Original article

Molecular detection and identification of *Aspergillus* spp. from clinical samples using real-time PCR

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Summary

The definite and rapid diagnosis of invasive aspergillosis is necessary because of the high mortality caused. The objective of this study was to evaluate a real-time PCR assay to detect *Aspergillus* spp. in clinical samples, based on the Light Cycler technology. Specificity was assessed by using DNA extracted from pathogenic and non-pathogenic bacteria/fungi from Spanish Collection including: two *Aspergillus flavus*, four *Aspergillus fumigatus*, two *Aspergillus nidulans*, two *Aspergillus niger* and two *Aspergillus terreus* isolates. The analytical sensitivity was evaluated with different inocula (10^1–10^5 conidia ml^{-1}), and serially diluted DNA of *A. fumigatus.* To assess clinical applicability, samples from patients at risk were analysed. Species identification was determined by analysing the melting curves. Reactions using genomic DNA from other species of different genera than *Aspergillus* were negative (specificity: 100%). Analytical sensitivity was 60 fg using DNA and 5–20 conidia using conidial suspensions. The linear range was from 60 to 6×10^7 fg. The Tm ranged from 67.34 to 70.7 °C for the different *Aspergillus* spp. studied. Nine hundred and forty-eight consecutive blood samples from 127 patients were processed. In total, 10 (1%) of 948 samples from blood samples were PCR-positive. The real-time PCR assay provides a high sensitivity and specificity for detection of fungal DNA and rapidly identifies most of clinically relevant *Aspergillus* species.

Key words: Invasive fungal infection, *Aspergillus* spp., real-time PCR.

Introduction

Invasive aspergillosis (IA) is the main cause of mortality because of the infection in leukaemic patients and allogeneic stem cell transplant recipients.[1] The average death rate is approximately 50% in leukaemic patients and 90% in allogeneic bone marrow transplant recepients.[2] This poor prognosis is due in part to a failure to get an early diagnosis, which in turn results in delays in initiating antifungal therapy.

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Making a definitive diagnosis of fungal infections, especially in the inmunocompromised host, remains an everlasting problem. Signs and symptoms of fungal infections, if fever there exist, are usually non-specific. A histopathological diagnosis cannot be made in most cases in thrombocytopenic and critically ill patients. A culture may yield the aetiological agent, but it is slow, the sensitivity differs according to the site where the specimen is obtained and, finally, contamination sometimes cannot be ruled out.[3]

Recently, two developments relating to the diagnosis of invasive aspergillosis have occurred. First, the standardisation of criteria for determining the category of this disease according to the European Organization for Research and Treatment of Cancer/Mycosis (EORTC) and Mycoses Study Group of National Institute of Allergy and Infectious Diseases (NIAID) consensus

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definitions[4] has allowed comparison of results from different studies to be undertaken. The second development is the generation of PCR assays based on real-time technologies that are able to quantify *Aspergillus* DNA.

This report describes the development of an assay that uses a manual method for extraction of *Aspergillus* DNA, in combination with the real-time PCR LightCycler System. The whole assay takes approximately 3 h to perform. To assess its clinical applicability, a large number of blood samples from patients with suspected invasive fungal infection were analysed by the assay.

Materials and methods

Standard fungal strains used in the study were *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger* ATCC 1640, *Aspergillus nidulans* and *Aspergillus terreus*, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019. Other species of pathogenic and non-pathogenic fungal and bacterial were used: *Candida guilliermondii*, *Candida glabrata, Candida tropicalis*, *Fusarium* spp., *Acremonium* spp., *Penicillium* spp., *Rhodotorula* spp., *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Bacillus megaterium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Aeromonas* spp., *Yersinia enterocolitica* and *Enterococcus faecalis* (Table 1). Clinical isolates were obtained from patients attended at University Hospital of Valme and University Hospital of Rocio, from Seville and Hospital of SAS in Jerez, Cadiz, Spain.

Sample preparation

Before DNA extraction, *Aspergillus* cultures were grown on Sabouraud-dextrose agar for 72 h at 35 °C and *Candida* cultures were grown for 48 h at 30 °C. Fungal suspensions in saline were adjusted (0.5× MacFarland standard) to a concentration of 1×10^6–5×10^6 cells ml^{-1}. The same procedure was used for the bacterial but using specific culture medium 10-fold serial dilutions (10^6–10^1 cells) were prepared to test the sensitivity and specificity of the assay.

Table 1 Fungal and bacterial strains tested by real-time PCR

Fungal and bacterial used	
Candida guilliermondii	*Pseudomonas aeruginosa*
Candida glabrata	*Proteus mirabilis*
Candida tropicalis	*Bacillus megaterium*
Fusarium spp.	*Staphylococcus aureus*
Acremonium spp.	*Staphylococcus epidermidis*
Penicillium spp.	*Aeromonas* spp.
Trichophytum rubrum	*Yersinia enterocolitica*
Rhodotorula spp.	*Enterococcus faecalis*

For determination of the detection limit in blood, EDTA anti-coagulated whole-blood samples (5 ml) from healthy volunteers were spiked with serial dilutions of *Aspergillus* (1.5–10^6–10^1 cells ml^{-1}). DNA was extracted and analysed according to the protocol described below.

For specificity testing of the probes, cells from the following fungi and bacteria were tested: *C. guilliermondii*, *C. glabrata*, *C. tropicalis*, *Fusarium* spp., *Acremonium* spp., *Penicillium* spp., *Rhodotorula* spp., *P. aeruginosa*, *P. mirabilis*, *B. megaterium*, *S. aureus*, *S. epidermidis*, *Aeromonas* spp., *Y. enterocolitica* and *E. faecalis* (Table 1).

Preparation of total genomic DNA from clinical samples

Five millilitre of blood specimens were incubated initially with a hypotonic red cell lysis buffer (RCLB) as described previously.[5] Following lysis of the erythrocytes, the samples were centrifuged at 2500 *g* for 10 min. The pellets were transferred to l.5-ml Eppendorf tubes containing glass beads (1.180 μm; Sigma, Deissenhofen, Germany) and vortexed thoroughly for 3 min; 200 μl of the supernantant was used to extract DNA following instructions guide (QIAamp®DNA minikit; Qiagen, Hilden, Germany). Before extraction, the samples were treated with lyticase (180 U) and incubated for 30 min at 37 °C. DNA was extracted from bacterial, fungal and blood samples (total genomic DNA as described above) using the QIAmp DNA kit following manufacturer's instruction.

Primer and hybridisation probes for LightCycler-based amplification of *Aspergillus* DNA

The primers and hybridisation probes for *Aspergillus* spp. (Table 2) were as described by Loeffer *et al.* [6] and were synthesised by Tibmolbiol (Berlin, Germany). Each PCR included a negative control consisting of water without template DNA and a positive control containing fungal DNA to monitor possible contamination.

Table 2 Primers and probes used in DNA amplification

	Secuences (5′–3′)
Primers	
RNA 18S	5′-ATTGGAGGGCAAGTCTGGTG-3′
RNA 18S	5′-CCGATCCCTAGTCGGCATAG-3′
Probes	
Asp FL	5′-GTTCCCCCCACAGCCAGTGAAGGC-3′flu
Asp LC	5′-Red640-TGAGGTTCCCCAGAAGGAAAGGTGCAGC-3′

PCR amplification and detection

The LightCycler system (Roche, Mannheim, Germany) was used for amplification of *Aspergillus* DNA. LightCycler hot-start PCR was performed in glass capillaries with a LightCycler Fast Start DNA Master Hybridisation Probes kit (Roche) as specified by the manufacturer. The PCR master mix (15 μl) contained 1× Fast Start reaction mixture with Fast Start *Taq* DNA polymerase, reaction buffer, dNTPs, 1.6 μl of 25 mmol l^{-1} $MgCl_2$, 1.2 μl of each primer (3 μmol l^{-1}) and 2 μl (2 μmol l^{-1}) of each hybridisation probe.

PCR was performed in a final volume of 20 μl (10 μl of master mix + 10 μl of DNA extract) with 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C, 10 s at 58 °C and 20 s at 72 °C, with a temperature transition rate (TTR) of 20 °C s^{-1}. The PCR was followed by a melting temperature analysis cycle comprising 95 °C for 10 s (TTR of 20 °C s^{-1}), 50 °C for 60 s (TTR of 20 °C s^{-1}) and 75 °C for 0 s (TTR of 0.1 °C s^{-1}) to check the specificity of the PCR product.

Each PCR included a negative control consisting of water without template DNA to monitor possible contamination. Furthermore, DNA extracts from clinical samples were analysed in parallel with an extraction control and a PCR positive control containing fungal DNA.

Patients and clinical samples

Between 1 June 2004 and 31 July 2006, the real-time PCR assay was used to analyse 948 clinical samples from 127 patients at risk for fungal infection, or from patients for whom confirmation of fungal infection before, during and after treatment was required. Classification of opportunistic invasive fungal infections in immunocompromised patients with cancer or haematopoietic stem-cell transplants was according to the EORTC/NIAID criteria.[4]

All samples were screened for *Aspergillus* to the species level in the real-time PCR assay. The samples analysed comprised blood (3 ml of EDTA blood from children, 5 ml of EDTA blood from adults). The samples were either analysed directly on arrival at the laboratory or were kept refrigerated overnight or stored frozen at −80 °C until analysis.

Results

Sensitivity of the PCR assay

The *in-vitro* sensitivity obtained with serially diluted genomic DNA from all species of *Aspergillus* tested was 60 fg. The linear range of the assay was from 60 to 6×10^7 fg for *A. fumigatus* DNA. When conidia dilutions were used, the minimal amount of DNA detected was equivalent to 15 conidia per PCR reaction.

Table 3 Mean and SD of Ct (the PCR cycle number at which an increase in reporter fluorescence above the baseline signal can first be detected) obtained of serial dilution of conidial suspension

	10^4	10^5	10^6
Mean	28.4	27.1	24.3
SD	0.60	1.6	0.80
Coefficient of variation (%)	2.1	5.9	3.2

SD, standard deviation.

As a measure of interday reproducibility, the coefficient of variation of the Ct (the PCR cycle number at which an increase in reporter fluorescence above the baseline signal can first be detected) obtained of serial dilution of conidial by PCR real time assay was between 2.1 and 5.9% (Table 3).

Specificity of the identification

To test the specificity of the LigtCycler PCR assay, DNA from a range of fungal and bacterial pathogens were used. All PCRs with the other fungal and bacterial strains were negative indicating that specificity of the assay was a 100%.

Species were identified by melting curve analysis (Fig. 1). All strains were differentiated based on the Tm values (Table 4), with the single exception of *A. fumigatus* and *A. flavus*, which showed identical Tm.

Results with inoculated blood

When the study was performed with inoculated blood samples, the results showed a minimum detection limit of 20 ufc ml^{-1} per reaction. We only spiked the blood samples with *A. fumigatus* because it is the most frequent fungi involved in IA.

PCR results with clinical samples

In total, 948 samples of whole blood samples collected in EDTA vacutainers from 127 patients were investigated. The main characteristics of the patients are summarised in Table 5.

There were two cases of proven IA, three cases of probable IA and 17 cases of possible IA. The proven IA was based on histopathological examinations from needle aspiration of lung sample, that showing hyphae with evidence of associated tissue damage. Only patients

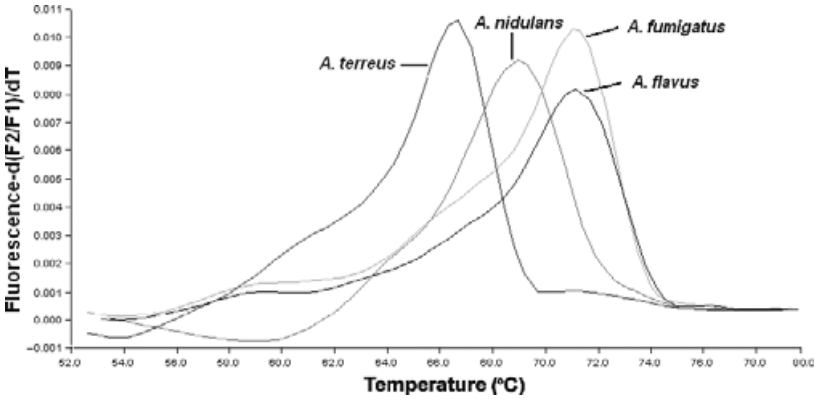

Figure 1 Melting curves for *Aspergillus* isolates.

Table 4 Melting temperatures for *Aspergillus* spp.

Aspergillus isolates	Temperature (°C)
A. fumigatus	71.18
A. terreus	66.48
A. niger	67.31
A. nidulans	68.9
A. flavus	71.03

Table 5 Data for the patients (n = 127) tested by PCR

Parameter	Value
Age, median (Q1–Q3)	45.4 (30.1–58.4)
Male sex (%)	64 (49.4)
Inclusion criteria (%)	
SCT*	70 (58.4)
Neutropenia	46 (36.4)
Prolonged steroid use	11 (5.2)
SCT	
Allogénic	52 (74)
Not allogénic	18 (26)
Enfermedad subyacente (%)	
Leukaemia	47 (41.5)
Lymphoma	29 (22.1)
Myeloma	22 (16.9)
Aplasia	10 (5.2)
Others	19 (14.3)

*SCT, stem cell transplantation.

with proven/possible IA, have at least one specimen test positive by *Aspergillus* PCR. Single positive PCR results among multiple samples were considered positive results. *Aspergillus* DNA was detected by PCR in 10 (1%) of 948 samples from blood samples. The blood samples collected per patient were: average 6.3, range: 4–9 samples. The 10 PCR positive samples were obtained consecutively. There were not a negative PCR result between the two positives. These samples belonging to two patients with proven IA (seven samples) and two patients with possible IA (three samples). There were no PCR positive results between patients with probable IA (Table 6). The 10 positive samples came from four immunocompromised patients, three allogeneic stem cell transplantation (SCT) patients with leukaemic disease and one patient with neutropenia and respiratory symptoms. There were only two bronchoalveolar lavage (BAL) samples belonging to the patient who has respiratory symptoms because of the difficulty to obtain invasive samples in these immunocompromised patients. PCR positivity rates were higher in the proven IA categories and correlated well with radiological markers – two of the four patients with PCR positive, proven/probable IA had CT scans of the chest suggestive of possible fungal infection. Furthermore, the other case with probable IA whose CT scan findings were positive, had negative PCR results.

Table 6 Distribution of PCR positive results and proven/probable/possible invasive aspergillosis

Invasive aspergillosis	Patients	PCR positive results	Comments
Proven	2	7	Patient 1 : 6 samples Patient 2 : 1 sample
Probable	3	0	–
Possible	17	3	Patient 3 : 2 samples Patient 4 : 1 sample

Discussion

Of the various molecular approaches used for the diagnosis of IA, real-time PCR looks the one capable of analysing blood, other body fluids and biopsy samples more efficiently. In these study, we analysed the sensitivity and specificity of LightCycler PCR *in vitro* assay and the possibility to apply this method to diagnosis of IA using clinical samples.[7–10]

To test the specificity of the LightCycler PCR assay, DNA from a range of fungal and bacterial pathogens were used. All PCRs with the other fungal and bacterial strains were negative (Table 1) indicating a specificity of 100% for hybridisation probes, similar to the results obtained by Skladny *et al.* [11].

The real-time PCR assay described in the present study shows a high sensitivity of detection 60 fg equivalent to 5–20 conidia per reaction and was achieved by optimising DNA extraction method. The DNA extraction method used for fungi was crucial, because DNA extracted from different body fluids contains not only human DNA, but possibly also fungal, bacterial, viral and parasite DNA. The total amount of DNA may vary greatly from sample to sample. Inhibition may occur when using the LightCycler probe system if the total amount of DNA is >500 ng per sample. Therefore, the DNA concentration should be measured routinely for all samples by spectrophotometry, with dilution of samples before PCR analysis when necessary. The analytical sensitivity of previous published assays varies by several orders of magnitude, most ranging between 1 fg and 10 pg of DNA. The great variation published is a consequence of the different methodologies used.[12] We obtained only 60 fg probably because of the clearance of extracellular DNA as a consequence of the DNA extraction procedure applied to the blood samples.

Aspergillus was detected by PCR in 10 (1%) of 948 samples from four patients with haematological malignancy. Nevertheless, as stated by Einsele *et al.* [13], the low number of positive PCR results could be explained because all patients were receiving antifungal therapy with antifungal agents because of the risk of IA. Little is known about the effects of antifungal therapy on the detection of DNA of *Aspergillus* spp. in blood samples. *Aspergillus* was detected only from total blood samples and BAL samples. PCR positivity results predated all other criteria of IA, including clinical suspicion of IA, abnormalities on CT scan and mycological/histological diagnosis of IA.

PCR was positive in the two BAL samples obtained from these patients. These kinds of samples are very difficult to obtain from these patients. It is well known that blood cultures are normally negative in patients with proven aspergillosis, and it was therefore not possible to compare the PCR-positive results for *Aspergillus* with blood cultures.

DNA may have been detected at an early stage of infection in some patients, i.e. before clinical signs of disease. 'Infection' and 'disease' might not be synonymous when DNA detection is used for screening. The pathogenesis of IA is poorly understood. Spores are inhaled and may form hyphae when deposited in the alveoli of the lungs. Fungal DNA (spores and/or hyphae) may be released into the bloodstream at this stage, with clinical signs, but also without real evidence of disease, which does not equate to an absence of disease. The spread of *Aspergillus* through the bloodstream to internal organs may take place long before the patient develops clinical symptoms.[14]

The goal of diagnostic research in invasive mycoses should be to detect the presence of fungus as early as possible. With a rapid and highly sensitive assay, such as a real-time PCR, it may be possible to identify infected patients at an early stage as we have demonstrated in this study. If so, real-time PCR may be useful also for excluding patients at risk; thus, the negative predictive value might be of great importance.

In conclusion, the real-time PCR assay described in this study provides high sensitivity and specificity for the *in vitro* detection of fungal DNA in blood samples within 6 h and identifies most *Aspergillus* spp. The ability to detect and distinguish between the various clinically relevant *Aspergillus* species is of great diagnostic value, as certain species vary in their resistance to antifungal therapy and are associated with increased virulence and higher mortality. This is mainly the case for *A. terreus* and *A. nidulans*, which are frequently resistant to amphotericin B.[15] Further clinical studies are needed to elucidate the true potential of such new techniques for different patient groups.

Because of the low incidence of proven IA in immunocompromised patients, the prospective clinical validation of this assay requires large numbers of patients at high risk for fungal infection. The clinical validation of the assay in a prospective multicentre study to define the predictive value of the assay and its experimental validation with a histologically defined animal model of aspergillosis are in progress. There is a need for prospective studies to evaluate the potential benefits of early therapy, based on real-time PCR, in patients at risk for invasive *Candida* and *Aspergillus* infection.

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