Journal of Medical Microbiology (2006), **55**, 1187–1191 **DOI** 10.1099/jmm.0.46510-0

Comparison of DNA extraction methods for *Aspergillus fumigatus* using real-time PCR

Lisa J. Griffiths,[1] Martin Anyim,[2] Sarah R. Doffman,[3] Mark Wilks,[1,4] Michael R. Millar[1,4] and Samir G. Agrawal[2]

Correspondence
Mark Wilks
m.wilks@qmul.ac.uk

[1]Department of Microbiology, Barts and The London NHS Trust, Royal London Hospital, London, UK

[2,4]Centre for Haematology[2] and Centre for Infectious Disease[4], Institute for Cell and Molecular Sciences, Barts and The London School of Medicine and Dentistry, London, UK

[3]Department of Respiratory Medicine, Barts and The London NHS Trust, St Bartholomew's Hospital, London

Newer methods such as PCR are being investigated in order to improve the diagnosis of invasive aspergillosis. One of the major obstacles to using PCR to diagnose aspergillosis is a reliable, simple method for extraction of the fungal DNA. The presence of a complex, sturdy cell wall that is resistant to lysis impairs extraction of the DNA by conventional methods employed for bacteria. Numerous fungal DNA extraction protocols have been described in the literature. However, these methods are time-consuming, require a high level of skill and may not be suitable for use as a routine diagnostic technique. Here, a number of extraction methods were compared: a freeze–thaw method, a freeze–boil method, enzyme extraction and a bead-beating method using Mini-BeadBeater-8. The quality and quantity of the DNA extracted was compared using real-time PCR. It was found that the use of a bead-beating method followed by extraction with AL buffer (Qiagen) was the most successful extraction technique, giving the greatest yield of DNA, and was also the least time-consuming method assessed.

Received 5 January 2006
Accepted 9 May 2006

INTRODUCTION

Aspergillus fumigatus is a ubiquitous saprophytic fungus with a worldwide distribution. It is usually non-pathogenic, rarely causing disease in immunocompetent humans (Latgé, 1999). However, over the last 15 years this picture has changed dramatically. An increase in the number of immunocompromised patients has changed the perception of the pathogenic nature of *A. fumigatus* from a relatively inert fungus to one of the most prevalent fungal pathogens, causing severe and often fatal invasive infections (Denning, 1998; Latgé, 1999).

Clinical signs and symptoms of invasive aspergillosis (IA) are non-specific. Those patients most at risk for fungal infections often show little or no evidence of any systemic infective processes (Tomee & van der Werf, 2001). Lung biopsy remains the 'gold standard' investigation in forming a firm diagnosis of IA, allowing demonstration of septate, branching hyphae in tissue. However, profound neutropenia and thrombocytopenia are often contraindications to biopsy in the at-risk population (Soubani & Chandrasekar, 2002). Culture of sputum and broncho-alveolar lavage yield positivity rates of only 30 and 30–50 %, respectively, in patients with confirmed IA (Denning, 1998; Soubani & Chandrasekar, 2002). The presence of fungi in a broncho-alveolar lavage sample is more highly predictive of infection than isolation from sputum. In patients with haematological malignancy the isolation of A*spergillus* spp. from sputum has a positive predictive value for infection of 80–90 % (Denning, 1998; Soubani & Chandrasekar, 2002) and should therefore always be assumed to be pathogenic in this patient group until proven otherwise. Blood cultures are usually negative, even when techniques such as lysis centrifugation are carried out (Denning, 2000; Mandell *et al.*, 2000; Collier *et al.*, 1998); therefore, microbiological cultures have a limited role in forming a diagnosis of IA.

High-resolution computed tomography scanning is a useful clinical investigation that supports the diagnosis of IA. However, the halo sign, which is highly suggestive of IA, only appears in 33–60 % of patients with IA (Singh & Paterson, 2005). In order to be clinically useful, a computed tomography scan must be performed within 1 week of the first symptoms, as 75 % of halo signs disappear within 1 week (Singh & Paterson, 2005). The air crescent sign, which correlates with neutrophil recovery (Soubani & Chandrasekar, 2002), is also highly suggestive of IA. However, this is a late sign of the infection (Singh & Paterson, 2005). These diagnostic limitations result in frequent

Abbreviations: C_t threshold cycle; IA, invasive aspergillosis.

empirical use of systemic antifungal agents, with significant costs and potential toxic side effects. The inability of traditional diagnostics to give an efficient and conclusive diagnosis of IA has led investigators to look at newer emerging molecular technologies. PCR is one such approach. Detecting the presence of fungal DNA in the blood may improve the diagnosis of aspergillosis.

One of the major hurdles of using PCR to diagnose aspergillosis is extraction of the DNA. The presence of a complex, sturdy cell wall that is resistant to lysis impairs extraction of the DNA by conventional methods employed for bacteria. Several fungal DNA extraction protocols have been described in the literature (Al-Samarrai & Schmid, 2000; Einsele *et al.*, 1997; Griffin *et al.*, 2002; Müller *et al.*, 1998; Velegraki *et al.*, 1999; Williamson *et al.*, 2000). However, these methods are time-consuming, require a high level of skill and may not be suitable for use as a routine diagnostic technique.

In order to extract DNA from fungal cells, it is necessary to disrupt the cell wall. This can be achieved in a number of ways. In previous studies, freeze–thawing of microbes that are resistant to standard cell lysis, using liquid nitrogen or dry ice, has been shown to be successful (Griffin *et al.*, 2002; Johnson *et al.*, 1995; Loeffler *et al.*, 2001). Homogenization has also been used to extract fungal DNA incorporating the use of glass-bead beating (Müller *et al.*, 1998; Smit *et al.*, 1999). Enzyme extraction is another method. Overnight incubation with lyticase breaks down components of the fungal cell wall, releasing fungal DNA (Williamson *et al.*, 2000). Whilst these protocols give good results, they are complex, time-consuming and require a high skill level. We have amended and simplified these previously published techniques (Griffin *et al.*, 2002; Müller *et al.*, 1998; Williamson *et al.*, 2000), omitting steps and using alternative buffers to make them more suitable for use in the routine laboratory. DNA yield and suitability for downstream amplification was compared by real-time PCR using the primers and probe from a previously published method (Challier *et al.*, 2004).

The aim of this study was to compare six different extraction methods using three different operators with different levels of technical expertise. The methods compared were: (i) a freeze–thawing method (Griffin *et al.*, 2002); (ii) a freeze–boiling method (Griffin *et al.*, 2002); (iii) an enzyme extraction method (Williamson *et al.*, 2000); (iv) a bead-beating method (Müller *et al.*, 1998); (v) a mixture of bead beating and enzyme extraction (Müller *et al.*, 1998; Williamson *et al.*, 2000); and (vi) a bead-beating method using a different lysis buffer (in-house method). Each lysis procedure was followed by DNA purification using the Qiagen DNA Mini kit, as recommended by Loffler *et al.* (1997).

METHODS

A pure culture of *A. fumigatus* obtained from a patient at the Royal London Hospital was grown on Saboraud's dextrose agar (Oxoid) with chloramphenicol for 3 days at 37 °C. A 1 cm^2 area of culture was removed from the agar, added to 0·9 % sterile saline (Oxoid) and vortexed. Tenfold serial dilutions were prepared and conidia were counted using a Kova Glasstic Slide 10 with grid (Hycor), giving starting concentrations of 3 $\log_{10}$ to 7 $\log_{10}$ conidia ml^{-1}. Ten microlitres of each suspension was added to 600 µl sorbitol buffer (1 M sorbitol, 100 mM EDTA, 14 mM β-mercapthoethanol), blood or 360 µl AL buffer (Qiagen) from which DNA was to be extracted.

DNA extracted from each dilution was compared using real-time PCR to ascertain which method gave the best-quality DNA suitable for PCR. PCR was carried out using primers and a method described previously (Challier *et al.*, 2004). DNA was confirmed as belonging to *A. fumigatus* by amplification using a set of 18S broad-spectrum primers (Innis *et al.*, 1990), followed by sequencing of the product.

Extraction methods

Each extraction method was carried out in triplicate by three different operators of differing technical experience, ranging from no previous DNA extraction experience to a highly skilled technician.

Each procedure was followed by the addition of 20 µl proteinase K at a concentration of 20 mg ml^{-1} (Qiagen) and incubation for 2 h at 55 °C. DNA purification using the QIAamp DNA Mini kit (Qiagen) was carried out following the tissue protocol with the amendment of using a 100 µl elution volume.

Method 1: freeze–thaw. Ten microlitres of each dilution of conidia was added to 600 µl sorbitol buffer. Samples were frozen to −70 °C for 10 min and then thawed at room temperature.

Method 2: freeze–boil. Ten microlitres of each dilution of conidia was added to 600 µl sorbitol buffer. Samples were frozen to −70 °C for 10 min and then boiled at 100 °C for 2 min.

Method 3: enzyme digestion. Ten microlitres of each dilution of conidia was added to 600 µl sorbitol buffer. Lyticase (200 U; Sigma) was added and samples were incubated at 37 °C for 30 min.

Method 4: bead beating. Ten microlitres of each dilution of conidia was added to 600 µl sorbitol buffer. Beads (0·5 g, 0·5 mm diameter; Biospec) were added and the mixture was shaken in a Mini-BeadBeater-8 (Biospec) for 3 min on the 'homogenize' setting.

Method 5: enzyme digestion and bead beating. Ten microlitres of each dilution of conidia was added to 600 µl sorbitol buffer. Lyticase (200 U; Sigma) was added and samples were incubated at 37 °C for 30 min. The mixture was centrifuged at 11 000 ***g*** for 10 min. The supernatant was discarded and the pellet was resuspended in 360 µl AL buffer and 20 µl proteinase K at a concentration of 20 mg ml^{-1} (both from Qiagen). Beads were added and shaken in a Mini-BeadBeater-8, as in Method 4.

Method 6: bead beating with a different lysis buffer. Ten microlitres of each dilution of conidia was added to 360 µl AL buffer and 20 µl proteinase K at a concentration of 20 mg ml^{-1} (both from Qiagen). Beads were then added and shaken in a Mini-BeadBeater-8, as in Method 4.

Automated extraction. In addition to the manual methods described above, two commercial automated extraction platforms, EZ1 (Qiagen) and MagNA Pure (Roche), were evaluated. Extractions were carried out following the manufacturer's instructions.

Extraction from whole blood. Whole blood was collected from a healthy volunteer in a 4 ml EDTA vacutainer (BD) containing

7·2 mg EDTA. *A. fumigatus* fungal saline suspensions were prepared as described for the previous extractions, giving starting concentrations of 3 $\log_{10}$ to 7 $\log_{10}$ conidia ml^{-1}. Ten microlitres of each suspension was added to 600 µl EDTA/whole blood. This mixture was centrifuged at 11 000 ***g*** for 10 min. The supernatant was discarded, the pellet was resuspended in 360 µl AL buffer (Qiagen) and the DNA was extracted using Method 6.

PCR protocol. The 25 µl PCR mixture contained 1 × Taqman universal PCR Master Mix (Applied Biosystems), 500 nM each primer, 100 nM probe and 7 µl DNA. DNA was amplified in an ABI Prism 7900HT sequence detector (Applied Biosystems) in optical 384-well plates. Cycling conditions were 95 °C for 10 min, followed by 40 amplification cycles of 15 s of denaturation at 95 °C and 1 min of hybridization and elongation at 60 °C. The primers and probe were from a previously published method (Challier *et al.*, 2004). The threshold cycle (C_t) value, which is inversely proportional to the log of the amount of target DNA initially present, was calculated using SDS software version 2.0 (Applied Biosystems). All samples were run in triplicate with negative and positive controls. The median value of the triplicate results was recorded.

RESULTS AND DISCUSSION

The threshold was set manually to be above all of the negative controls. Therefore, all samples that were above the threshold were deemed positive and the C_t value was recorded. All samples extracted by each individual operator were run in triplicate and the median value was recorded, giving three different figures per extraction. The median of these three values was then taken to give an overall figure for comparison (Fig. 1).

Method 1 (freeze–thaw) had a lower limit of detection from a starting amount of 10^3 conidia. There was also substantial variation among the three operators (data not shown) and the extraction process took around 4 h to complete. Method 2 (freeze–boil) failed to give any positive signal. This method also took 4 h to complete. Method 3 (enzyme extraction) had a lower limit of detection from a starting amount of 10^3 conidia and was extremely reproducible, showing little variation among operators (data not shown). This method took 3 h to complete. The detection limit of Method 4 (bead beating) was from a starting amount of 10^2 conidia and took 2·5 h to complete. Method 5 could detect DNA from a starting amount of 10^3 conidia and there was a great deal of variation among operators (data not shown). This method took 3·5 h to complete. Method 6 was the most sensitive technique with a limit of detection from a starting amount of 10 conidia. There was little variability among operators (Fig. 2) and the whole process was completed within 2·5 h.

Method 6 was carried out by the most experienced operator using spiked whole blood. Detectable DNA could still be extracted from a starting amount of 10 conidia (Fig. 3).

The automated methods that were tested gave a lower yield of DNA over a range of concentrations than the manual Method 6, with the C_t values obtained consistently being three to four cycles higher than the equivalent sample that was extracted manually (data not shown). DNA extraction using the MagNA Pure system took 3 h to complete, which was equivalent to the time taken for the manual extraction. The EZ1 system took approximately 30 min from start to finish. The reduction in extracted DNA yield negated any advantages gained by automation. Additional pretreatment steps required that might improve the DNA yield from the robots would increase the time and complexity of the extraction procedure, thus reducing any advantages gained from automation.

Traditionally, purification of DNA has involved extraction methods that use toxic chemicals such as phenol/

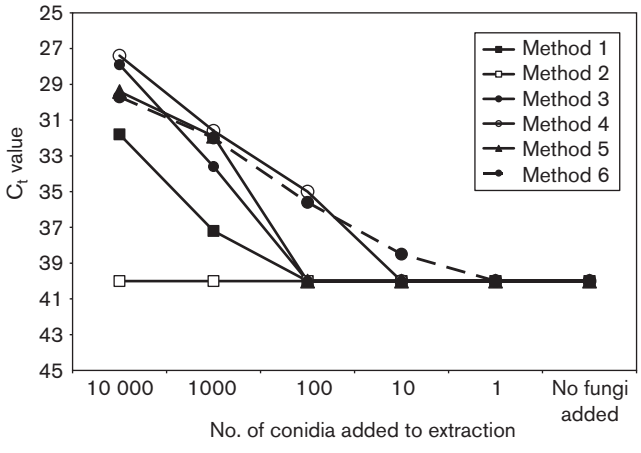

Fig. 1. Relationship between the number of *A. fumigatus* conidia and the median C_t value obtained after DNA extraction by six different methods from saline suspensions of *A. fumigatus* added to various extraction buffers (see text for details). Each line represents the median value of the results obtained by three different operators.

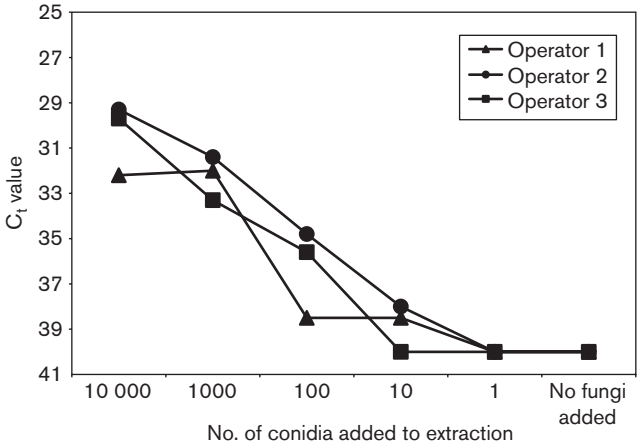

Fig. 2. Levels of DNA detected (C_t value) after extraction by different operators carrying out Method 6.

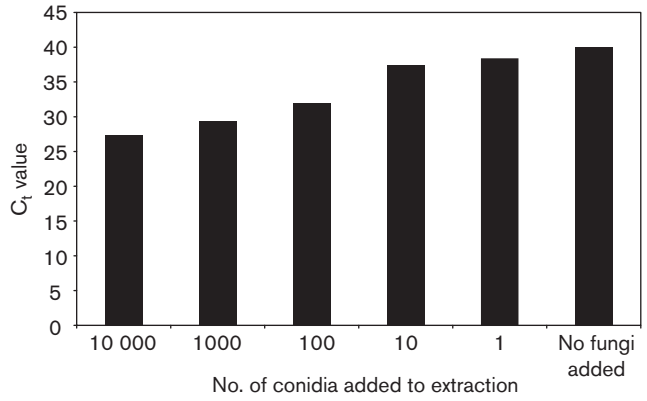

Fig. 3. Levels of DNA detected (C_t value) after extraction from whole blood using Method 6.

chloroform and are technically difficult to execute. In recent years, commercial kits have been used more frequently and have been shown to be less technically demanding and to be safer and quicker without loss of sensitivity (Loffler *et al.*, 1997). Loffler *et al.* (1997) compared a number of commercial kits and the Qiagen DNA Mini kit was reported to be the best available for the extraction and purification of DNA from fungi. Extra steps are still required initially to lyse the cell prior to purification, as fungal cell walls are extremely strong and difficult to lyse by traditional extraction techniques.

These difficulties in lysing the cell wall have led to time-consuming, expensive and complex lysis methods. These include overnight incubations with enzymes (Williamson *et al.*, 2000) and the use of liquid nitrogen with many intermediate steps (Einsele *et al.*, 1997). These methods are not practical in the context of a routine diagnostic laboratory where high-throughput, reproducible results are required. The high level of technical expertise required and the time-consuming nature greatly increase the labour costs associated with extraction. The use of enzymes and consumables such as liquid nitrogen also significantly increase the cost of the method. The increased number of steps associated with these methods increases the chance of contamination occurring. Here, we have described a comparison of six methods that are suitable for use in a routine laboratory.

In this comparison of various cell lysis techniques, the bead-beating methods (Methods 4 and 6) were shown to give the greatest yield of DNA, and were the most reproducible and the quickest and simplest to perform. This DNA extraction procedure took 3 h to perform, in contrast with the freeze–thaw method, which took over 4 h to complete. The bead-beating method showed the least variability among the three operators, indicating not only that it is highly reproducible but also that it is not reliant on technical expertise, as there was little difference between the results obtained from the operator with little or no experience and those from the most experienced operator. By using a detergent buffer (Method 6) instead of sorbitol buffer (Method 4), the DNA yield was further improved. The freeze–boil method proved to be the least reliable technique, resulting in little, if any, DNA.

The Mini-BeadBeater-8 is a cell disrupter that violently agitates up to eight standard microcentrifuge tubes at a time. It is aerosol-free, thus reducing the opportunity for cross-contamination. A high-throughput version of the Mini-BeadBeater-8 is available that can homogenize up to 192 samples. The capital cost of these machines may be an issue, as the Mini-BeadBeater-8 costs approximately £880 and the Mini-BeadBeater-96 costs approximately £2600. Most of the ongoing costs of extracting the DNA are due to operator time. Consumable costs for this method are low compared with alternative methods that utilize enzymes or liquid nitrogen. The high capital cost of the equipment would quickly be outweighed by savings in labour and consumables due to the simple and rapid method described here.

The other potential obstruction to the use of this machine is the noise that it generates, requiring it to be placed in a separate room or ear defenders to be provided when it is in use. However, once these problems are overcome, this machine disrupts fungal cells quickly and efficiently.

Extraction Method 6, when applied to the extraction of fungal DNA from spiked whole blood, had a limit of detection of 10 conidia extracted from blood. Einsele *et al.* (1997) reported that the extraction method they utilized was able to detect 10 conidia ml^{-1}, which is in line with our own findings. However, our extraction method is far simpler. This is a good limit of detection. However, clinically IA may present with fungal loads of <1 conidia ml^{-1}. In this study, we used a volume of 600 µl, which may result in infections being missed due to the use of a small volume. The method we describe here could be used on larger volumes of blood. This could be achieved using larger capacity spin columns such as the DNA Midi kit (Qiagen), which can accept blood volumes of up to 2 ml, or the DNA Maxi kit (Qiagen), which has a capacity of 4 ml blood.

In conclusion, we have reported here a rapid, reproducible and simple method for the extraction of DNA from fungi. This is a method that can be used in the routine laboratory.

REFERENCES

Al-Samarrai, T. H. & Schmid, J. (2000). A simple method for extraction of fungal genomic DNA. *Lett Appl Microbiol* **30**, 53–56.

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

Collier, L., Sussman, M., Balows, A., Wilson, G. S. & Topley, W. W. C. (1998). *Topley & Wilson's Microbiology and Microbial Infections*, 9th edn. London: Arnold.

Denning, D. W. (1998). Invasive aspergillosis. *Clin Infect Dis* **26**, 781–803.

Denning, D. W. (2000). Early diagnosis of invasive aspergillosis. *Lancet* **355**, 423–424.

Einsele, H., Hebart, H., Roller, G. & 8 other authors (1997). Detection and identification of fungal pathogens in blood by using molecular probes. *J Clin Microbiol* **35**, 1353–1360.

Griffin, D. W., Kellogg, C. A., Peak, K. K. & Shinn, E. A. (2002). A rapid and efficient assay for extracting DNA from fungi. *Lett Appl Microbiol* **34**, 210–214.

Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (editors) (1990). *PCR Protocols: a Guide to Methods and Applications*. San Diego: Academic Press.

Johnson, D. W., Pieniazek, N. J., Griffin, D. W., Misener, L. & Rose, J. B. (1995). Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. *Appl Environ Microbiol* **61**, 3849–3855.

Latgé, J.-P. (1999). *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* **12**, 310–350.

Loeffler, J., Hebart, H., Cox, P., Flues, N., Schumacher, U. & Einsele, H. (2001). Nucleic acid sequence-based amplification of *Aspergillus* RNA in blood samples. *J Clin Microbiol* **39**, 1626–1629.

Loffler, J., Hebart, H., Schumacher, U., Reitze, H. & Einsele, H. (1997). Comparison of different methods for extraction of DNA of fungal pathogens from cultures and blood. *J Clin Microbiol* **35**, 3311–3312.

Mandell, G. L., Bennett, J. E. & Dolin, R. (editors) (2000). *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases*, 5th edn. Philadelphia & London: Churchill Livingstone.

Müller, F.-M. C., Werner, K. E., Kasai, M., Francesconi, A., Chanock, S. J. & Walsh, T. J. (1998). Rapid extraction of genomic DNA from medically important yeasts and filamentous fungi by high-speed cell disruption. *J Clin Microbiol* **36**, 1625–1629.

Singh, N. & Paterson, D. L. (2005). *Aspergillus* infections in transplant recipients. *Clin Microbiol Rev* **18**, 44–69.

Smit, E., Leeflang, P., Glandorf, B., van Elsas, J. D. & Wernars, K. (1999). Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Appl Environ Microbiol* **65**, 2614–2621.

Soubani, A. O. & Chandrasekar, P. H. (2002). The clinical spectrum of pulmonary aspergillosis. *Chest* **121**, 1988–1999.

Tomee, J. F. C. & van der Werf, T. S. (2001). Pulmonary aspergillosis. *Neth J Med* **59**, 244–258.

Velegraki, A., Kambouris, M., Kostourou, A., Chalevelakis, G. & Legakis, N. J. (1999). Rapid extraction of fungal DNA from clinical samples for PCR amplification. *Med Mycol* **37**, 69–73.

Williamson, E. C., 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. *Br J Haematol* **108**, 132–139.