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

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Summary

Invasive aspergillosis (IA) is a considerable clinical problem in neutropenic patients with haematological malignancies but its diagnosis remains difficult. We prospectively evaluated a LightCycler™ polymerase chain reaction (PCR) assay, a nested-PCR assay and a galactomannan (GM) enzyme-linked immunosorbent assay (ELISA) to validate their significance in diagnosing IA. During 205 treatment episodes in 165 patients from six centres, a nested-PCR assay and GM testing was performed at regular intervals. Positive nested-PCR results were quantified by a LightCycler™ PCR assay. Patient episodes were stratified according to the 2002 European Organization for Research and Treatment of Cancer/Mycosis Study Group consensus criteria and the PCR and serology results were correlated with the clinical diagnostic classification. Sensitivity and specificity rates for the nested-PCR assay were up to 63·6% [95% confidence interval (CI): 30·8–89%) and 63·5% (95% CI: 53·4–72·7%) respectively, and 33·3% and 98·9% (95% CI: 7·5–70·1% and 94·2–99·9%) for GM respectively. The LightCycler™ PCR assay yielded positive results in 21·4%, lacking discrimination by quantification across the different clinical categories. In this prospective comparison, PCR was superior to GM with respect to sensitivity rates. In patients at high risk for IA, positive results for *Aspergillus* by PCR of blood samples are highly suggestive for IA and contribute to the diagnosis.

Keywords: invasive aspergillosis, polymerase chain reaction, LightCycler™ PCR, haematological malignancies, haematological stem cell recipients.

Systemic fungal infections, especially infections with *Aspergillus* spp., are a frequent cause of death in patients with prolonged periods of neutropenia, patients with acute leukaemias and in haematological stem cell transplant recipients (Denning & Stevens, 1990; Groll *et al*, 1996; Wald *et al*, 1997; Yuen *et al*, 1997; Lin *et al*, 2001; Chan *et al*, 2002; Ruhnke & Maschmeyer, 2002).

Infections with *Aspergillus* spp. are difficult to diagnose, the gold standard of diagnosis is based on histo- or cytopathological proof of hyphae or positive culture results from normally sterile sites, which are rarely achieved.

Standardized criteria for the diagnostic probability of invasive fungal infections were established by a consensus

committee composed of members of the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer (EORTC), Brussels, Belgium, and of the Mycosis Study Group of the National Institutes of Allergy and Infectious Diseases (NIAID-MSG), Bethesda, MD, USA, as guidelines only for clinical research (Ascioglu *et al*, 2002).

In recent years, non-invasive diagnostic tools, such as polymerase chain reaction (PCR) assays and serological tests have been established to aid clinical decision-making. Several studies have shown the clinical validity of *Aspergillus* PCR assays (Einsele *et al*, 1997; Skladny *et al*, 1999; Hebart *et al*, 2000; Buchheidt *et al*, 2001, 2002; Lass-Floerl *et al*, 2001; Ferns *et al*, 2002; Raad *et al*, 2002).

The aim of this study was to further investigate the clinical value of a LightCycler™ (Roche Applied Science, Mannheim, Germany)-mediated quantitative PCR assay (Spiess *et al*, 2003) compared with the nested-PCR assay previously established in our laboratory (Skladny *et al*, 1999) and *Aspergillus* serology testing for the diagnosis of invasive aspergillosis (IA) and to investigate its role in the monitoring of antifungal treatment. Serial blood samples from patients with haematological malignancies were analysed prospectively by the nested-PCR assay, by the LightCycler™ PCR assay and by a galactomannan (GM) enzyme-linked immunosorbent assay. All assays were evaluated for sensitivity and specificity in the detection of IA after classification of patient episodes according to the 2002 guidelines established by the EORTC/MSG (Ascioglu *et al*, 2002).

Patients and methods

Patients and samples

Blood samples, bronchoalveolar lavage (BAL) and other samples (cerebrospinal fluid, ascites, sputum, pleural fluid) were obtained from 165 patients from five university hospitals (Mannheim, Cologne, Rostock, Ulm and Bochum) and the Bone Marrow Transplant Unit at the Deutsche Klinik für Diagnostik, Wiesbaden, all in Germany, between 1 March 2001 and 30 September 2002. We included patients with haematological malignancies undergoing intensive chemotherapy or recipients of haematopoietic stem cell transplantation that fulfilled host factor criteria according to Ascioglu *et al* (2002). Overall, 1522 samples were tested for the presence of *Aspergillus* DNA by the nested-PCR assay (Skladny *et al*, 1999). All samples were obtained under sterile conditions and sent to the laboratory immediately. At least three samples (either blood, BAL or other) were required per patient episode for inclusion in the study. Samples were obtained in the course of disease(mean, every 3 d). Table I summarizes the characteristics of the patients included in the study. The mean number of samples per patient episode in different patient groups is shown in Table II. In general, episodes were defined according to periods of inpatient treatment.

Table I. Patient characteristics and distribution of treatment episodes.

Number of patients	165
Number of treatment episodes	205
Age (median years, range)	46 (17–81)
Sex (male/female)	123/82
Number of samples	
All	1522
Blood	1503
BAL	13
Other (ascites, CSF, sputum, pleural fluid)	6
Mean number of samples/patient episode	7·8
Treatment episodes	
Induction chemotherapy for acute leukaemia, CML-BC, MDS	89
Chemotherapy for malignant lymphoma	8
Autologous PBSCT	3
Allogeneic HSCT	104
Other	1 (SAA)
Total	205

BAL, bronchoalveolar lavage samples; CSF, cerebrospinal fluid; PCR, polymerase chain reaction; CML-BC, chronic myeloid leukaemia-blast crisis; MDS, myelodysplastic syndrome; PBSCT, peripheral blood stem cell transplantation; BMT, bone marrow transplantation; SAA, severe aplastic anaemia.

Postallogeneic PBSCT, BMT, all patient episodes >100 d after transplantation and other host factors according to Ascioglu *et al* (2002) if not proven invasive aspergillosis.

Serology

For serological testing a serum GM antigen enzyme-linked immunosorbent assay (ELISA) (Bio-Rad, Munich, Germany) was applied as recommended by the manufacturer. Positivity was defined as ≥2 serial samples with optical density (OD) value >1·5 times the cut-off index, as recommended by the manufacturer and with OD >0·7 times the cut-off index, as suggested by Herbrecht *et al* (2002) respectively. Overall, 1228 serum samples from 177 patient episodes were analysed by GM.

DNA preparation, nested-PCR assay and LightCycler™ PCR assay

DNA preparation, the nested-PCR and the LightCycler PCR assay were performed as described previously (Skladny *et al*, 1999; Spiess *et al*, 2003). Nested-PCR-negative samples were not analysed by LightCycler™ PCR since this technique was shown to be less sensitive than the nested-PCR assay (Spiess *et al*, 2003). The same DNA extracts were used for both the nested-PCR and the LightCycler PCR assay; DNA samples were stored at −80°C. With the exception of two samples, LightCycler™ PCR was performed in triplicate for all samples. The arithmetic mean was calculated in all samples with two or more positive results.

The definition of IA was based on the 2002 EORTC/MSG guidelines (Ascioglu *et al*, 2002). Decisions about whether

EORTC/MSG 2002	None	Proven	Probable	Possible	Total
Number of samples	628	67	46	781	1522
Number of patient episodes	104	8	3	90	205
Mean number of samples/patient episode (range)	6·4 (3–17)	8·4 (3–25)	17 (13–20)	8·98 (3–41)	7·77 (3–41)
Number of PCR-positive samples	50	8	5	56	119
Number of PCR-negative samples	578	59	41	725	1403

Table II. Distribution of samples and number of polymerase chain reaction (PCR)-positive and PCR-negative samples in the different patient groups considering the EORTC/MSG 2002 criteria.

patients had proven, probable or possible IA were made strictly independent from the PCR results. The PCR results were evaluated by correlating them with the clinical classification. Results of serological diagnostic techniques and postmortem histological examination were included for clinical classifications.

Statistical analysis

For the calculation of sensitivity of the PCR assay, only episodes with proven and probable IA were assumed to be true positive episodes. Episodes with possible IA were non-conclusive and were therefore not considered in the calculation of sensitivity and specificity of the PCR assay. For the calculation of specificity, episodes without evidence of IA were assumed to be true negative episodes. Furthermore, we calculated the probability of an episode being PCR-positive depending on the number of samples obtained per episode. Statistical analysis was performed with SPSS Advanced Statistics, version 10.0.6., Chicago, SPSS 1999 (software). *P*-values ≤0·05 were considered significant.

Results

Patients

Overall, 205 patient episodes from 165 patients were analysed (Table I). The mean number of samples analysed per patient episode was similar in all patient groups (Table II).

Correlation between the number of samples per episode and frequency of positive nested-PCR results

In order to assess the influence of the number of PCR samples tested per episode on the number of positive PCR results, patient episodes were divided into different groups according to the number of PCR samples per episode. The percentage of episodes with one and two or more positive PCR results respectively, was determined. With an increasing number of PCR samples tested per episode, the frequency of one or more PCR results being increased positively (Fig 1). If more than 25 samples per episode were tested, 100% of patient episodes had at least one positive PCR result; if more than 30 PCR tests were performed in an episode, 100% of patient episodes had two or more positive PCR results.

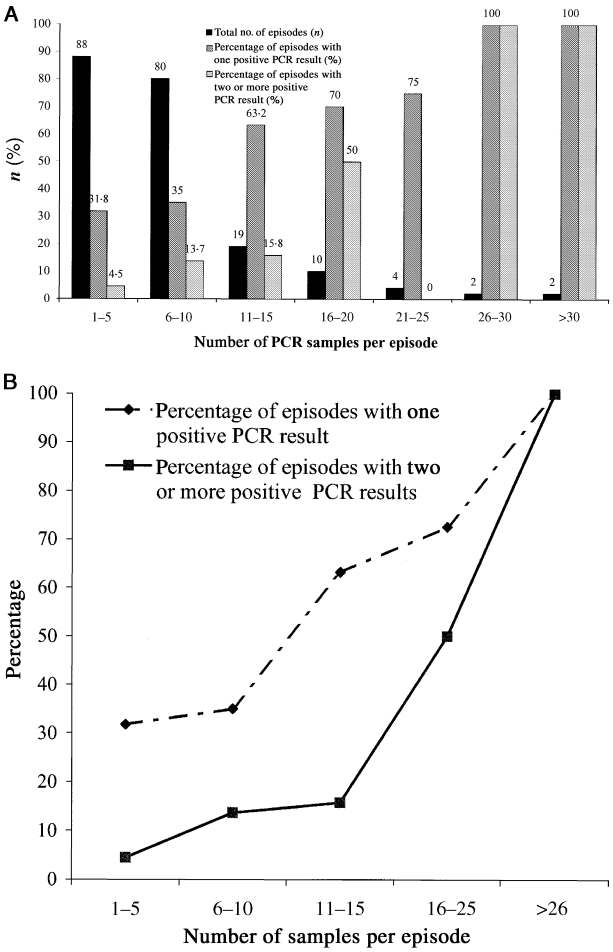

Fig 1. (A) Percentage of polymerase chain reaction (PCR)-positive episodes depending on the number of PCR results obtained per episode. (B) Correlation between the number of samples obtained per episode and the percentage of PCR-positive episodes.

Correlation of PCR results with clinical data

Figure 2 shows the distribution of patients according to the EORTC criteria and the PCR results in the different groups.

Applying the EORTC/MSG 2002 criteria, 104 (50·7%) patients had no evidence of IA, eight (3·9%) had proven IA, three (1·5%) had probable and 90 (43·9%) possible IA. Four of eight patients with proven IA were PCR-positive. All of the three patients with probable IA were PCR-positive. Considering the patients with proven and probable IA, the sensitivity

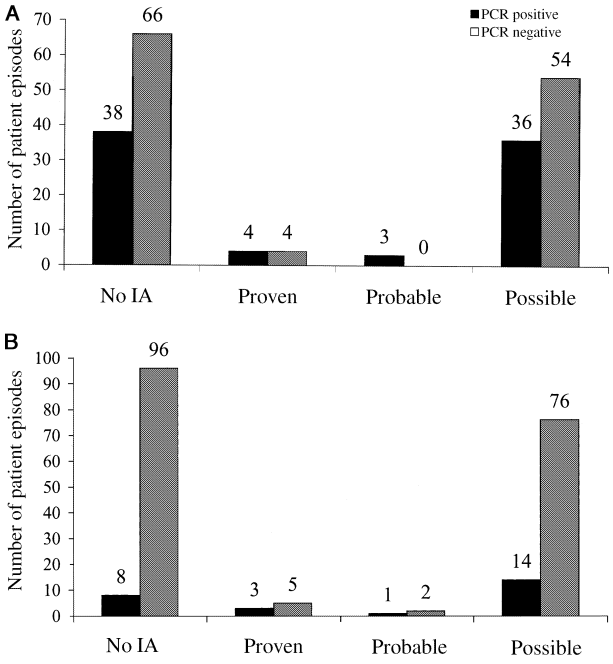

Fig 2. (A) Polymerase chain reaction (PCR)-positive (one or more positive PCRs) and PCR-negative episodes in patient groups, according to the probability of invasive aspergillosis (IA). (B) PCR-positive (two or more positive PCRs) and negative episodes in patient groups, according to the probability of IA.

rate was 63·6% (95% CI: 30·8–89·1%). Of 104 patients without evidence of IA, 38 had positive and 66 had negative PCR results (specificity rate 63·5%, 95% CI: 53·4–72·7%; Fig 2A).

Classifying only patients with ≥2 positive PCR results as 'PCR-positive', three of eight patients with proven IA and one of three patients with probable IA had two or more positive PCR results (sensitivity rate 36·4%, 95% CI: 10·9–69·2%). Eight of 104 patients without evidence of IA were PCR-positive and 96 were PCR-negative (specificity rate 92·3%, 95% CI: 85·4–96·6%; Fig 2B).

Aspergillus serology

In 177 patient episodes serological testing (GM) was performed along with the nested-PCR testing. Two of these patients were classified as having probable IA because of the serology result. Results are shown in Fig 3. For 1999 and 2002 criteria, the sensitivity and specificity rate were 33·3% (95% CI: 7·5–70·1%) and 98·9% (95% CI: 94·2–99·9%) respectively. If, according to Herbrecht *et al* (2002), a cut-off of 0·7 OD was applied, the results did not change. For calculation of the sensitivity rate patient episodes with proven and probable IA were considered.

Quantification of Aspergillus-DNA by LightCycler™ PCR

From 119 nested-PCR-positive samples, 117 were further analysed by LightCycler™ PCR assay; two samples did not

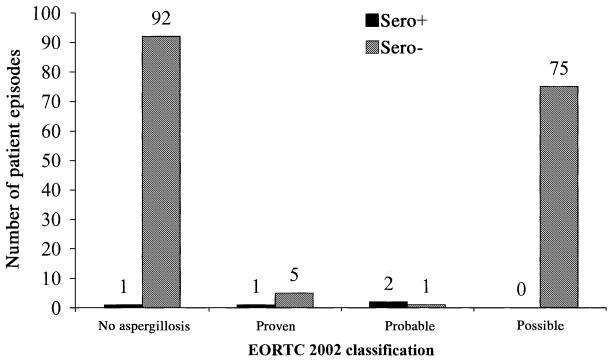

Fig 3. *Aspergillus* serology (galactomannan test) in patient groups, according to the probability of invasive aspergillosis (IA).

yield sufficient DNA for further analysis. From the 117 nested-PCR-positive samples analysed, 25 (21·4%) were positive by LightCycler™ PCR, 89 (76·1%) were negative by LightCycler™ PCR and in three samples analysis by LightCycler™ PCR was not conclusive. There were no LightCycler™ PCR-positive samples in the group of patients with proven IA, seven LightCycler™ PCR-positive samples occurred in the group without evidence of IA (range: 3·7–90 909 copies/ml), two in the group with probable IA (range: 5270–1 902 099 copies/ml) and 16 in the group with possible IA (range: 52–4 410 667 copies/ml). Since only 21·4% of nested-PCR-positive samples were positive by LightCycler™ PCR, a cut-off level to distinguish between infection and colonization could not be established.

Discussion

Underlining the diagnostic difficulties, the proportion of patient episodes with proven or probable IA in our study population was rather small (eight episodes with proven IA and three episodes with probable IA, according to EORTC/MSG 2002 criteria). IA could be proven in three patients only at autopsy, in one patient IA was definitely proven after partial lung resection 3 months after the acute episode and in two other patients IA was histologically proven by biopsy late in the course of the disease, 2 months after detection of the initial lung infiltrates by a computed tomography (CT) scan. The classification of fungal infection in a clinical setting without postmortem results according to the criteria proposed by the EORTC/MSG 2002 is therefore likely to lead to an underestimate of proven and probable IA (Ascioglu *et al*, 2002).

Recently, the use of various PCR tests has been reported for detection of IA, but the clinical use of these tests remains unclear. We have previously demonstrated a high correlation between positive histology, culture, or chest CT findings and nested-PCR results (Skladny *et al*, 1999). In a further study, our PCR assay had sensitivity and specificity rates of 100% and 92·6% respectively, when used to detect *Aspergillus* DNA in BAL samples, and the rates were 91·7% and 81·3% respectively, when used for the detection of *Aspergillus* DNA in blood

samples (Buchheidt *et al*, 2001). Compared with previous results, the sensitivity of our PCR assay in the present study is lower (63·5%). Similar results have been reported recently in various studies with different PCR assays (Williamson *et al*, 2000; Lass-Floerl *et al*, 2001). We are aware of the difficulties that arise from the calculation of sensitivity from a small number of episodes with proven and probable IA, as in this study. However, including the large group of patient episodes with possible IA does not lead to reliable results concerning sensitivity and specificity. The discrepancies concerning sensitivity and specificity encountered in the various studies evaluating PCR tests as a diagnostic tool for IA may be due to different definitions of proven and probable IA while different diagnostic and changing therapeutic strategies using novel antifungal agents also influence results of PCR testing (Caillot *et al*, 1997; Ruhnke & Maschmeyer, 2002). In our study, all the PCR results were negative in four of 11 episodes with proven and probable IA. In three of these episodes blood samples were obtained during antifungal treatment. The influence of antifungal treatment on PCR results has not been clarified up to now, but it has been assumed that antifungal treatment leads to clearance of fungi from blood without clearance of fungi from the lungs (van Burik *et al*, 1998; Lass-Floerl *et al*, 2001). Encapsulation of the process or a low level of angioinvasion must also be considered (Verweij *et al*, 2000). Therefore, PCR negativity may be due to blood sampling during the absence of DNAaemia despite the presence of IA, i.e. in between episodes of transient DNAaemia. Short episodes of transient DNAaemia have been reported (Lass-Floerl *et al*, 2001) and recent work (Girmenia *et al*, 2001) demonstrates that *Aspergillus* fungaemia is rare (Duthie & Denning, 1995). In an animal model of mice and rabbits with IA, *Aspergillus* PCR was positive in only 25% of blood samples (Loeffler *et al*, 2002). Moreover, there are reports that the half-life of circulating DNA is short, probably <5 min (Bretagne *et al*, 1998).

In our laboratory, a LightCycler™ PCR assay for quantification of *Aspergillus* DNA was established to distinguish between contamination, colonization and infection (Spiess *et al*, 2003). Quantification of *Aspergillus* DNA was attempted in all samples with a nested-PCR-positive result but was successful in only 21·4% of the samples analysed, lacking discrimination by quantification across the different clinical categories.

There are two explanations for these results: the level of *Aspergillus* DNA detectable in a sample by the nested-PCR assay may be lower than the detection threshold of the LightCycler™ PCR assay. Furthermore, other species than *A. fumigatus* are detectable by the nested-PCR assay (Skladny *et al*, 1999) but not by the *A. fumigatus*-specific LightCycler™ PCR assay performed here. Data published by Marr *et al* (2002) confirm an increasing incidence of non-*fumigatus* *Aspergillus* species in haematologic stem cell transplantation (HSCT) recipients.

Our study found a substantial number of episodes with positive PCR results (38 episodes with at least one positive PCR and eight episodes with two or more positive PCR results) among 104 patient episodes classified as not having IA. Recently, others have reported that a single PCR-positive result was never associated with fungal disease and resolved without antifungal treatment (Lass-Floerl *et al*, 2001). In another study, four of 19 patients with unlikely IA were PCR-positive (Williamson *et al*, 2000). Whether PCR positivity in patients without evidence of IA is due to contamination or due to transient DNAaemia remains unclear. Since *Aspergillus* species is a ubiquitously spread organism, contamination is a considerable risk in spite of appropriate precautions in handling samples. Another explanation for PCR positivity without stringent evidence of IA may be an earlier, CT scan-based application of a more effective antifungal treatment, which makes definite proof of IA more difficult.

In 177 episodes *Aspergillus* serology (GM) results were available. Sensitivity rates were substantially lower than those for *Aspergillus* PCR testing (33·3% vs. 63·6%), whereas specificity was high at 98·8%. Other authors report a comparable low sensitivity for serum samples (38% sensitivity rate) but a higher sensitivity rate of 90% for samples from the respiratory tract (Siemann & Koch-Dorfler, 2001). In a study by Pinel *et al* (2003), the specificity was high at 99·6%, but the sensitivity was only 50%. Herbrecht *et al* (2002) found a sensitivity rate of 64·5% for proven IA but only 16·4% for probable IA. In contrast, there are two reports of sensitivity rates between 89·7 and 92·6% and specificity rates between 95·4 and 98·1% (Maertens *et al*, 1999, 2001). One possible explanation for these high sensitivity rates is that a large number of samples per episode (between 17·7 and 36·5) in the episodes with probable and proven IA was tested (Maertens *et al*, 2001) whereas in the episodes without IA, fewer samples were tested (mean 10·5). In our study, the frequency of one or more positive samples increased with an increasing number of samples tested per episode (Fig 1). The higher sensitivity rates found by Maertens *et al* (2001) may therefore be due to the higher number of samples tested in the group of patients with proven and probable IA.

So far, *Aspergillus* PCR from blood samples alone cannot prove or rule out *Aspergillus* infection, but positive PCR results are suggestive in high-risk patients and, together with other diagnostic tools and the clinical findings, contribute to the diagnosis. However, an adequate interpretation of PCR results is important. From our results we conclude, in accordance with others (Williamson *et al*, 2000; Lass-Floerl *et al*, 2001) that one single positive blood PCR result is non-conclusive. Serial sampling appears to be necessary to maximize detection (Maertens *et al*, 2001). One or several negative blood PCR results do not definitively exclude IA. Concerning further clinical decision-making, clinical investigations including PCR assays to substantiate or refute the diagnosis should continue (Stevens *et al*, 2000), because of high specificity rates (92·3%) of the PCR assay if a patient has two or more positive PCR results.

More investigations under well-defined conditions are under way to further evaluate the clinical significance of PCR-mediated detection of *Aspergillus* DNA in different specimens (e.g. BAL samples) and to assess influencing factors, such as antifungal treatment or modalities of appropriate sampling.

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