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Role of prospective screening of blood for invasive aspergillosis by polymerase chain reaction in febrile neutropenic recipients of haematopoietic stem cell transplants and patients with acute leukaemia

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Summary

Guidelines for the use of polymerase chain reaction (PCR)-based assays to aid the diagnosis of invasive aspergillosis (IA) in high-risk haematology patients have not been formulated. We prospectively evaluated a nested PCR assay to detect *Aspergillus* in blood during 95 febrile neutropenic episodes, in patients with haematological malignancy and haematopoietic stem cell transplant (HSCT) recipients. PCR results were correlated with the diagnostic classification of the 2002 European Organisation for Research and Treatment of Cancer/Mycosis Study Group. When two-positive results were used to define an episode as 'PCR positive', the sensitivity, specificity, positive-predictive value and negative predictive value for 'proven'/'probable' IA ($n = 13$) were 100%, 75·4%, 46·4% and 100%, respectively. Consecutive positive results occurred in 61·5% of these 13 episodes. Overall, PCR positivity preceded standard diagnosis by a mean of 14 d and the median time between positive results was shorter than that in other categories of IA. All 13 episodes occurred in the setting of allogeneic HSCT recipients and acute leukaemia. If 'eligibility' for antifungal therapy were based on two-positive-PCR tests, use of empiric treatment could have been reduced by up to 37%. The nested PCR assay is a practical screening test for excluding IA. Patients with consecutive positive results or intermittent-positive results (within 14 d) warrant immediate investigations for IA and the initiation of antifungal therapy.

Keywords: invasive aspergillosis, polymerase chain reaction, haematopoietic stem cell transplant recipients, haematological malignancies.

Invasive aspergillosis (IA) is an important clinical entity in haematopoietic stem cell transplant (HSCT) recipients and patients undergoing chemotherapy for haematological malignancies (Denning, 1998; Latgé, 1999; Marr *et al*, 2002a,b). Despite advances in antifungal therapy, mortality because of IA remains high (56–90%) (Lin *et al*, 2001; Marr *et al*, 2002b). This is partly because of difficulties in establishing a reliable diagnosis early enough for successful intervention (Latgé, 1999; Jones & McClintock, 2003). Definitive diagnosis depends on visualisation of *Aspergillus* spp. in affected tissue and/or culture of the organism from normally sterile sites, which are rarely achieved (Latgé, 1999).

In recent years, rapid, 'in-house', polymerase chain reaction (PCR)-based assays have been developed to detect *Aspergillus* DNA in clinical specimens but are not standardised. The majority target multi-copy genes, including the rDNA gene cluster and the mitochondrial gene (Einsele *et al*, 1997; van Burik *et al*, 1998; Skladny *et al*, 1999; Hebart *et al*, 2000; Hendolin *et al*, 2000; Williamson *et al*, 2000; Raad *et al*, 2002). Evaluation of these assays using whole blood specimens has shown a wide range of sensitivities (57–100%) and specificities (56–100%), with a lower limit of detection of 1–10 colony forming units (cfu) of *Aspergillus conidia* per millilitre of blood.

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A few prospective studies have evaluated the clinical utility of different PCR assays in HSCT recipients and/or patients with haematological malignancies, with variable results. In general, sensitivities and negative predictive values (NPVs) have been moderate to high (63·6–100%, and 98–100%, respectively), but specificities only moderate (65–81·3%) (Buchheidt *et al*, 2001, 2004; Ascioglu *et al*, 2002). Additional concerns have been the interpretation of a single-positive-PCR result and intermittent-positive-PCR results. Furthermore, the occurrence of positive-PCR results in patients considered to have 'possible', but not 'probable' or 'proven', IA is not readily explained (Hebart *et al*, 2000; Williamson *et al*, 2000; Lass-Flörl *et al*, 2001).

More recently, real-time PCR assays have been developed allowing the rapid DNA amplification, quantification of fungal load, and the potential for contamination to be greatly reduced (Löeffler *et al*, 2000; Kami *et al*, 2001; O'Sullivan *et al*, 2003; Pryce *et al*, 2003; Sanguinetti *et al*, 2003; Spiess *et al*, 2003; Buchheidt *et al*, 2004; Challier *et al*, 2004). However, these are not as sensitive as nested PCR assays (Spiess *et al*, 2003; Buchheidt *et al*, 2004; Halliday *et al*, 2005) and the majority are specific for *A. fumigatus*, which in Australia accounts for approximately 85% of cases (Australian Mycoses Interest Group, Australasian Society for Infectious Diseases, Sydney, Australia).

In this study, we modified the sensitive *Aspergillus*-specific PCR assay of Skladny *et al* (1999) and evaluated its utility for the early detection of IA by screening blood from febrile neutropenic adults and children undergoing chemotherapy for haematological malignancy and HSCT recipients. The PCR results were correlated with clinical data.

Patient and methods

Patients

Patients admitted to the Haematology and Bone Marrow Transplant (BMT) services at Westmead Hospital (June 2002 to December 2002) and the Haematology and Oncology Services at the New Children's Hospital at Westmead (August 2002 to July 2003) were eligible for inclusion if they were undergoing intensive (induction or salvage) chemotherapy for haematological malignancy or were recipients of allogeneic or autologous HSCT, and had developed febrile neutropenia. The study was performed in accordance with the guidelines of the Institutional Review Committee at the New Children's Hospital and with the guidelines for quality assurance activities at Westmead Hospital. Informed consent was obtained in all cases.

Case definitions

'At-risk' episodes were defined as febrile neutropenia (neutrophil count $<0{\cdot}5 \times 10^9$/l) occurring after chemotherapy or in the early post-transplant period (usually until at least day 30) for HSCT recipients. In general, episodes corresponded to periods of inpatient treatment.

Classification of IA

Invasive aspergillosis was classified according to the European Organisation for Research and Treatment of Cancer (EORTC)/Mycology Study Group (MSG) criteria (Ascioglu *et al*, 2002). Proof of infection ('proven' IA) was based on: histopathological or cytopathological evidence of hyphae with evidence of associated tissue damage, or a positive culture from a normally sterile and clinically or radiologically abnormal site consistent with infection. 'Probable' IA required at least one host risk factor, one microbiological criterion, and one major (or two minor) clinical criteria (Ascioglu *et al*, 2002). 'Possible' IA required at least one host factor, plus one microbiological criterion or one major (or two minor) clinical criteria (Ascioglu *et al*, 2002). 'No' IA was defined as no microbiological or clinical evidence of *Aspergillus* infection. These case definitions were applied at the end of the 'at-risk' episode and were made independently of the PCR results. The day of diagnosis of IA was defined as the day on which the first diagnostic culture, microscopic, or diagnostic examination was performed.

Antifungal prophylaxis and management of febrile neutropenia

Patients undergoing HSCT usually received fluconazole prophylaxis from the onset of conditioning therapy. Patients undergoing chemotherapy for haematological cancer did not receive prophylaxis. Antimicrobial therapy for febrile neutropenia was instituted according to the Infectious Diseases Society of America (IDSA) Fever and Neutropenia Guidelines (Hughes *et al*, 2002). Empiric antifungal therapy, usually with amphotericin B, was initiated when patients remained febrile despite 96 h of broad-spectrum antibiotics. Protocol investigations for patients with suspected IA included high-resolution thoracic-computerised tomography (HRCT) scanning and bronchoalveolar lavage (BAL).

Blood sampling and results

Blood samples were collected from consecutive inpatients into EDTA-containing tubes twice weekly. After discharge from hospital, blood was drawn weekly at patient review. Only patients from whom at least three blood samples were obtained per febrile neutropenic episode were included in the analysis. PCR results were not communicated to the treating physicians and decisions about patient management were made strictly independent of the PCR results. The PCR results were evaluated by correlating them with the clinical classification.

Aspergillus PCR assay

DNA extraction Chemicals were obtained from Sigma Chemical Co., St Louis, MO, USA and BDH Laboratory

Supplies, England. DNA extractions were performed in a class II laminar flow cabinet using the GenElute Mammalian Genomic DNA kit (Sigma-Aldrich Co) with some modifications. Blood samples (500 μl) were lysed with three volumes of erythrocyte lysis buffer [0·155 mol/l NH_4Cl; 0·01 mol/l NH_4HCO_3; and 0·1 mmol/l EDTA (pH 7·4)] (Skladny *et al*, 1999) for 10 min at −20°C and centrifuged 4000 ***g*** for 10 min. The pellets resuspended in 200 μl of sorbitol buffer (1 mol/l sorbitol; 100 mmol/l EDTA; and 0·1% 2-mercaptoethanol) (Williamson *et al*, 2000) and 200 U of lyticase (Sigma-Aldrich Co). After incubation at 37°C for 60 min, spheroplasts were precipitated by centrifugation at 5400 ***g*** for 5 min, resuspended in 180 μl of Lysis Solution T (GenElute Mammalian Genomic DNA kit) and 20 μl of Proteinase K (Sigma-Aldrich Co), and incubated at 55°C for 60 min. The DNA was extracted according to the manufacturer's instructions with a final elution volume of 120 μl. To monitor for contamination, a 500 μl aliquot of molecular biology grade H_2O was extracted alongside each specimen. All DNA samples and negative controls were stored at −20°C until use.

Nested PCR The PCR primers and amplification conditions were modified from those of Skladny *et al* (1999). First- and second-round PCR amplification reactions were performed in a final volume of 25 μl. The first-round PCR reaction mix contained 1 × PCR reaction buffer [10 mmol/l Tris–HCl (pH 8·3); 50 mmol/l KCl; 1·5 mmol/l $MgCl_2$; 0·001% gelatin] (Applied Biosystems, Foster City, CA, USA), 1 mmol/l MgAc, 200 μM of the deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics, Mannheim, Germany), 1·2 μM concentrations of primers AFU 7S (5′-CGGCCCTTAAAT-AGCCCG) and AFU 7AS (5′-GACCGGGTTTGACCAA-CTTT), 1·25 U *Taq* DNA polymerase (Applied Biosystems) and 10 μl of DNA. The second-round PCR reaction mix contained 1 × TTH buffer [10 mmol/l Tris–HCl (pH 8·3); 50 mmol/l KCl, 1·5 mmol/l $MgCl_2$; 0·01% Tween 20; 0·01% gelatin; 0·01% Nonidet P-40], 3 mmol/l MgAc, 200 μM of dNTPs, 0·4 μM concentrations of primers AFU 5S (5′-AGGGCCAGCGAGTACATCACCTTG) and AFU 5AS (5′-GGGRGTCGTTGCCAACYCYCCTGA), 0·5 U DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland) and 2 μl of the first-round PCR product. The first-round PCR amplification conditions were 94°C for 2 min followed by 26 cycles of 94°C for 40 s, 65°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The second-round PCR amplification conditions were 96°C for 2 min followed by 30 cycles of 96°C for 20 s, 65°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. A negative control of molecular biology grade water and an inhibition control to exclude the presence of inhibitory substances were included for each specimen during PCR amplification. The inhibition control was composed of an equal mix of specimen DNA and *Aspergillus* DNA. PCR products were separated by 1·5% agarose gel electrophoresis, stained with ethidium bromide and visualised by ultra-violet transillumination. To minimise the contamination, DNA extractions and the first- and second-round PCRs were performed in three separate rooms. Gels were run in a fourth room, with a unidirectional workflow between the four rooms.

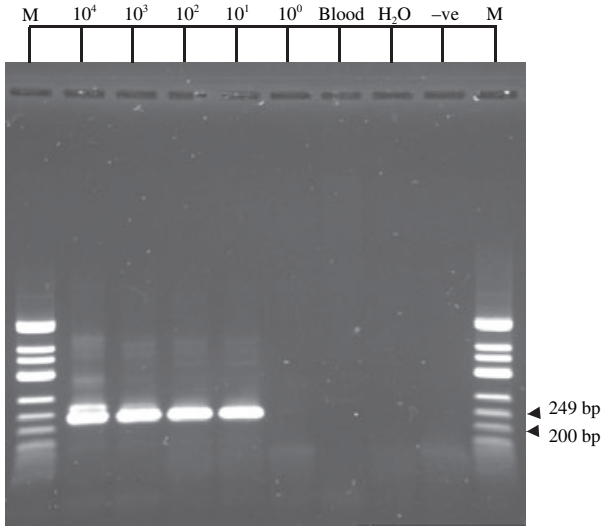

Fig 1. Peripheral blood samples from healthy donors seeded with serial dilutions of *A. fumigatus* conidia (10^4 – 10^0 cfu/ml). A signal derived from 10^1 cfu/ml was detectable by ethidium bromide staining of an agarose gel. DNA from unseeded blood and negative reagent controls during DNA extraction (H_2O) and PCR amplification (−ve) resulted in no bands. The HinfI (Promega, Madison, WI, USA) ladder was used as a molecular size marker (M).

Sensitivity and specificity of the PCR assay The sensitivity of the assay was determined to be 10 cfu/ml of blood, based on the DNA extracted from normal blood samples that had been seeded with serial dilutions of *A. fumigatus conidia* (10^4–10^0 cfu/ml) (Fig 1). To test for specificity, DNA was extracted from reference fungal strains obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and clinical strains obtained from the Mycology Laboratory at Westmead Hospital. Organsisms tested included: *Candida albicans* (ATCC 90028), *C. glabrata* (ATCC 90030), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), *C. tropicalis*, *Cryptococcus neoformans* var. *neoformans* (ATCC 90112), *C. gattii* (ATCC 32608), *A. fumigatus* (ATCC 204305), *A. flavus* (ATCC 204304), *A. terreus*, *A. nidulans*, *A. niger*, *Penicillium chrysogenum*, *Scedosporium prolificans*, *S. apiospermum*, *Fusarium solani*, *F. oxysporum*, *Rhizopus oryzae*. Only template DNA from *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. terreus* and *P. chrysogenum* was amplified. *P. chrysogenum* is phylogenetically very closely related to *Aspergillus* spp., but is not a human pathogen and should not be detectable in blood. Amplified PCR products from 14 clinical blood samples were randomly chosen for sequencing to verify their identity. For all samples, DNA sequencing showed

99–100% homology to the expected fragment of the 18S rRNA gene from numerous *Aspergillus* spp. in the GenBank database.

Statistical analysis

The sensitivity, specificity, positive-predictive value (PPV) and NPV of the assay were calculated using the three different definitions of disease status for an episode of febrile neutropenia (Method A/B, C and D) as described previously by Maertens *et al* (2001). Method A/B excluded 'possible' cases, defining episodes of 'proven' IA and 'probable' IA as true positives and 'no' IA as true negatives. Methods C and D included episodes of 'possible IA' as either true positives or true negatives, respectively. An episode was considered to be PCR positive if two blood specimens tested positive. The confidence intervals (CIs) were calculated using CI Analysis (CIA) software, version 1·0, London, England. Chi-squared tests were used to assess the association between *Aspergillus* PCR-positive results and categorical variables. A *P*-value of <0·05 was considered significant.

Results

A total of 998 blood samples from 29 adults (272 samples) and 36 children (726 samples) were collected during 95 episodes of febrile neutropenia. Patient information and clinical categories of IA are summarised in Table I. Graft-*versus*-host disease (GVHD) was present during 25 episodes in allogeneic HSCT recipients; corticosteroid therapy was administered in 22 of the 25 episodes. All 'probable' ($n = 8$) or 'proven' ($n = 5$) febrile neutropenic episodes of IA occurred in allogeneic HSCT recipients (eight of 13 episodes, 61·5%) or patients with acute leukaemia who had received intensive chemotherapy (five of 13 episodes, 38·5%). In contrast, in 12 of 14 febrile neutropenic episodes in autologous HSCT recipients there was no evidence of IA and two were classified as 'possible' IA. PCR results by diagnostic category of IA for these patients are summarised in Table II.

Correlation of PCR results with clinical features

A positive-PCR test was documented in 64 of 95 (67·4%) episodes. The percentage of positive results was greater in episodes of 'probable/proven' IA than 'possible' or 'no' IA ($P < 0·001$, see Table II). Twenty-five of 64 (39·1%) episodes were PCR positive only once and these were confined to patients with 'no' or 'possible' IA (see Table III). There was a relationship between sample size and likelihood of a positive result. All of the episodes with 20 samples tested ($n = 10$) were positive at least once and in 90% of these episodes two or more assays were positive. Time lines of the PCR results for each febrile neutropenic episode showed that the positive results were not random as they clustered together during an episode, regardless of the EORTC/MSG 2002 classification (Ascioglu *et al*, 2002) (data not shown).

Table I. Patient characteristics.

Patient characteristic	Number
Number of patients	65
Number of febrile neutropenic episodes	95
Adults ($n = 29$)	
Age, median years (range)	37·8 (16–62)
Sex (male:female)	23:6
Children ($n = 36$)	
Age, median years (range)	6·6 (0·8–16)
Sex (male:female)	32:4
Underlying condition (febrile *neutropenic episodes)	
Induction/salvage chemotherapy for acute leukaemia ($n = 27$)	
AML	17
ALL	9
APML	1
Autologous HSCT ($n = 14$)	
BMT	10
PBSCT	4
Allogeneic HSCT ($n = 53$)	
Matched sibling donor (BMT)	31
Matched unrelated donor	
UCBT	3
BMT	19
Severe aplastic anaemia	1
EORTC/MSG categorisation of invasive aspergillosis (Episodes)†	
Proven IA	5
Probable IA	8
Possible IA	21
No evidence of IA	61
Total	95

AML, acute myeloid leukaemia; ALL, acute lymphocytic leukaemia; APML, acute promyelocytic leukaemia; HSCT, haematopoietic stem cell transplant; BMT, bone marrow transplant; PBSCT, peripheral blood stem cell transplantation; UCBT, umbilical cord blood transplant.

*The median duration of neutropenia was 29·4 d (range 15–62 d).

†Seven of the episodes with probable or proven IA (53·8%) were associated with GVHD requiring corticosteroid therapy.

Sensitivity, specificity, positive and negative predictive values

The data summarised in Table IVA are based on defining a 'PCR-positive' episode by at least two-positive tests. The sensitivity and predictive values of the assay calculated by method A/B changed significantly when episodes of 'possible' IA were included in either the 'true-positive' or 'true-negative' categories, using Methods C and D, respectively.

Using a more stringent definition of two consecutive positive-PCR results (two-positive results within a week) to classify an episode as 'PCR positive', the sensitivity and NPV were reduced and the specificity and PPV were increased using Method A/B (Table IVB). If 'possible' cases of IA were included as 'true negatives', the specificity decreased to 91·5% but the sensitivity remained unchanged.

Table II. Underlying disease and PCR results in the different patient groups according to the EORTC/MSG 2002 classification.

		EORTC/MSG 2002 Patient group		
	No IA	Possible	Probable/proven	Total
Underlying disease				
Acute leukaemia	16	6	5	27
Autologous HSCT	12	2	0	14
Allogeneic HSCT	32	13	8	53
Severe aplastic anaemia	1	–	–	1
Samples and PCR results				
Number of samples	602	222	174	998
Number of febrile neutropenic episodes	61	21	13	95
Mean number of samples/febrile neutropenic episode (range)	10·4 (3–47)	10·6 (3–34)	13·4 (5–24)	10·5 (3–47)
Number of PCR-positive samples	65	42	50	157
Number of PCR-negative samples	537	180	124	841
Percentage of samples positive	10·8%	18·9%	28·7%*	15·7%
Median time in days to first two PCR-positive results (range) †	14 (7–66)	21 (3–27)	13 (3–79)	-
Median time in days between any PCR-positive results (range) [interquartile range]	21 (6–77) [13–32]	14 (2–70) [7–25]	10 (3–80) [5–18]	– [–]

*Compared with possible and no IA groups, $P < 0{\cdot}001$.
†Median time measured from beginning of the febrile neutropenic episodes.

Table III. Correlation of PCR results with different patient groups according to EORTC/MSG 2002 criteria.

	EORTC/MSG 2002, (*n*, %) [Therapy:no therapy]		
PCR results all episodes ($n = 95$)	No IA	Possible	Probable/proven
PCR negative ($n = 31$)	29 (47·5) [9:20]	2 (9·5) [2:0]	0 (0) [–]
PCR-positive once ($n = 25$)	17 (27·9) [9:8]	8 (38·1)* [8:0]	0 (0) [–]
Intermittent PCR positive ($n = 24$)	15 (24·6) [9:6]	4 (19·1) [3:1]	5 (38·5) [5:0]
Consecutive PCR positive ($n = 15$)	0 (0) [–]	7 (33·3) [6:1]	8 (61·5) [8:0]
Total	61 (100%)	21 (100%)	13 (100%)

*Disseminated *Fusarium* infection was found at post-mortem in one patient.

Table IV. Sensitivity, specificity and predictive values of the PCR assay according to the different definitions of disease status using the criteria of ≥2 positive-PCR results to classify an episode as 'PCR positive' (A), and two consecutive positive-PCR results to classify an episode as 'PCR positive' (B).

	Method A/B	Method C	Method D
(A)			
Sensitivity	100 (75·3–100)	70·6 (52·3–84·9)	100 (75·3–100)
Specificity	75·4 (62·7–85·5)	75·4 (62·7–85·5)	74·7 (63·3–84)
positive-predictive value (PPV)	46·4 (27·5–66·1)	61·5 (44·6–76·6)	40·6 (23·7–59·4)
NPV	100 (92·3–100)	82 (69·6–91·1)	100 (93·6–100)
(B)			
Sensitivity	61·5 (31·6–86·1)	44 (27·2–62·1)	61·5 (31·6–86·1)
Specificity	100 (94·1–100)	100 (94·1–100)	91·5 (83·2–96·5)
PPV	100 (63·1–100)	100 (78·2–100)	53·3 (26·6–78·7)
NPV	92·4 (83·2–97·5)	76·3 (65·4–85·1)	93·8 (86·0–97·9)

Method A/B defines episodes of 'proven' and 'probable' IA as true positives and 'no' IA as true negatives; Method C defines episodes of 'proven', 'probable' and 'possible' IA as true positives and 'no' IA as true negatives; Method D defines episodes of 'proven' and 'probable' IA as true positives and 'possible' and 'no' IA as true negatives.
All values given are percentages.
Values in parentheses are 95% CI.

In all episodes of 'probable' ($n = 8$) or 'proven' IA ($n = 5$), at least two PCR results (range 2–7) were positive. Consecutive positives were noted in eight of 13 (61·5%) episodes with intermittent positives in the remaining five. Consecutively, positive-PCR results were not observed in episodes classified as 'no' IA, but were noted in seven of 21 (33·3%) episodes of 'possible' IA (Table III). In two of the seven episodes, more than 20 samples had been collected.

Effect of empirical and targeted antifungal therapy on PCR results

Overall, there was no correlation between a positive-PCR result and concurrent antifungal therapy (see Table III). Antifungal therapy effective against *Aspergillus* spp. was administered in 12 of 13 episodes of 'probable' or 'proven' IA. Despite intensive therapy, the PCR remained intermittently positive in all 12 episodes. Antifungal therapy was not administered in the 13th episode.

Clinical implications of a positive-PCR result

Polymerase chain reaction positivity was the earliest indicator of IA by a mean of 14 d (range 3–30 d) in seven episodes (in the remaining six, PCR was performed at approximately the same time as the clinical diagnosis was made). The median time to the first two-positive-PCR results, measured from the beginning of the febrile neutropenic episode, was not affected by category of IA, though the median time and the interquartile (IQ) range in time between positive results were reduced in episodes of proven/probable IA (see Table II). Empiric antifungal agents were prescribed in 27 of 61 (44·3%) episodes considered unlikely to be associated with IA. If, however, the criterion of two-positive-PCR results (i.e. a PCR-positive episode) was used to guide the management, only 15 episodes would have been treated, a net reduction of 20%. Nineteen of 21 episodes (90·5%) of 'possible' IA received antifungal therapy; using the above criteria, only 11 of 21 (52·4%) would have been 'eligible' to receive therapy, a reduction of 38·1% (Table III). If episodes occurring in autologous HSCT recipients were omitted from the analysis, the reduction in empiric antifungal use would have been 22·5% and 36·9% in cases of 'no' IA and 'possible' IA, respectively (data not shown).

Discussion

The value of PCR-based tests in clinical decision-making in cases of suspected IA remains a topic of debate. This large study indicated that screening of blood for *Aspergillus* DNA by a sensitive nested-PCR assay improved the early diagnosis of IA in febrile neutropenic recipients of allogeneic HSCT and patients with acute leukaemia. The sensitivity of the PCR assay, using the criterion of two-positive tests and excluding 'possible' cases from the analysis, was 100% in patients with 'proven' or 'probable' IA (EORTC/MSG 2002 criteria) (Ascioglu *et al*, 2002), and preceded standard diagnosis of IA by a mean of 14 d. Importantly, the NPV was 100%, demonstrating the potential value of the test for excluding IA in high-risk patients.

Reported sensitivities of PCR assays for the diagnosis of IA have ranged from 36% to 100% (Buchheidt *et al*, 2001, 2004; Hebart *et al*, 2000; Williamson *et al*, 2000; Lass-Flörl *et al*, 2001; Raad *et al*, 2002). These studies are not directly comparable because different case definitions and criteria for a 'PCR-positive' episode, which influence sensitivity (Maertens *et al*, 2001; Buchheidt *et al*, 2004), have been used. Furthermore, many authors did not comment on the performance of PCR assays when results from patients with 'possible' IA were taken into account and others excluded these cases from the analysis on the basis of uncertainty of the clinical diagnosis (Williamson *et al*, 2000; Ferns *et al*, 2002; Buchheidt *et al*, 2004; Lass-Flörl *et al*, 2004). Calculation of test performance has necessarily been based on the low numbers of patients with proven or probable IA with its attendant problems, and sample sizes in some have been small. The lack of standardisation of 'in-house' PCR assays also contributes to differences in test performance in the different studies.

We determined that the sensitivity, specificity, PPV and NPV of the PCR assay were 100%, 75%, 46% and 100% when two-positive-PCR results were used to classify an episode as 'PCR positive'. Predictably, the test characteristics changed when 'possible' cases were included as 'true negatives' or 'true positives' (Table IV). Assuming that some 'possible' cases represent occult infection, it could be predicted that the sensitivity, specificity, PPV and NPV were >71%, equal to 75% and >41% and 82% respectively as these were the boundaries defined by including 'possible' cases as 'true positives' or 'true negatives' (Table IVA). Using a more stringent criterion of two consecutive positive-PCR results to define a 'PCR-positive' episode and excluding 'possible' cases, the specificity of our test increased from 75% to 100%, but at the expense of sensitivity (reduced from 100% to 61·5%).

The benefit of a sensitive PCR assay for screening high-risk patients for IA is counterbalanced by potential 'false-positive' results (McClintock *et al*, 2004). As discussed above, occult infection may have been responsible for 'false-positive' results in our study, for example in episodes of 'possible' IA. Transient *Aspergillus* DNAemia has been attributed to sub-clinical infection masked by empirical therapy or neutrophil recovery, and to hepatic clearance of fungal elements from the blood (Ansorg *et al*, 1994). We and others (Buchheidt *et al*, 2004) have also shown that the likelihood of a positive-PCR result increased with the number of specimens obtained per episode of febrile neutropenia. Our finding that clustering of PCR-positive results during febrile neutropenic episodes was not random, supports the notion that transient DNAemia occurred in at least some of the episodes classified as unlikely or 'possible' IA. Environmental contamination by ubiquitous fungal spores and/or non-specific amplification products are other possible causes of 'false-positive' results. This was not

likely to have occurred in the present study as strict precautions were taken, including performing DNA extractions under laminar airflow conditions, processing each specimen with its own negative control during DNA extraction and PCR amplification, and performing all procedures in a unidirectional workflow pattern. Additionally, a positive control of *A. fumigatus* DNA was included in each PCR run.

Polymerase chain reaction positivity may also be affected by antifungal therapy. Others have reported that such therapy was associated with conversion of PCR tests to negative (Lass-Flörl *et al*, 2001, 2004; Buchheidt *et al*, 2004; Yamakami *et al*, 1998). Studies using quantitative real-time PCR assays have correlated clearance of fungal DNA from blood with successful antifungal therapy. In the present study, a positive-PCR test *per se* did not correlate with concurrent receipt of antifungal drugs. Furthermore, although the majority of patients with proven or probable IA were receiving antifungal therapy, PCR results remained consecutively or intermittently positive. Single PCR-positive results usually occurred in patients with no evidence of IA, supporting the notion that a single-positive result is not clinically significant (Williamson *et al*, 2000; Lass-Flörl *et al*, 2001).

It is noteworthy that no episodes of 'probable' or 'proven' IA occurred in autologous HSCT recipients, although two episodes in one patient were classified as 'possible' IA. Autopsy revealed disseminated adenovirus infection in this patient. Conversely, 'probable' or 'proven' IA developed in approximately 15% of allogeneic HSCT recipients and 19% of patients undergoing intensive chemotherapy for acute leukaemia. These results suggest that autologous HSCT recipients are at lower risk for developing IA and are consistent with observations in the USA (Marr *et al*, 2002b; Singh & Paterson, 2005).

The place of routine screening for IA by PCR-based methods has not been resolved by studies conducted to date. Many of these have been compromised by small sample sizes. Even larger studies, including the present one, have involved a relatively small number of patients with 'proven' or 'probable' IA. It has been argued that a test such as the *Aspergillus* PCR assay, with its significant 'false-positive' rate, is of doubtful clinical value and conversely, that the same test is worthwhile because of its high NPV in cases of 'proven' or 'probable' IA. Although there is agreement that PCR positivity is an early indicator of IA [in the present study, PCR results were positive a mean of 14 d (range 3–30) before the diagnosis was made by standard methods, (Hebart *et al*, 2000; Williamson *et al*, 2000)], an effect on clinical outcomes has not been established. Such studies are important because routine screening by PCR tests has significant resource implications for the diagnostic laboratory. We calculated the potential effect on use of empirical antifungal therapy in our febrile neutropenic allogeneic HSCT and acute leukaemic patient population of requiring at least two-positive-PCR tests to initiate antifungal drug therapy. The net reduction in 'empirical' antifungal use would have been approximately 37% in cases of 'possible' IA and 23% in cases of 'no' IA.

On the basis of this study and the published literature, we have formulated the following guidelines. Twice weekly screening of blood from recipients of allogeneic HSCT and patients receiving intensive therapy for acute leukaemia is to be initiated at the onset of fever and neutropenia. PCR test-directed actions are then indicated in patients with persistent fever and neutropenia despite broad-spectrum antibacterial therapy as follows: *Note:* patients meeting current criteria for 'probable'/'proven' IA should commence antifungal therapy independent of the PCR results.

1 Consistently negative-PCR results: investigate/treat for other causes of fever and/or lung infiltrates.
2 Single-positive-PCR result: repeat test on a second blood sample.
3 Consecutive positive-PCR results: initiate antifungal therapy and undertake immediate additional investigations for IA (HRCT scan, BAL studies).
4 Intermittently positive-PCR results (within 2 weeks): As for three, on the basis that the median time between positive results was 10 d [interquartile (IQ) range 5–18 d] for 'probable'/'proven' IA and 21 d (IQ range 13–32 d) for the group 'no' IA.
5 Intermittently positive-PCR results (longer than 2 weeks apart): investigate for IA by standard tests; withhold antifungal therapy-pending results.

Whether empiric antifungal therapy should be instituted outside these proposed guidelines should be determined by the clinical state of the patient. A reappraisal of the use of empirical antifungal therapy in high-risk haematology patients is needed with the shift to *Aspergillus* species as the predominant pathogens. This has been highlighted by recent studies addressing the benefits of empiric treatment, which have been inconclusive, as summarised by de Pauw (2005). There is a need to attempt to distinguish between patients truly in need of antifungal therapy and those who are not, in the context of growing experience in the use of more sensitive diagnostic tests for IA. The proposed algorithm in this study has the potential to reduce the use of empiric antifungal therapy and hence toxicity and cost. Indeed, if two-positive-PCR results is used to guide management, an estimated reduction in the use of empirical therapy of up to 37% may be achieved. Our study indicates that no patient with 'proven' or 'probable' IA had only a single-positive-PCR test. On the other hand, larger, prospective studies are required to determine the clinical impact of a pre-emptive strategy incorporating PCR-based diagnosis of IA compared with the use of empirical antifungal therapy.

In summary, the use of *Aspergillus* PCR tests enables IA to be identified earlier than conventional diagnostic tests and interim guidelines for its use as a screening test have been proposed. To determine if early diagnosis has an impact on the mortality of IA, or on the use of empirical antifungal therapy, larger numbers of patients at risk need to be studied in carefully designed, randomised-clinical trials.

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References

Ansorg, R., Heintschel von Heinegg, E. & Rath, P.M. (1994) *Aspergillus* antigenuria compared to antigenaemia in bone marrow transplant recipients. *European Journal of Clinical Microbiology and Infectious Diseases*, **13,** 582–589.

Ascioglu, S., Rex, J.H., de Pauw, B., Bennett, J.E., Bille, J., Crokaert, F., Denning, D.W., Donnelly, P., Edwards, J.E., Erjavec, Z., Fiere, D., Lortholary, O., Maertens, J., Meis, J.F., Patterson, T.F., Ritter, J., Selleslag, D., Shah, M., Stevens, D.A. & Walsh, T.J. (2002) Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and haemopoietic stem cell transplants: an international consensus. *Clinical Infectious Diseases*, **34,** 7–14.

Buchheidt, D., Baust, C., Skladny, H., Ritter, J., Suedhoff, T., Baldus, M., Seifarth, W., Leib-Mosch, C. & Hehlmann, R. (2001) Detection of *Aspergillus* species in blood and bronchoalveolar lavage samples from immunocompromised patients by means of 2-step polymerase chain reaction: clinical results. *Clinical Infectious Diseases*, **33,** 428–435.

Buchheidt, D., Hummel, M., Schleiermacher, D., Spiess, B., Schwerdtfeger, R., Cornely, O.A., Wilhelm, S., Reuter, S., Kern, W., Südhoff, T., Mörz, H. & Hehlmann, R. (2004) Prospective clinical evaluation of a LightCycler™-mediated polymerase chain reaction assay, a nested-PCR assay and a galactomannan enzyme-linked immunosorbent assay for detection of invasive aspergillosis in neutropenic cancer patients and haematological stem cell transplant recipients. *British Journal of Haematology*, **125,** 196–202.

van Burik, J.A., Myerson, D., Schreckhise, R.S. & Bowden, R.A. (1998) Panfungal PCR assay for detection of fungal infection in human blood specimens. *Journal of Clinical Microbiology*, **36,** 1169–1175.

Challier, S., Boyer, S., Abachin, E. & Berche, P. (2004) Development of a serum-based Taqman real-time PCR assay for the diagnosis of invasive aspergillosis. *Journal of Clinical Microbiology*, **42,** 844–846.

Denning, D.W. (1998) Invasive aspergillosis. *Clinical Infectious Diseases*, **26,** 781–803.

Einsele, H., Hebart, H., Roller, G., Loffler, J., Rothenhofer, I., Muller, C.A., Bowden, G., Burik, J., Engelgard, D., Kanz, L. & Schumacher, U. (1997) Detection and identification of fungal pathogens in blood by using molecular probes. *Journal of Clinical Microbiology*, **35,** 1353–1360.

Ferns, R.B., Fletcher, H., Bradley, S., Mackinnon, S., Hunt, C. & Tedder, R.S. (2002) 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. *British Journal of Haematology*, **119,** 720–725.

Halliday, C., Wu, Q.X., James, G. & Sorrell, T. (2005) Development of a nested qualitative real-time PCR assay to detect *Aspergillus* DNA in clinical specimens. *Journal of Clinical Microbiology*, **43,** 4366–4368.

Hebart, H., Löeffler, J., Meisner, C., Serey, F., Schmidt, D., Böhme, A., Martin, H., Engel, A., Bunjes, D., Kern, W.V., Schumacher, U., Kanz, L. & Einsele, H. (2000) Early detection of *Aspergillus* infection after allogeneic stem cell transplantation by polymerase chain reaction screening. *The Journal of Infectious Diseases*, **181,** 1713–1719.

Hendolin, P.H., Paulin, L., Koukila-Kahkola, P., Antilla, V.-J., Malmberg, H., Richardson, M. & Ylikoski, J. (2000) Panfungal PCR and multiplex liquid hybridisation for detection of fungi in tissue specimens. *Journal of Clinical Microbiology*, **38,** 4186–4192.

Hughes, W.T., Armstrong, D., Bodey, G.P., Bow, E.J., Brown, A.E., Calandra, T., Feld, R., Pizzo, P.A., Rolston, K.V.I., Shenep, J.L. & Young, L.S. (2002) 2002 guidelines for use of antimicrobial agents in neutropenic patients with cancer. *Clinical Infectious Diseases*, **34,** 730–751.

Jones, B.L. & McClintock, L.A. (2003) Impact of diagnostic markers on early antifungal therapy. *Current Opinion in Infectious Disease*, **16,** 521–526.

Kami, M., Fukui, T., Ogawa, S., Kazuyama, Y., Machida, U., Tanaka, Y., Kanda, Y., Kashima, T., Yamazaki, Y., Hamaki, T., Mori, S., Akiyama, H., Mutou, Y., Sakamaki, H., Osumi, K., Kimura, S. & Hirai, H. (2001) Use of real-time PCR on blood samples for diagnosis of invasive aspergillosis. *Clinical Infectious Diseases*, **33,** 1504–1512.

Lass-Flörl, C., Aigner, J., Gunsilius, E., Petzer, A., Nachbaur, D., Gastl, G., Einsele, H., Löeffler, J., Dierich, M.P. & Würzner, R. (2001) Screening for *Aspergillus* spp. using polymerase chain reaction of whole blood samples from patients with haematological malignancies. *British Journal of Haematology*, **113,** 180–184.

Lass-Flörl, C., Gunsilius, E., Gastl, G., Bonatti, H., Freund, M.C., Gschwendtner, A., Kropshofer, G., Dierich, M.P. & Petzer, A. (2004) Diagnosing invasive aspergillosis during antifungal therapy by PCR analysis of blood samples. *Journal of Clinical Microbiology*, **42,** 4154–4157.

Latgé, J.-P. (1999) *Aspergillus fumigatus* and aspergillosis. *Clinical Microbiology Reviews*, **12,** 310–350.

Lin, S.J., Schranz, J. & Theutsch, S.M. (2001) Aspergillosis case-fatality rate: Systematic review of the literature. *Clinical Infectious Diseases*, **32,** 358–366.

Löeffler, J., Henke, N., Hebart, H., Schmidt, D., Hagmeyer, L., Schumacher, U. & Einsele, H. (2000) Quantification of fungal DNA by using fluorescence resonance energy transfer and the LightCycler system. *Journal of Clinical Microbiology*, **38,** 586–590.

Maertens, J., Verhaegen, J., Lagrou, K., Van Eldere, J. & Boogaerts, M. (2001) Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. *Blood*, **97,** 1604–1610.

Marr, K.A., Carter, R.A., Boeckh, M., Martin, P. & Corey, L. (2002a) Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. *Blood*, **100,** 4358–4366.

Marr, K.A., Carter, R.A., Crippa, F., Wald, A. & Corey, L. (2002b) Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. *Clinical Infectious Diseases*, **34,** 909–917.

McClintock, L.A., Jordanides, N.E., Allan, E.K., Copland, M., Stewart, K., Parker, A., Devaney, M., Holyoake, T.L. & Jones, B.L. (2004) The use of risk group stratification in the management of invasive fungal infections: a prospective validation. *British Journal of Haematology*, **124,** 403–404.

O'Sullivan, C.E., Kasai, M., Francesconi, A., Petraitis, V., Petraitiene, R., Kelaher, A.M., Sarafandi, A.A. & Walsh, T.J. (2003) Development and validation of a quantitative real-time PCR assay using fluorescence resonance energy transfer technology for detection of *Aspergillus fumigatus* in experimental invasive pulmonary aspergillosis. *Journal of Clinical Microbiology*, **41,** 5676–5682.

de Pauw, B.E. (2005) Between over- and undertreatment of invasive fungal disease. *Clinical Infectious Diseases*, **41,** 1251–1253.

Pryce, T.M., Kay, I.D., Palladino, S. & Heath, C. (2003) Real-time automated polymerase chain reaction (PCR) to detect *Candida albicans* and *Aspergillus fumigatus* DNA in whole blood from high-risk patients. *Diagnostic Microbiology and Infectious Disease*, **47,** 487–496.

Raad, I., Hanna, H., Sumoza, D. & Albitar, M. (2002) Polymerase chain reaction on blood for the diagnosis of invasive pulmonary aspergillosis in cancer patients. *Cancer*, **94,** 1032–1036.

Sanguinetti, M., Posteraro, B., Pagano, L., Pagliari, G., Fianchi, L., Mele, L., La Sorda, M., Franco, A. & Fadda, G. (2003) Comparison of real-time PCR, conventional PCR, and galactomannan antigen detection of enzyme-linked immunosorbent assay using bronchoalveolar lavage fluid samples from hematology patients for diagnosis of invasive pulmonary aspergillosis. *Journal of Clinical Microbiology*, **41,** 3922–3925.

Singh, N. & Paterson, D.L. (2005) *Aspergillus* infections in transplant recipients. *Clinical Microbiology Reviews*, **18,** 44–69.

Skladny, H., Buchheidt, D., Baust, C., Krieg-Schneider, F., Seifarth, W., Leib-Mosch, C. & Hehlmann, R. (1999) Specific detection of *Aspergillus* species in blood and bronchoalveolar lavage samples of immunocompromised patients by two-step PCR. *Journal of Clinical Microbiology*, **37,** 3865–3871.

Spiess, B., Buchheidt, D., Baust, C., Skladny, H., Seifarth, W., Zeilfelder, U., Leib-Mosch, C., Morz, H. & Hehlmann, R. (2003) Development of a LightCycler PCR assay for detection and quantification of *Aspergillus fumigatus* DNA in clinical samples from neutropenic patients. *Journal of Clinical Microbiology*, **41,** 1811–1818.

Williamson, E.C.M., Leeming, J.P., Palmer, H.M., Steward, C.G., Warnock, D., Marks, D.I. & Millar, M.R. (2000) Diagnosis of invasive aspergillosis in bone marrow transplant recipients by polymerase chain reaction. *British Journal of Haematology*, **108,** 132–139.

Yamakami, Y., Hashimoto, A., Yamagata, E., Kamberi, P., Karashima, R., Nagai, H. & Nasu, M. (1998) Evaluation of PCR for detection of DNA specific for *Aspergillus* species in sera of patients with various forms of pulmonary aspergillosis. *Journal of Clinical Microbiology*, **36,** 3619–3623.