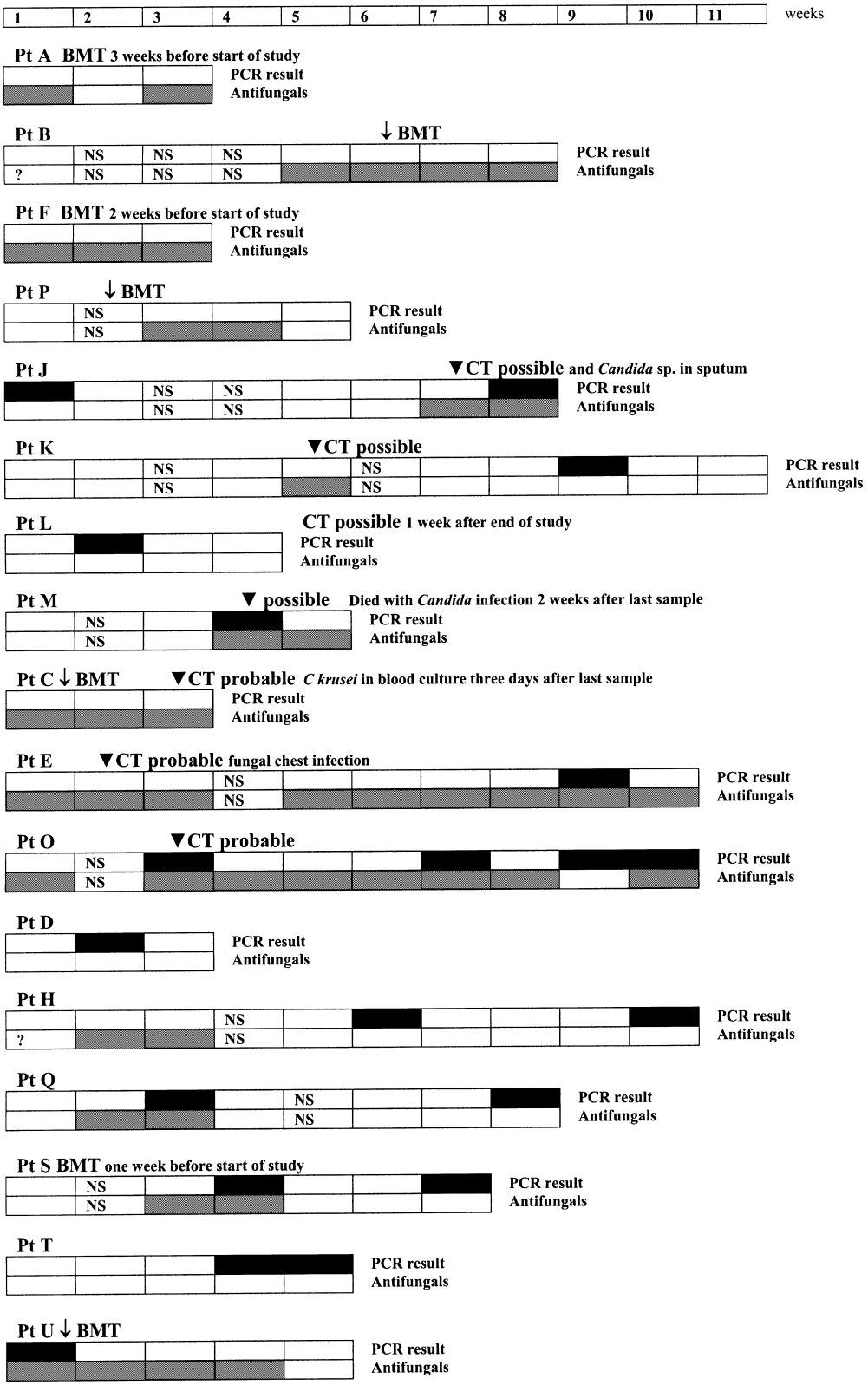

Fig 3. PCR results and antifungal data on 17 patients with the timing of the diagnosis of a possible or probable *Aspergillus* infection (from CT scan) and BMT dates indicated. Black boxes, PCR positive; white boxes, PCR negative or no antifungal therapy; grey boxes, antifungal therapy; BMT↓, timing of BMT; ▼CT probable or possible, diagnosis and timing of first CT scan reported; Pt, patient; NS, no sample; ?, antifungal status unknown.

good sensitivity of amplification. We chose to design a nested PCR, detecting a small mitochondrial region specific for *Aspergillus* species, because no cross-reactivity with other fungi and yeasts in this amplified region had been reported (Bretagne *et al*, 1995). Also no reaction with human DNA was observed. A nested PCR was developed to enable a more sensitive and specific test to be developed even though nested PCRs are known to pose contamination problems. Specificity of the PCR was confirmed by sequencing. Both *A. fumigatus* and *A. flavus* DNA samples were sequenced and the *A. fumigatus* sequence was as predicted (135 bp, GenBank database), and a 121 bp product matching the sequence size was observed for *A. flavus*, although there is no published database sequence for this region.

Whole blood (2 ml) was chosen as the starting material. As there is still uncertainty as to where the *Aspergillus* infection is located within the blood, it would seem wise to take whole blood. This is supported by limited study (Löffler *et al,* 2000b). A recent study using similar primers (Verweij *et al,* 2000) could not detect DNA in plasma samples from a patient with chronic granulomatus disease and proven *Aspergillus* infection. Positive results were only seen in samples taken from the site of infection, as if encapsulation of the infection in this disease had prevented leakage of the infection into the blood. Doubling or trebling of the volume of blood taken to 4 ml or 6 ml may increase the sensitivity. Other studies have used 5 ml (Hebart *et al,* 2000a,b) or 6 ml (Van Burik *et al*, 1998) or 10 ml (Lass-Flörl *et al,* 2001) of whole blood. The sensitivity of the PCR when testing the spiked blood cultures in this study was at least equivalent or greater than that reported (Bretagne *et al*, 1995; Yamakami *et al*, 1996; Einsele *et al*, 1997; Fletcher *et al*, 1998; Van Burik *et al*, 1998; Skladny *et al*, 1999; Jaeger *et al,* 2000; Löffler *et al,* 2000a).

However, even this level of sensitivity may not be satisfactory. It does appear that, as in case of patient O, the infection is detected one week and not the next. This phenomenon has been reported (Van Burik *et al*, 1998; Yamakami *et al*, 1998; Lass-Flörl *et al,* 2001). The ability of the PCR to detect *Aspergillus* may have been affected by the presence of antifungal drugs.

In two cases where a probable infection was suspected, antifungals were given throughout the study. Only on one occasion was the PCR result positive. None of these samples were PCR inhibitory. Only one patient with a probable infection had high enough levels of *Aspergillus* species to be detected throughout, during treatment, and even then not every week.

Two of the four patients (K and L) with a possible infection were only positive once (no antifungals on week of testing), whereas the other two patients (J and M) were given antifungals at the time of diagnosis of a possible infection and were PCR positive around that time so it was likely that both had high enough levels of *Aspergillus* to be detected in the presence of antifungals. A better correlation between possible infection and PCR-positive results was seen in this group than the probable group, which may reflect the more limited antifungal treatment. Also in the group of six patients where no fungal infection was suspected and PCR positivity occurred occasionally, two had no treatment and four patients were treated intermittently, again suggesting a higher detection rate linked to the absence of antifungals. As none of the samples from the 17 patients were above the cut-off when tested in the Sanofi antigen ELISA, it is probable that low levels of *Aspergillus* were present. This perhaps emphasizes the need to produce a highly sensitive *Aspergillus* PCR that will detect infection even in the presence of antifungals.

In conclusion, the benefits of PCR screening may be quite limited if testing takes place once treatment has started. Consistently positive results were only seen in one patient where probable aspergillosis was diagnosed. In both probable and possible groups of patients, no clear pattern emerged between PCR positivity and disease. Treatment with antifungal agents may have been responsible for reducing aspergillosis to subdetectable levels. In practice, many haematology patients are started on antifungal agents empirically for unresolving febrile neutropenia, and there is an increasing tendency to use antifungal agents prophylactically, which may further limit the role of PCR screening.

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