

Detection of *Aspergillus fumigatus* PCR products by a microtitre plate based DNA hybridisation assay

H A Fletcher, R C Barton, P E Verweij, E G V Evans

Abstract

Aims—To develop a DNA based plate hybridisation assay for the detection of polymerase chain reaction (PCR) products amplified from *Aspergillus fumigatus* DNA; and to determine the sensitivity of this technique and compare it with Southern blotting.

Methods—A half-log dilution series of DNA extracted from *A fumigatus* was amplified with specific primers, one of which was 5' end labelled with biotin. PCR products were subsequently detected by agarose gel electrophoresis, Southern blotting, and binding of the products to a streptavidin coated microtitre well, followed by non-radioactive colorimetric detection. Amplification was carried out 10 times for each DNA dilution and a plot of initial DNA concentration against signal intensity was made.

Results—A DNA concentration of 1.5 pg could be detected by agarose gel electrophoresis and Southern blotting with a non-radioactively labelled aspergillus specific probe; 1.5 pg was detectable by streptavidin binding of the PCR products to a microtitre plate. The signal from the microtitre plate detection was proportional to the amount of DNA in the PCR reaction on a log-log scale between 100 and 1 pg of DNA.

Conclusions—A DNA based plate hybridisation assay for the detection of *A fumigatus* PCR products is as sensitive as Southern blotting. However, results are obtained in three hours rather than the three days required for agarose gel electrophoresis, blotting, hybridisation, and detection.

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Keywords: aspergillosis; polymerase chain reaction; enzyme linked immunosorbent assay

Aspergillus fumigatus is a saprophytic fungus that can cause serious infections in patients, particularly those undergoing organ and bone marrow transplants and those with haematological malignancies. Invasive pulmonary aspergillosis is one of the commonest causes of death from infection in the immunocompromised host,¹ with a mortality rate of up to 90%. Although 16 aspergillus species are known to cause infection,² the major species involved in aspergillosis is *Aspergillus fumigatus*. *A fumigatus* is ubiquitous in the environment and can be isolated from numerous sources such as air

conditioning systems, soil, rotting compost, and fireproofing material.¹ The route of entry is thought to be by the inhalation of airborne conidia, which are relatively small in diameter and can penetrate deep into the lung.³

The main difficulty in the management of invasive pulmonary aspergillosis is the diagnosis of the infection at an early stage of the disease, essential for successful treatment and the avoidance of empirical therapy. Establishing a clinical diagnosis of fungal infection in the immunocompromised host is difficult as the clinical presentation of invasive pulmonary aspergillosis is often non-specific and a positive aspergillus culture is found in only one third of patients with invasive aspergillosis before death.⁴

Serological tests for antibodies are usually not indicated in immunocompromised patients, while antigen detection using the latex agglutination test for galactomannan is at most 70% sensitive and 86% specific.^{5,6} Enzyme linked immunosorbent assay (ELISA) tests for galactomannan are more sensitive but have a lower specificity.⁵ Attention is therefore now turning to molecular methods for the diagnosis of invasive aspergillosis by the detection of *Aspergillus* DNA.⁷

Polymerase chain reaction (PCR) assays targeting several regions of the *A fumigatus* genome have been developed in various laboratories.⁸⁻¹⁵ Many have shown that PCR is a more sensitive method than culture for detecting *A fumigatus* in bronchoalveolar lavage fluid or sputa,¹¹⁻¹⁵ and more recently blood.¹⁶ Detection of PCR product has mainly been by ethidium bromide stained agarose gel electrophoresis and Southern blotting. These methods of detection, although sensitive, are time consuming and the interpretation of results may be subjective. Microtitre plate based DNA hybridisation assays have been developed for the detection of chlamydia and candida DNA.^{17,18} The aim of our study was to develop a microtitre plate based assay for the rapid, quantitative detection of aspergillus PCR products.

Methods

CULTURE

Aspergillus fumigatus strain LM004, isolated from a patient with invasive aspergillosis, was grown at 37°C on a Sabouraud agar slope for two days. Spores were harvested by gently agitating with 1 ml 0.5% Tween 20, then 100 µl of spore suspension was added to 50 ml Sabouraud liquid medium and incubated overnight in a 37°C shaker at 120 rpm.

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ISOLATION OF DNA

Mycelial growth was obtained by filtration of the culture with a 0.45 µm membrane filter. Using liquid nitrogen and pestle and mortar, 0.3 g of filtered mycelia was ground to a powder. Powdered mycelia were resuspended in 500 µl lysis buffer (400 mM Tris-HCl, pH 8.0, 60 mM EDTA, 150 mM NaCl, and 1% lauroyl sulphate) and transferred to a 1.5 ml microfuge tube; 100 µl of 5 M sodium perchlorate were added and the suspension was incubated at 65°C for 20 minutes, after which 1 ml of chloroform (at -20°C) was added and the suspension was incubated at room temperature for 10 minutes with occasional shaking. After centrifugation for five minutes at 1.2×10^4 g the upper layer was transferred to a clean tube and 40 µl of silica suspension were added (Nucleon II; Scotlab, Strathclyde, UK). Following centrifugation for five minutes to pellet the silica, 500 µl of the supernatant was transferred to another clean tube and 1 ml of 100% ethanol (at -20°C) was added to precipitate the DNA. The precipitate was pelleted by centrifugation, washed with 70% ethanol (at -20°C), and resuspended overnight in 100 µl of 10 mM Tris-OH/1 mM EDTA, pH 7.5 (TE). Spectrophotometry (Gene Quant RNA/DNA Calculator, Pharmacia, Cambridge, UK) was used to determine the DNA concentration of each sample.

ISOLATION OF DNA FROM CLINICAL MATERIAL

DNA was isolated from the bronchoalveolar lavage fluid of HIV positive patients as described by Verweij *et al*⁹; 100 µl of resuspended pellet were added to a microcentrifuge tube two thirds filled with 0.5 mm zirconium beads. Cell destruction was achieved

by shaking at 6000 rpm for 160 seconds in a Mini Beadbeater (Biopspec Products, Bartlesville, Oklahoma, USA). Nucleic acids were purified with L6 buffer (10 M guanidinium thiocyanate, 0.1 M Tris-HCl, pH 6.4, 0.2 M EDTA, 2.6% (wt/vol) Triton X-100) and 40 µl silica particles (Sigma, St Louis, Missouri, USA) and then washed twice with L2 buffer (10 M guanidinium thiocyanate, 0.1 M Tris-HCl, pH 6.4). After washing twice with 70% (vol/vol) ethanol and once with 100% acetone, DNA was resuspended for one hour at 56°C in 100 µl of sterile distilled water.

POLYMERASE CHAIN REACTION

A region of the fungal 18S ribosomal DNA was amplified. Fifty-microlitre reaction mixtures were set up consisting of buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 1.0% Triton X-100; Promega, Southampton, UK); 1.0 mM MgCl₂ (Sigma-Aldrich Chemical Company, Poole, UK); 0.2 mM dNTP (Immugen International, Sunderland, UK), and 1 U of Taq DNA polymerase (HT Biotechnology, Cambridge, UK); 80 pmol each of primer,¹¹ Asp1 5'-CGGCCCTTAAATAGCCCGGTC-3', and biotin labelled Asp2 (5'-biotin) 5'-ACCCCCCTGAGCCAGTCCG-3' (Cruachem, Glasgow, UK), and 5 µl of template DNA were then added to the reaction mix. Amplification was performed in a programmable thermal cycler (Progene; Techne, Cambridge, UK) under the following conditions: five minutes at 94°C followed by 40 cycles of one minute at 94°C, one minute at 62°C, and one minute at 72°C. Negative controls of reaction mix without template DNA were included in each reaction.

ANALYSIS OF AMPLIFIED DNA

Aliquots of product (10 µl) were analysed by electrophoresis on a 1.5% agarose gel and visualised by ultraviolet light after staining with 0.5 µg/ml ethidium bromide. Gels were blotted overnight onto a nylon membrane which was hybridised with a digoxigenin-11-dUTP (DIG) labelled aspergillus specific oligonucleotide probe,¹¹ (Asp-p) 5'-ATGGAAGTGCGCGCAATAAC-3'. Hybridisation (0.6 M NaCl, 0.06 M sodium citrate, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulphate, 1% blocking reagent, 10 pmol/ml probe) for four hours at 46°C was followed by colorimetric detection.²⁰ Briefly, after washing the membrane in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5; 0.3% (vol/vol) Tween 20), it was blocked with 1% wt/vol blocking reagent. After binding of the anti-digoxigenin-AP antibody, colour substrate solution was added and the colour allowed to develop. Blocking reagent, antibody, and colour substrate solutions were supplied by Boehringer Mannheim UK (Diagnostics and Biochemicals, Lewes, East Sussex, UK).

MICROTITRE PLATE DETECTION OF PCR PRODUCTS

Amplified product was also detected in a streptavidin coated microtitre plate format similar to that described by Roymans *et al*.¹⁷

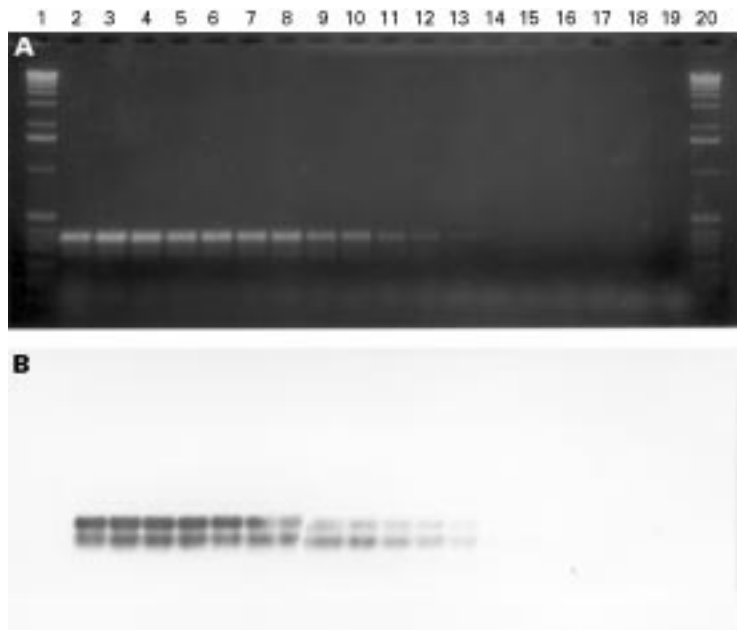


Figure 1 A half-log dilution series of *A. fumigatus* DNA was amplified with primers Asp 1 and Asp 2 and detected by agarose gel electrophoresis (A) and Southern blotting (B). Lanes 1 and 20, 1 kb molecular weight marker. Lane 2, 3.63 µg; lane 3, 1.15 µg; lane 4, 363 ng; lane 5, 115 ng; lane 6, 36.3 ng; lane 7, 11.5 ng; lane 8, 3.63 ng; lane 9, 1.15 ng; lane 10, 363 pg; lane 11, 115 pg; lane 12, 36.3 pg; lane 13, 11.5 pg; lane 14, 3.63 pg; lane 15, 1.15 pg; lane 16, 363 fg; lane 17, 115 fg; lanes 18 and 19, negative polymerase chain reaction controls.

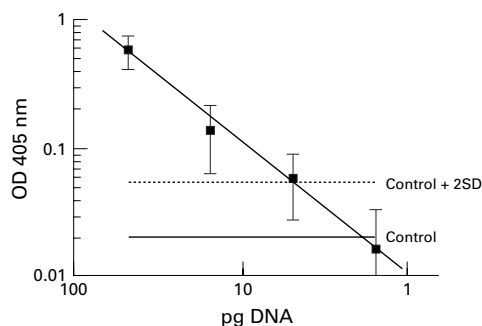


Figure 2 A half-log dilution series of *A fumigatus* DNA was amplified, captured by streptavidin binding to a microtitre well, and detected colorimetrically. Each dilution was amplified 10 \times and the log₁₀ mean OD₄₀₅ was plotted against the log₁₀ of the original DNA concentration. The mean of 30 negative controls plus 2 standard deviations was included as the cut off for a positive polymerase chain reaction result.

Briefly, 20 μ l of biotin labelled product was diluted 1/10 in 1 \times SSC–0.5% Tween 20; 200 μ l of diluted product were added to a 96 well streptavidin coated microtitre plate (Boehringer Mannheim UK) and incubated for 30 minutes at 37°C on a microtitre plate shaker at 1000 rpm (IKA Labortechnik, Staufen, Germany). Antibody and buffers for detection were supplied as part of the PCR-ELISA DIG detection kit (Boehringer Mannheim UK). The method for detection was adapted as follows: wells were washed (Well Wash 4; Denley Instruments, Billingshurst, Sussex, UK) four times with washing buffer and incubated for 10 minutes at room temperature with 200 μ l of 0.1 M NaOH. Wells were washed four times, then incubated with shaking for one hour at 37°C with 200 μ l of 10 pmol/ml DIG labelled Asp-p probe. Wells were washed four times to remove unbound probe and incubated with shaking for 30 minutes at 37°C with 200 μ l of 0.1 U/ml anti-digoxigenin peroxidase diluted in conjugate buffer. After washing four times the hybridisation signal was detected by incubating wells with shaking for 20 minutes at 37°C with 200 μ l mg/ml of 2,2 azino-di [3-ethylbenzthiazoline sulphonate] (ABTS). The absorbance of the wells was read at 405 nm (Titertek Multiskan MC; IQ (Bio), Cambridge, UK). The absorbance of ABTS alone was subtracted from the value of each test sample.

Table 1 Specificity of microtitre plate detection of *A fumigatus* DNA from bronchoalveolar lavage specimens

Patient	(Myc) bacteriology	Parasitology	Virology (culture)	Mycology	PCR	Blot	Microtitre plate detection
1	<i>Haemophilus influenzae</i>	-	-	<i>Penicillium</i> spp	+	-	-
2	<i>Mycobacterium</i> spp*	PCP	CMV, RSV	<i>Penicillium</i> spp	+	-	-
3	-	PCP	CMV	-	-	-	-
4	-	-	CMV	-	-	-	-
5	-	-	HSV1	-	-	-	-
6	-	-	-	<i>A fumigatus</i>	+	+	+
7	Hemolytic streptococci Lancefield group G, <i>Staphylococcus aureus</i>	PCP	CMV, HSV1	<i>C albicans</i>	-	-	-
8	<i>Mycobacterium tuberculosis</i>	-	-	-	-	-	-

*Atypical, non-specified mycobacterium.

PCP, *Pneumocystis carinii*; CMV, cytomegalovirus; RSV, respiratory syncytial virus; HSV1, herpes simplex virus type 1.

DETERMINATION OF DETECTION LIMITS

DNA of spectrophotometrically determined concentration was serially diluted beyond the limits of detection. PCR was performed as described above, and detection was performed by agarose gel electrophoresis, Southern blotting, and plate hybridisation methods. OD₄₀₅ values are expressed as the mean (SD).

Results

PRIMER SELECTION

Primers Asp1 and Asp2¹¹ were selected from a study of published reports on the basis of target suitability (rDNA of *Aspergillus* species) and reported sensitivity.

PCR DETECTION LIMITS

The limit of detection of aspergillus DNA by PCR using ethidium bromide stained agarose gel electrophoresis was 15 pg of DNA (fig 1A). Southern blotting and hybridisation with an *Aspergillus* specific probe increased the sensitivity of detection to 1.5 pg (fig 1B). The two bands seen on the ethidium bromide stained gel and Southern blot presumably represent a polymorphism in some copies of the rDNA repeat target. When extracted DNA was stored dissolved in TE at 4°C it was found that after seven to 14 days' storage the detection limit of the dilution series fell 10-fold. Storing DNA at 4°C as a precipitate in 100% ethanol and dissolving in TE shortly before amplification maintained the sensitivity of detection, a reproducible detection limit being obtained after four months of storage.

MICROTITRE PLATE OD₄₀₅ FOR PCR DETECTION

Using microtitre plate detection, the mean (SD) OD₄₀₅ of 30 PCR negative controls was 0.02 (0.017), and an OD₄₀₅ value of 0.056 (mean negative control plus 2 standard deviations) was taken as the cut off point for a positive result (fig 2). To optimise the microtitre plate detection, a half log dilution series of *A fumigatus* DNA was amplified and detected under various conditions. Hybridisation for one hour at 37°C with 10 pmol/ml probe was found to be optimal. For example, increasing hybridisation time to 90 minutes decreased the mean OD₄₀₅ of PCR product amplified from 15 pg DNA by 0.79. The speed and type of shaking strongly affected the results: it was found that reciprocal shaking at 1000 rpm gave the strongest signal. Increasing shaking speed to 1100 rpm produced more variable results, and tilting the plate at 50 rpm decreased the mean OD₄₀₅ of 15 pg amplified DNA by 1.09.

Under optimal conditions, using a serial dilution of *Aspergillus* DNA a mean of 5 pg could be detected (fig 2). The signal from the microtitre plate detection was proportional to the amount of DNA in the PCR reaction on a log-log scale between 100 and 1 pg of DNA. DNA amounts greater than 100 pg produced an OD₄₀₅ reading of more than 2.00 and were therefore off the linear part of the graph in fig 2.

DETECTION OF DNA FROM CLINICAL MATERIAL

Of the eight bronchoalveolar lavage samples extracted, three were positive and five negative

for aspergillus DNA by PCR. Microtitre plate hybridisation gave an OD₄₀₅ >2.00 for sample 6; all other samples were negative. Southern blotting was also positive for sample 6 and this was the only sample from which *A fumigatus* was cultured (table 1).

Discussion

Current tests used for the diagnosis of invasive aspergillosis lack sensitivity and specificity, and positive results are often obtained too late to influence patient management. The sensitivity of the PCR for the detection of aspergillus DNA in our assay is very high. Fifteen picograms of DNA can be detected by agarose gel electrophoresis, and Southern blotting using an aspergillus specific probe increases the sensitivity 10-fold. This sensitivity is comparable with that obtained by other groups researching the PCR detection of *A fumigatus* DNA.^{8 10-12 14 15} Microtitre plate detection of PCR products has the same sensitivity and specificity as Southern blotting.^{17 18} Our study confirms the sensitivity of microtitre plate detection of *A fumigatus* DNA. However, results are obtained in one day rather than the three days required for agarose gel electrophoresis, blotting, hybridisation, and detection. This format does not affect the specificity of the assay since no cross reactivity has been observed with DNA extracts from bronchoalveolar lavage. Patients culture positive for *Candida albicans*, *Penicillium sp*, *Mycobacterium tuberculosis* and other upper respiratory tract pathogens (table 1) were negative by microtitre plate detection. In addition, the microtitre plate format of the assay will allow the rapid incorporation of the test into diagnostic laboratories routinely performing ELISA antigen assays.¹⁸ A large scale prospective study comparing the microtitre plate detection with other diagnostic tests on specimens from patients at risk of invasive aspergillosis is required to determine the usefulness of this assay and is currently underway.

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