

Assessment of the lightcycler PCR assay for diagnosis of invasive aspergillosis in paediatric patients with onco-haematological diseases

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Summary

A reliable diagnosis of invasive aspergillosis (IA) is hampered by the difficulty in obtaining suitable tissue samples. To evaluate the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the LightCycler PCR for the diagnosis of IA, 536 blood samples were collected over a 22-month period from 62 paediatric patients (median age 10 years, range 1–18) considered at risk of IA. The galactomannan antigen (GM) and fungal DNA were assessed on serial blood samples. IA was diagnosed in eight of 62 patients (13%): proven, five, probable, three. Sensitivity, specificity, PPV and NPV of LightCycler PCR varied according to the number of positive samples used to define positivity: 88%; 37%; 17% and 95% for single sample positivity; and 63%, 81%, 33% and 94% for serial sample positivity respectively. The concordance between positivity of LightCycler PCR assay and the diagnosis of IA was 79%. The single positivity of LightCycler PCR assay showed a good sensitivity for the diagnosis of IA in paediatric patients. The high NPV makes LightCycler PCR a promising tool in addition to GM testing to design a strategy of pre-emptive antifungal therapy, although further validation studies are needed.

Key words: Paediatric malignancy, invasive aspergillosis, LightCycler PCR, galactomannan antigen.

Introduction

Aspergillus is a saprophytic fungus whose natural ecological niche is the soil and whose conidia are inhaled into the lower respiratory tract.¹ *Aspergillus* spp. causes severe and often fatal disease in immunocompromised hosts.^{2,3} The incidence of invasive aspergillosis (IA) appears to be increasing, the annual figures being 7.3–10.5%.^{4,5}

Early and prompt diagnosis of IA still represents a challenge today because results from culture methods are delayed and biopsy is often hindered by the difficulty in performing invasive procedures in critically ill

patients; moreover, clinical and radiological signs do not allow a diagnosis of certainty.^{6–8}

Recently, the detection of the galactomannan antigen (GM test) in serum has raised much interest as a non-invasive tool for the diagnosis of IA, although the sensitivity and specificity of this test may vary considerably according to the patient population and the cut-off level used.^{8–10}

To date, IA has been less frequently investigated in paediatric patients than in adults, and interestingly, differences have been reported for paediatric IA concerning the radiological presentation (frequency of halo sign at lung CT scan) or GM test.¹¹ Herbrecht *et al.* found a higher rate of false positive GM results in children than in adults with an overall specificity of 94.8% for the GM test in adults compared with only 47.6% for the group of 42 children analysed.¹²

Lately, the determination of fungal DNA in the blood by qualitative and quantitative PCR has shown promising results as an alternative or complementary non-invasive method to the GM test for the diagnosis of IA.

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However, differences in PCR technique and divergent results have limited its application in the routine diagnostic procedures for AI.^{9,13–15}

LightCycler PCR is a rapid, standardised and highly reproducible technique of DNA amplification and analysis in an *in vitro* closed system that minimises the risk of carry-over contamination and allows both real-time fungal species identification and DNA load quantification.^{13,16–18}

The aim of this study was to evaluate the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of LightCycler PCR for the diagnosis of IA in paediatric patients undergoing intensive chemotherapy or haematopoietic stem cell transplantation (HSCT).

Design and methods

The study was conducted in the Paediatric Haematology-Oncology Department of the University of Padua from January 2004 to October 2005. Eligible patients were considered at risk of IA according to the following criteria: (a) persisting fever whilst severely neutropenic despite 72–96 h of broad-spectrum antibiotics; (b) occurrence of fever, with or without neutropenia, in patients who had undergone allogeneic HSCT and whilst on steroid treatment for acute or chronic graft vs. host disease; (c) any signs or symptoms suggestive of IA or other invasive fungal infection such as fever, cough or dyspnoea with pulmonary infiltrates, seizures and skin nodules in patients undergoing intensive or high-dose chemotherapy for acute leukaemia, non-Hodgkin lymphoma, relapsed solid tumour or HSCT. The follow-up data are as of 28 February 2006.

The study was approved by Ethics Committee of the University Hospital of Padua.

General management of patient at risk of IA

Severely neutropenic patients were nursed in reverse-barrier isolation rooms, whilst patients undergoing HSCT were nursed in high-efficiency particulate-filtered air (HEPA) cubicles. Published recommendations were used for the diagnosis and treatment of febrile episodes together with the use of chest computed tomographic (CT) scanning early in the course of the febrile episode for patients not responding to broad spectrum antibiotics.¹⁹

All study patients were assessed for serum GM by a double-sandwich enzyme-linked immunosorbent assay (GM ELISA, Platelia Aspergillus, Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France). The GM test

was considered positive if the index value was ≥ 0.5 in two consecutive tests or ≥ 0.8 in a single test respectively.²⁰

A standard approach for the empirical treatment of fever based on broad spectrum antibiotics and, for non-responding patients, liposomal amphotericin B was used.^{21,22} Fluconazole or itraconazole prophylaxis was administered to HSCT patients during the pre-engraftment and to neutropenic leukaemia patients.^{23,24}

Definitions

The diagnosis and definition of IA was made according to published EORTC/MSG criteria.²⁵ Severe neutropenia was defined as an absolute neutrophil count $< 0.5 \times 10^9 \text{ l}^{-1}$. The response to antifungal treatment was defined according to the criteria of Denning *et al.* [26].

Collection of clinical samples

Three milliliter of blood in an EDTA-containing vacutainer tube for LightCycler PCR and 3 ml of serum for GM testing were collected prospectively from all patients at entry into the study and then repeated within 3–5 working days. The patients who met the EORTC/MSG criteria for probable or proven IA at initial diagnostic work-up continued blood sampling for LightCycler PCR and GM testing once or twice a week until they tested negative or until resolution of IA or death.

DNA preparation from clinical samples

All manual steps of the extraction procedure were performed in a class 2 laminar flow cabinet.

Red blood cells lysis

One milliliter of whole blood was mixed with 10 ml of red blood cell lysis solution (10 mmol l^{-1} Tris pH 7.6; 5 mmol l^{-1} MgCl_2 ; 10 mmol l^{-1} NaCl) and incubated for 15 min at room temperature. Unlysed cells were centrifuged at 5000 *g* for 10 min. The pellet was again mixed with 10 ml of red blood cell lysis solution and again spin-dried 5000 *g* for 10 min. The pellet was finally re-suspended in 100 μl of sterile water.

DNA extraction and purification

The DNA was extracted and purified using the DNA-Blood kit (Qiagen, Hilden, Germany) on a PerkinElmer Packard MULTIPROBE II HT EX liquid handling system according to the manufacturer's instructions.

Quantitative real-time PCR

A quantitative PCR assay with the LightCycler (Roche Diagnostics, Mannheim, Germany) amplification and detection system was established.

Primer and hybridisation probes

Primers and probes used to bind an *Aspergillus*-specific conserved region of the 18S rRNA gene were designed based on Loeffler *et al.* [16].

Real-time PCR conditions

The PCR mixture contained 3 mmol l⁻¹ MgCl₂; 2 pmol of probes, 12.5 pmol of primers, 1× LightCycler Fast-Start DNA Master Hybridisation Probes. The final volume of the reaction was 20 µl. Reactions, performed using a LightCycler, were subjected to 45 cycles of amplification: denaturation (1 s at 95 °C), annealing (15 s at 62 °C), extension (25 s at 72 °C).

Standard curve

The 504 bp region of the *Aspergillus* DNA target of the PCR reaction was cloned into pCR2.1 (Invitrogen, San Diego, CA, USA). The resulting plasmid was amplified in *Escherichia coli*, purified using the Qiagen Plasmid Midi Kit (Qiagen), and finally quantified using a NanoDrop DN-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A standard curve was obtained subjecting seven known different amounts (50–5 × 10⁷ copies range) of the plasmid containing the DNA target region to duplicate PCR reactions. The standard curve was linear over the entire range of values.

Sensitivity

The sensitivity of the system was measured in reconstruction experiments where healthy donor blood samples spiked with known amounts of *Aspergillus fumigatus* conidia were subjected to DNA extraction and real-time PCR. The lowest amount of detectable fungal DNA per milliliter was that equivalent to 10 conidia.

Statistical analysis

Percentage, median and range were used as appropriate to describe continuous and categorical variables.

To calculate the performance of LightCycler PCR, we used in part the simulation previously reported by Maertens *et al.* [27] to analyse the GM test. Proven IA and probable IA were considered true-positive, whereas possible IA and at-risk patients were considered true-negative. Furthermore, the performance of LightCycler PCR was analysed according to the number of positive

results used to define the positivity of the test: one single and at least two positive consecutive results respectively.

Kaplan–Meier methods were used to calculate the 3-month-survival and overall survival (OS) probability (SAS Institute, Cary, NC, USA, version 8.2). The date of recruitment of patients into the study was used as the starting point for survival analysis.

Results

Over the 22-month study period, 62 patients met the inclusion criteria and their main demographic and clinical characteristics are shown in Table 1. During this period, 536 blood samples were collected from 62 patients for analysis with LightCycler PCR. The median number of samples per patient was six, range 2–47, whilst the mean number was 8.6.

A diagnosis of IA was made in eight of 62 patients (13%). There were five patients with proven IA and three with probable IA. Table 2 summarises the main demographic and clinical data of these patients. Four of

Table 1 The main demographic and clinical characteristics of patients recruited into the study are shown

Total number of patients	62
Gender M/F	37/25
Age	Median 11.1, range 1.3–18
Underlying disease	
Leukaemia	32
Acute lymphoblastic leukaemia	14
Acute myeloid leukaemia	12
Chronic myeloid leukaemia	6
Lymphoma	12
Non-Hodgkin lymphoma	11
Hodgkin lymphoma	1
Solid tumours	11
Neuroblastoma	5
Peripheral neuroectodermal tumour	2
Osteosarcoma	2
Rhabdomyosarcoma	1
Wilms tumour	1
Miscellaneous ¹	2
Non-malignant disease ²	5
Phase of therapy	
Primary induction chemotherapy	22
Reinduction chemotherapy after relapse	8
HSCT	31
Uncontrolled underlying disease	1
Severe neutropenia (neutrophils <0.5 × 10 ⁹ /l)	43

¹Miscellaneous: myelodysplastic syndrome (1), myxoid chondrosarcoma (1).

²Non-malignant diseases: sickle cell disease (1), aplastic anaemia (3), mucopolysaccharidosis (1).

Table 2 Characteristics of the patients diagnosed with invasive aspergillosis during the study period

Pts	Sex	Age (years)	Disease	Phase of therapy	Neutrophils at diagnosis of IA ($\times 10^6 \text{ l}^{-1}$)	Type of IA	Microbiological data	Antifungal therapy (duration in days)	Surgery for IA	3 month-outcome of IA	Status at last follow-up and cause of death
1	M	10.4	ALL	HSCT	<100	Proven	GM test: pos <i>Aspergillus</i> hyphae on lung after surgery	153	Right middle lobectomy and left apical wedge resection Left lobectomy	Alive, CR	Died of <i>Pseudomonas auruginosa</i> sepsis while on relapse of ALL, after 9 months Alive at 12 months
2	F	9	AML	Consolidation	<100	Probable	GM test: neg <i>Aspergillus fumigatus</i> on nasal swab	142	No	NA	Died of Gram-negative sepsis after 2 months
3	F	17	CML	HSCT	<100	Probable	GM test: neg <i>Aspergillus</i> hyphae on bronchoalveolar lavage	66	No	NA	Died of IA while severely aplastic after 3 weeks
4	M	15.9	NHL	Primary Induction CT	<100	Proven	GM test: pos <i>Aspergillus fumigatus</i> on lung and liver at autopsy	31	No	NA	Alive at 15 months
5	M	8	ALL	HSCT	<500	Proven	GM test: neg <i>Aspergillus fumigatus</i> on biopsy of skin lesion	173	No	NA	Died of IA after 2 weeks
6	M	5.2	AML	Reinduction CT after relapse	<100	Probable	GM test: pos	15	No	NA	Alive after 7 months
7	F	17.2	AML	Consolidation	<100	Proven	GM test: neg <i>Aspergillus</i> hyphae on lung after surgery	105	Left inferior lobectomy	Alive, CR	Alive after 14 months
8	F	10.9	AML	Consolidation	<100	Proven	GM test: pos <i>Aspergillus</i> hyphae on lung after surgery	282	Right inferior lobectomy	Alive, CR	NA, not applicable

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; NHL, non-Hodgkin lymphoma; HSCT, haematopoietic stem cell transplantation; CT, chemotherapy; GM test, galactomannan test; pos, positive; neg, negative; IA, invasive aspergillosis; CR, complete remission; NA, not applicable.

the eight patients (50%) were positive on GM testing (all had at least two positive samples). In this group of patients, the GM index value at diagnosis of IA was >5 (three patients), and 1.2 (one patient) respectively. The GM test became positive 3 days before the LightCycler test in three patients and on the same day as the LightCycler test in the fourth patient.

The remaining 54 patients did not ever meet the EORTC/MSG criteria for IA. One of them had a single positive result on GM testing, although he had not been concurrently treated with piperacillin/tazobactam, which has been shown as a cause for false positivity.²⁸

LightCycler PCR

Seven of eight (88%) patients with a diagnosis of proven or probable IA according to EORTC/MSG criteria had at least one positive sample on the LightCycler PCR assay, whilst five of them (63%) had at least two positive samples. The eighth patient resulted negative on LightCycler whilst serum GM test was positive. In this group, 31 blood samples tested positive to LightCycler, the median number of positive samples per patient being three, range 1–10.

Considering the patients with ≥ 2 positive samples, the concordance of LightCycler PCR and the diagnosis of proven and probable of IA was 79% (49/62).

Among 54 patients who did not meet the criteria for proven and probable IA 34 (63%) tested positive in at least one blood sample, whilst 10 (19%) of them had two or more positive samples. Overall, 46 blood samples tested positive to LightCycler, the median number of positive samples per patient being one, range 1–6.

Table 3 summarises the performance of the LightCycler PCR test according to the number of samples needed to define the positivity of LightCycler. The sensitivity was higher with one positive test, i.e. 88%; whilst the specificity being better with two consecutive positive results, i.e. 81%. In both instances, the NPV was high, i.e. 95% and 94% respectively.

Table 3 Sensitivity, specificity, positive (PPV) and negative (NPV) predictive value of LightCycler PCR

	Single LightCycler Positivity	≥ 2 LightCycler Positivity
Sensitivity	88% (7/8)	63% (5/8)
Specificity	37% (20/54)	81% (44/54)
PPV	17% (7/41)	33% (5/15)
NPV	95% (20/21)	94% (44/47)

Proven and probable cases are true positives; possible IA and at-risk cases are true negatives.

Fungal load by LightCycler PCR

Considering the first LightCycler positive test, the median amount of fungal DNA was equivalent to 6802 conidia per milliliter of blood, range 397–78 000 in the patients with IA vs. 15 840 conidia per milliliter of blood, range 1204–244 000 in the patients without IA, $P = 0.1$. A trend to higher amount of fungal DNA, although statistically not significant, was observed in a group of seven patients who were not receiving any antifungal drugs for prophylactic, empiric, or therapeutic purposes at the time of first positivity of LightCycler test: 59 800 vs. 15 720 DNA-equivalent conidia per milliliter of blood, $P = 0.3$.

Clinical outcome of patients with IA

The patients with proven or probable IA received antifungal therapy with liposomal amphotericin B, voriconazole, and caspofungin given as monotherapy or as combination therapy for a median of 124 days, range (15–282). Four of them underwent surgery as adjuvant therapy for IA. Four patients underwent lung surgery (lobectomy, three; lobectomy plus wedge resection, one). Before surgery, three patients were classified as probable IA and one patient as possible IA. Interestingly, all four patients were positive to LightCycler PCR in at least one sample.

After surgery, culture and histology resulted in an upgrade to proven IA in two of three patients with probable IA and in the patient with possible IA.

The 3-month survival for patients with IA was 63% (five of eight patients), confidence interval (CI) 29–96, vs. 94% for patients without IA (51 of 54 patients), CI 88–100, $P = 0.001$.

As of 28 February 2006, four of eight (50%) patients with IA and 37 of 54 (69%) of the patient without IA are alive. After a median follow-up of 343 days, range 16–726, the OS probability was 47%, CI 10–83 for the patients with IA and 62%, CI 47–77, for patients without IA, $P = 0.2$.

LightCycler PCR in patients without IA

According to the number of positive LightCycler PCR results one or more than one false-positive results were found in 63% and 19% of 54 patients respectively. Nineteen of them were HSCT patients. To exclude that the false-positive results could be because of the contamination of samples during manipulation, we analysed 30 negative control samples containing MilliQ

sterile water, which went through the complete lysis and DNA extraction procedure. None of them resulted positive.

Among 24 patients with one single positive result for LightCycler PCR, 14 (58%) were severely neutropenic at the time of the positive test, nine (38%) patients had radiological, although non-specific, signs of lung infection on chest X-ray or CT such as bronchial wall thickening, lung opacities or interstitial pneumonia or radiological evidence of sinusitis, and 10 (42%) were receiving prophylaxis or empiric treatment with itraconazole (three patients), liposomal amphotericin B (five patients) or caspofungin (two patients). Interestingly, one of these patients had been diagnosed with possible pulmonary aspergillosis 1 year previously on the basis of the appearance of multiple pulmonary infiltrates with halo sign at chest CT scan whilst the patient was severely neutropenic and persistently febrile.

In the 10 patients who had serial (two or more) false-positive LightCycler tests, seven (70%) of them were severely neutropenic at the time of the test, five (50%) had non-specific radiological lesions, and five (50%) were receiving empirical treatment with liposomal amphotericin B.

Discussion

The role of fungal DNA detection in blood by means of PCR as a non-invasive tool for diagnosing infection by *Aspergillus* is still a matter of investigation and currently not included in the EORTC/MSG criteria for defining IA.^{14,25} The introduction of real-time quantitative PCR technology based on fluorescent DNA hybridisation probes permits fast and automated processing of blood samples reducing both the risk of contamination during manipulation and the time to obtaining a result.^{9,15} Ideally, a highly sensitive assay such as PCR can detect very small quantities of *Aspergillus* DNA in the blood before the infection is clinically or radiologically evident, thereby making possible the early (pre-emptive) introduction of antifungal therapy.

Previous studies have reported a sensitivity of 55–100% and a specificity of 57–94% for the diagnosis of proven or probable IA by detection of *Aspergillus* DNA in blood by PCR.⁹ Comparison of sensitivity between PCR and GM testing has shown that the results are influenced by the type of sample. A higher sensitivity of the GM test compared with that of PCR was reported in four of five studies using serum or plasma. Conversely, four of the five studies using whole blood samples reported a better sensitivity for PCR than

GM, with large volume blood sampling (4–5 ml) and postamplification handling by PCR-ELISA methods being factors possibly involved in the superiority of PCR in this situation.¹⁵

Overall, the positivity of single-round LightCycler PCR resulted in a sensitivity of 88% and a specificity of 37%, whilst serial positivity of the assay was associated with a sensitivity of 63% and a specificity of 81% for the diagnosis of proven or probable IA. These results compare well with those reported in previous studies where LightCycler PCR had a sensitivity and a specificity of 63.6% and 63.5% for single positivity assay; and of 36.4% and 92.3% for serial positivity assay.^{17,29}

A recent retrospective study mostly in adult patients, showed a better performance of *Aspergillus* LightCycler PCR for the diagnosis of proven or probable IA, i.e. sensitivity, 92%; specificity 92–94%.¹³ The authors performed a nested PCR to enhance further the sensitivity of the LightCycler assay.

In our study, the sensitivity of LightCycler PCR for diagnosis of proven or probable IA was higher using a single positive result. We speculate that the performance of LightCycler PCR may be affected by broad use of antifungal drugs as prophylaxis or empirical therapy in patient at risk of IA. In favour of this hypothesis, there is the known negative effect of empirical antifungal therapy on performance of both PCR and GM assay.^{30–33} Accordingly, we found a higher fungal load in the group of seven patients who were not receiving any antifungal drugs at the time of first positivity of LightCycler test.

Interestingly enough, a high agreement (79%) between double LightCycler PCR positivity and diagnosis of proven or probable IA was observed. Only one patient with probable IA consistently tested negative by LightCycler PCR. Moreover, LightCycler positivity anticipated the up-grading to proven IA in the patient with possible IA before surgery.

The specificity and the PPV of LightCycler PCR were affected by false-positive results found in 34 of the 54 (63%) patients who did not ever meet the EORTC/MSG criteria for IA. The high homology of the probes used by Loeffler *et al.* [16] with fungal DNA of *Penicillium*, *Gymnoascus*, *Histoplasma*, *Paracoccidioides*, *Blastomyces* and other genera may have influenced the rate of false-positive results. Moreover, DNA fungal load was expressed as conidia equivalent as both viable and not viable conidia could be detected by our technique. This fact may, in part, explain the false-positive results in patients without IA as we excluded the sample contamination during the procedure. Respiratory tract colonisation by *Aspergillus*

is frequently observed in patients with neutropenia, mucosal barrier damage and severe immune deficit.³⁴ This may determine occasionally the passage of viable conidia to the blood. On the other hand, the broad use of antifungal drugs as prophylaxis or empirical therapy of invasive mycoses may result in the release of non-viable conidia in patients with *Aspergillus* infection at early stage.

Fourteen of 34 (41%) patients with false-positive result to LightCycler had radiological signs, albeit not specific, for IA of the lung or sinus infection and/or were receiving systemic antifungal therapy active against moulds. We were not able to ascertain whether these false-positive tests were an expression of subclinical aspergillosis in patients who had recovered successfully with empirical antifungal therapy, neutrophil reconstitution, and overcoming the critical phase of their disease. This finding concurs with the fact that IA may be clinically underdiagnosed.^{35,36}

The LightCycler PCR assay showed a high NPV for proven and probable IA both with single test positivity, 95%; and serial positivity, 94% (our data) to 99%.¹³ This finding renders the assay a very attractive tool to optimise the administration of antifungal therapy. Maertens *et al.* demonstrated that intensive monitoring of patients at risk of IA with serial GM, high-resolution chest CT and bronchoalveolar lavage enables identification of those patients who really need antifungal treatment (pre-emptive antifungal treatment).³⁷ In our study, four of eight patients with proven or probable IA resulted consistently negative for GM test, whilst only one of these eight patients was consistently negative to LightCycler PCR. The addition of LightCycler PCR to a surveillance program with GM may make IA very unlikely, if both tests are negative in an at risk patient and indicate that other causes of infection need to be considered and excluded. Whether this choice may have a positive impact on the duration of, and cost for antifungal therapy needs to be assessed in the future.

In conclusion, we found that LightCycler PCR assay has a good sensitivity (single positivity) and good specificity (serial positivity) for the diagnosis of proven and probable IA in paediatric onco-haematological patients; moreover, its high NPV makes it a suitable tool to help design strategies of pre-emptive antifungal therapy to replace empiric antifungal treatment.

Nevertheless, a standard and optimal protocol for all the phases of the assay and its validation on a larger number of patients are needed to include LightCycler PCR in future consensus criteria for the diagnosis of IA.

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