Don't throw your blood clots away: use of blood clot may improve sensitivity of PCR diagnosis in invasive aspergillosis

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ABSTRACT

Background: The diagnosis of invasive aspergillosis (IA) remains challenging and frequently is not made until after death. Histopathological examination remains central to confirmation of diagnosis but often requires invasive procedures to obtain tissue for the examination. Detection of aspergillus DNA by quantitative PCR (qPCR) offers the potential for earlier diagnosis due to higher sensitivity, but PCR in clinical use is poorly reproducible, with different centres reporting variable results and often using different extraction and analytical methods.

Aims: To optimise the performance of aspergillus PCR as a diagnostic modality.

Methods: A rat inhalation model of invasive aspergillosis was used to optimise the methodology of diagnostic aspergillus PCR. Infected animals were terminally bled at 4 days post-infection; samples of EDTA blood, serum and the residual clot were pooled for subsequent analysis. DNA was extracted from each fraction using a variety of methods and an optimised qPCR reaction using an *Aspergillus fumigatus* primer set performed.

Results: Significantly more aspergillus DNA was detected from the clot than EDTA and serum samples. Enzymatic and mechanical pretreatment reduced the yield of fungal DNA. There was some evidence that the average Ct values were greater for the EZ1 BioRobot than the MagNA Pure automated extractor, but this did not reach statistical significance at the 5% level ($p = 0.078$).

Conclusions: Automated extraction from the clot present in a blood sample will increase DNA yield and improve the diagnostic sensitivity of the test.

Invasive aspergillosis (IA) is a common infection in patients with haematological malignancies and those undergoing hematopoietic cell transplantation. Despite the availability of several active antifungal agents, IA continues to have a high mortality rate,[1] and the diagnosis of IA remains problematic,[2] with many cases not diagnosed before death.[3] Symptoms such as fever, cough, or chest pain are non-specific and many patients may be asymptomatic. Some radiological findings, such as the presence of a halo sign or cavitating nodules in the lungs may be strongly suggestive of aspergillosis, but these can also be found with other infections and are again not entirely specific.[4] The failure to make an accurate diagnosis often results in either a delay in the use of optimal antifungal therapy or the over-use of empirical antifungal therapy to treat non-resolving pyrexia in the neutropenic patient, with associated cost and potential toxicity.

Molecular diagnostic techniques using nucleic acid detection by PCR have been utilised to aid in the diagnosis of IA for more than 15 years, and are potentially more sensitive and rapid alternatives to conventional techniques for the diagnosis of IA.[5] However, little is known about the optimal sample required and processing necessary to maximise the recovery of aspergillus DNA; a wide variety of studies using different methodologies and demonstrating widely varying sensitivity and specificity have been published (table 1). Two problems may occur with this diagnostic test which could be related to the extraction method. Firstly, false negatives, which could be due to low recovery of any DNA present, the presence of PCR inhibitors or the presence of large quantities of human genomic DNA competing with the fungal target DNA. Secondly, false positives due to introduction of contamination during sample collection and DNA extraction resulting from the presence of fungi in the laboratory consumables; this is more likely to occur if complex DNA extraction steps are used. Our study utilises an animal model of aspergillus infection and examines for the first time the effect of different sample types and extraction methods to optimise the yield of aspergillus DNA in an in vivo experimental system.

METHOD

An established rat inhalation model of aspergillosis was used; each animal was weighed and immunosuppressed with 75 mg/kg cyclophosphamide and 16 mg/kg Depo-Medrone. They were then infected 2 days after immunosuppression by inhalation (1×10^9 spores/ml for 1 hour) and re-immunosuppressed with 75 mg/kg cyclophosphamide. The animals were also given prophylactic antibiotics (Baytril 10 mg/kg) when immunosuppression was commenced; a daily dose was given thereafter.

The animals were sacrificed 4 days after infection and terminal blood samples collected. Samples of EDTA blood, serum and the residual clot were pooled for subsequent analysis. DNA was extracted from each fraction using two automated extraction machines, EZ1 BioRobot (QIAGEN, Crawley, UK) and MagNA Pure (Roche, Burgess Hill, UK), with and without the pretreatment steps described below.

Prior to analysis the pooled clot was aliquoted into 400 µl volumes by gently homogenising the sample with a sterile scalpel and transferring into a graduated 1.5 ml micro-centrifuge tube.

Table 1 PCR clinical studies in stem cell transplantation and haematological malignancy

Study (first author, date)	Patients	Prov/prob IFI (%)	Assay	Sens.	Spec.	NPV	PPV
Williamson, 2000[6]	37	13 (35)	Nested PCR, Asp-specific	81	100	–	100
Kami, 2001[7]	122	33 (27)	Real-time, Asp-specific	79	92	92	79
Hebart, 2000[8]	92	14 (15)	PCR blot, Pan-Fungal/Asp specific	100	73	100	36.8
Hebart, 2000[9]	84	7 (8)	Real-time, pan-fungal	100	65	100	44
Lass-Flörl, 2001[10]	121	3 (2)	Nested PCR, Asp-specific	75	96	98	42.71
Jordanides, 2005[11]	78	8 (10)	Real-time, pan-fungal	80	69	98	15
Halliday, 2006[12]	95	13 (14)	Nested PCR, Asp-specific	100	75.4	100	46.5
Florent, 2006[13]	167	33 (20)	PCR-ELISA, Asp-specific	63.6	89.7	89.7	63.6
Bucheidt, 2002[14]	141	21 (15)	Nested PCR, Asp-specific (BAL samples used)	93.9	94.4	83.8	98.1
White, 2006[15]	203	14 (7)	Nested and Real-time PCR, Asp-specific	92.3	94.6	99.3	60

Prov/prob IFI, Proven/probable invasive fungal infection; Sens., sensitivity; Spec., specificity; NPV, negative predictive value; PPV, positive predictive value.

Enzymatic pretreatment of samples: 400 µl of sample was mixed with 1 ml of red cell lysis buffer (RCLB, 10 mM Tris, pH 7.6, 5 mM $MgCl_2$, 10 mM NaCl), centrifuged at 1500 *g* for 10 minutes and the supernatant discarded. Red cell lysis was repeated and the remaining pellet was resuspended in 1 ml of white cell lysis buffer (WCLB, 10 mM Tris, pH 7.6, 50 mM NaCl, 0.2% sodium dodecylsulphate, 10 mM EDTA); 10 µl of proteinase K (>600 mAU/ml) was added and then incubated at 56°C for 30 minutes. Following centrifugation at 8000 *g* for 10 minutes the supernatant was discarded; 200 µl or 400 µl of lysis buffer (from the automated extraction kits) was added, and the sample loaded into the machine for automated extraction.

Mechanical pretreatment of samples: 400 µl of sample was centrifuged at 8000 *g* for 10 minutes. The supernatant was discarded and ~50 µl of glass beads added. The sample was bead-beaten by vortexing for 30 seconds. The beads were washed by repeated pipetting of 200 µl or 400 µl of lysis buffer (from the automated extraction kits). The buffer was transferred into a fresh tube and 10 µl of proteinase K added. This was incubated for 15 minutes at 56°C before loading into the machine for automated extraction. Enzymatic and mechanical pretreatment: 400 µl of sample was mixed with 1 ml RCLB, centrifuged at 1500 *g* for 10 minutes and the supernatant discarded. Red cell lysis was repeated and the remaining pellet was resuspended in 1 ml WCLB. The solution was added to a sample containing 10 µl of proteinase K and incubated at 56°C for 30 minutes. Following centrifugation at 8000 *g* for 10 minutes the supernatant was discarded and ~50 µl of glass beads added. The sample was bead-beaten by vortexing for 30 seconds. The beads were washed by repeated pipetting of 200 µl or 400 µl of lysis buffer (from the automated extraction kits). The buffer was transferred into a fresh tube, 10 µl of proteinase K added, and incubated for 15 minutes at 56°C before loading into the machine for automated extraction.

Automated extractors were used as per manufacturer's instructions with 200 µl sample volume (EZ1 BioRobot), 400 µl sample volume (MagNA Pure) and a 100 µl elution.

All real-time PCR assays were performed using *Aspergillus fumigatus* specific primers (ASPCF, 5′-CTC GGA ATG TAT CAC CTC TCG G-3′; ASPCR, 5′-TCC TCG GTC CAG GCA GG-3′) and probe 5′-FAM-TGT CTT ATA GCC GAG GGT GCA ATG CG-TAMRA-3′ targeting 28S gene.[16] The 25 µl PCR mixture contained 1× QuantiTect PCR master mix (QIAGEN, Warrington, UK), 300 nM of each primer, 150 nM of probe and 7.5 µl of DNA. DNA amplification and fluorescence detection was performed using an ABI Prism 7000 (Applied Biosystems) under the following conditions: 50.0°C for 2 minutes, 95.0°C for 15 minutes followed by 40 cycles, 95.0°C for 0.15 seconds (denaturation), and 58°C for 1 minute (hybridisation and elongation), with fluorescence detected after each cycle.

RESULTS

Significantly more aspergillus DNA was detected from the clot than the EDTA sample when both the EZ1 BioRobot ($p<0.001$, 95% CI 2.1 to 5.4) and the MagNA Pure ($p = 0.001$, 95% CI 1.1 to 4.2) were used.

Significantly more aspergillus DNA was also detected from the clot than the serum sample when both the EZ1 BioRobot ($p<0.001$, 95% CI 2.24 to 6.48) and the MagNA Pure ($p = 0.004$, 95% CI 1.1 to 6.08) were used.

When the clot was processed using the EZ1 BioRobot system, enzymatic pretreatment, mechanical pretreatment and a combination of both pretreatments reduced the yield of aspergillus DNA compared with no pretreatment ($p = 0.003$, 95% CI 0.845 to 4.850; $p<0.001$, 95% CI 2.269 to 6.273; and $p<0.001$, 95% CI 3.164 to 7.168, respectively).

There was some evidence that the average Ct (threshold cycle) values were greater for EZ1 BioRobot than the MagNA Pure, but this did not reach statistical significance at the 5% level ($p = 0.078$).

Table 2 summarises these results.

DISCUSSION

Despite the availability of new mould-active antifungal agents such as extended spectrum azoles (voriconazole, posaconazole) and echinocandins, invasive aspergillosis remains a significant cause of death in neutropenic patients. Delay in starting appropriate antifungal therapy may contribute to the high mortality; this may be compounded by the lack of a reliable diagnostic test. Molecular diagnostic techniques to detect aspergillus DNA in blood using PCR represents a promising approach to facilitate rapid diagnosis, but published studies are very variable in both methods of extraction and the PCR protocol used (table 1).

It is likely that most well designed PCR reactions will reliably amplify any aspergillus DNA present so the variability between studies may be due to both patient factors, such as the time interval between the onset of disease, and sampling. The use of antifungal prophylaxis has been shown to decrease the sensitivity of the galactomannan assay[17] and could also therefore effect other diagnostic tests. However, the type of specimen analysed and extraction methods are likely to have the most impact on the performance of the test.

Table 2 Extraction methods and real-time PCR results

Extraction method	Mean Ct value*		
	EDTA blood	Serum	Clot
EZ1 robot (QIAGEN)			
Without pretreatment tissue kit	36.89	38.20	34.51
With enzymatic pretreatment	38.05	ND	37.02
With mechanical pretreatment	38.19	ND	37.19
With enzymatic and mechanical pretreatment	ND	ND	37.82
MagNA Pure robot (Roche)			
Without pretreatment	36.12	37.86	34.27
With enzymatic pretreatment	ND	ND	35.99
With mechanical pretreatment	38.79	ND	35.04
With enzymatic and mechanical pretreatment	38.19	ND	36.55

*Mean Ct value of three extractions with triplicate real-time PCR reactions.
ND, not detected at 40 cycles.

We initially examined EDTA whole blood, serum and clot samples in order to identify the most useful sample for PCR diagnosis. We found that when using the EZ1 BioRobot the average Ct value for the clot sample was 2.38 cycles lower than EDTA blood and 3.69 cycles lower than serum. This equates to a 7.9- and 12-fold increase in DNA yield from the clot, respectively; this was also seen when samples were processed using the MagNA Pure automated extraction method. There seemed to be no diagnostic benefit from analysing EDTA blood or serum in addition to the clot as EDTA and serum samples were positive for *Aspergillus* DNA only when the clot was also positive.

Although the fungal burden the animals were exposed to was far greater than levels expected to be found in the clinical setting, in routine clinical practise larger volumes of blood could be used to compensate.

Aspergillus spp. are ubiquitous in the environment, creating the potential for contaminating fungal DNA to enter the assay process. Our results suggest that performing additional steps using enzymes or mechanical pretreatment does not improve the yield of DNA for these samples.

The advantage of excluding these additional steps is twofold. Firstly, it reduces the opportunity for contaminating fungal DNA to enter the analytical process; and secondly, it makes sample preparation less time consuming and more straightforward for implementation in a clinical laboratory setting.

A number of commercial systems exist for extracting DNA. Our study evaluated two of the most widely used, QIAGEN EZ1 BioRobot (QIAGEN) and MagNA Pure (Roche). The DNA extraction protocol, both as specified by the manufacturer or modified with either additional mechanical, enzymatic or a combination of steps, showed some evidence that the average Ct values were greater for the EZ1 BioRobot than MagNA Pure, but this did not reach statistical significance at the 5% level ($p = 0.078$).

The automated extraction systems we examined do have a drawback of only allowing a small number of samples to be processed (6–8 samples per run); however, there are larger machines available which could be validated with clot samples, providing that they use similar chemistry/technologies.

In summary, our study has shown that unmodified commercial extraction systems using a clot as substrate yields the best results in terms of aspergillus DNA yield, with the dual benefits of both reducing processing times and decreasing the number of stages during which fungal contamination of the sample may occur.

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