Real-time PCR and quantitative culture for monitoring of experimental *Aspergillus fumigatus* intracranial infection in neutropenic mice

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The central nervous system (CNS) is the most common site of dissemination during *Aspergillus* infection. PCR has the potential to facilitate early diagnosis of CNS aspergillosis, which could assist in reducing disease mortality. In two experiments, neutropenic CD-1 male mice were infected intracranially with 5×10^6 conidia of *Aspergillus fumigatus*. At time points up to 120 h after infection, mice were euthanized and samples of blood, brain, spinal cord and cerebrospinal fluid (CSF) were taken. The brain fungal burden was determined by quantitative culture, and fungal DNA was detected by quantitative PCR. Plating for *A. fumigatus* from the brain confirmed that all mice had burdens of $\log_{10} > 3$ from 4 to 120 h after infection. *A. fumigatus* DNA was detected in blood (88 %), brain (96 %), CSF (52 %) and spinal cord (92 %) samples. The brain and spinal cord contained the highest concentrations of fungal DNA. Adapting the extraction protocol to maximize yield from small sample volumes (10 μ l CSF or 200 μ l blood) allowed PCR detection of *A. fumigatus* in infected mice, suggesting the use of CSF and blood as diagnostic clinical samples for CNS aspergillosis.

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INTRODUCTION

Invasive aspergillosis is a leading cause of mortality in immunocompromised hosts (Neofytos *et al.*, 2009). Central nervous system (CNS) aspergillosis has been diagnosed with increasing frequency over the past decade (Nadkarni & Goel, 2005; Schwartz & Thiel, 2009); parenchymal abscesses represent the majority of these cases, with true meningitis being rare. The majority of cases of CNS aspergillosis are the result of haematogenous spread from primary sites of infection, but neurosurgery and other invasive procedures have been reported as iatrogenic causes

of infection (Schwartz & Thiel, 2009). Analyses of patients with cerebral or disseminated aspergillosis have shown that the mortality rate ranges between 88 and 99 % (Schwartz & Thiel, 2009), with the survival from onset of symptoms in some patients being as short as 5 days (Pagano *et al.*, 1996).

Murine models play an important role in the evaluation and development of novel treatments and in methods to monitor treatment and diagnose infection (Capilla *et al.*, 2007; Clemons & Stevens, 2005, 2006). The urgent need for more-effective diagnosis of CNS aspergillosis has been reported (Nadkarni & Goel, 2005). The main strategies for detecting fungal pathogens include radiological imaging, microscopy and culture methods, as well as antigen detection in infected fluids or tissues (Hope *et al.*, 2005). For CNS aspergillosis, these methods are generally unreliable at

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; GM, galactomannan; ITS, internal transcribed spacer; PAS, periodic acid–Schiff; p.i., post-infection; qPCR, quantitative PCR.

the early stages of infection (Schwartz & Thiel, 2009). The detection of galactomannan (GM) in serum samples is widely used in Europe for screening patients at risk for invasive aspergillosis and has shown high specificity and sensitivity in several studies (Mennink-Kersten *et al.*, 2004). However, this assay is affected by the administration of antifungal drugs and cannot be used directly to quantify fungal burden, as the relationship between the fungus and release of GM in the host is poorly understood (Mennink-Kersten *et al.*, 2004). There is evidence that PCR can detect *Aspergillus* DNA in patient samples in the absence of GM antigenaemia (Cuenca-Estrella *et al.*, 2009). Early detection of *Aspergillus fumigatus* in patient cerebrospinal fluid (CSF) has been reported using the Platelia *Aspergillus* EIA kit for GM (Bio-Rad; Klont *et al.*, 2004) and by nested PCR (Hummel *et al.*, 2006). There is still a need for tools that enable earlier diagnosis of infection and that will allow optimal treatment with the prospect of improving the outcome of CNS aspergillosis. In the current study, we used a murine model of cerebral aspergillosis to examine the potential of real-time PCR as a tool to track dissemination of *A. fumigatus* during infection and to determine the suitability of minute volumes of CSF and blood as diagnostic samples for cerebral aspergillosis.

METHODS

Organisms and murine model. Neutropenic 6-week-old male CD-1 mice were infected intracranially with 5×10^6 conidia of *A. fumigatus* (strain 10AF) while under isoflurane anaesthesia, as described previously (Chiller *et al.*, 2002; Singh *et al.*, 2005). The mice were neutropenic throughout the course of the study. Intracranial infection was chosen to ensure the development of cerebral aspergillosis, which would not be guaranteed through an intranasal infection model. Mice were pre-assigned to specific sample time points to exclude bias, with five mice per time point and four uninfected control mice. All animal studies were carried out under a protocol approved by the Institutional Animal Care and Use Committee of the California Institute for Medical Research.

At 4, 8, 24, 72 and 120 h post-infection (p.i.), mice were anaesthetized using isoflurane and exsanguinated for collection of whole blood. CSF samples were collected from the mice after they had been euthanized. In brief, skin and musculature were dissected away to expose the skull, and CSF was sampled from the cisterna magna with a finely drawn glass capillary by direct puncture. CSF samples were dispensed into a screw-cap tube and frozen at -80°C . Brain and spinal cord were dissected from the skull following CSF collection.

Quantitative plating. After blood and CSF samples had been taken, the fungal burden in the brain was determined by quantitative culture, as described previously (Chiller *et al.*, 2002; Clemons & Stevens, 2009; Singh *et al.*, 2005).

DNA extraction. *A. fumigatus* DNA was extracted from murine CSF (10 μl) using a QIAamp DNA Micro kit (Qiagen) and from brain homogenate (500 μl), blood (200 μl) and spinal cord homogenate (50 μl) using a High Pure PCR Template Purification kit (Roche), with the following modifications. CSF with kit buffers ATL and AL (containing carrier RNA) were added to 1.5 ml tubes with proteinase K [600 milliabsorbance units (mAU) ml^{-1}] and incubated at 56°C for 30 min. For brain, blood and spinal cord samples, binding buffer

and proteinase K (600 mAU ml^{-1}) were added (the buffer volumes needed for brain homogenate and blood were twice the amount described in the manual) to the samples and incubated at 70°C for 10 min for blood, 90 min for spinal cord and overnight for brain homogenate. The different treatment times were chosen due to the relative complexities of the tissues, with brain and spinal cord requiring longer treatment than blood. After proteinase K treatment, all samples were added to tubes of Magna Lyser Green Beads (Roche) and vortex mixed for 90 s; 300 μl chloroform:isoamyl alcohol (24:1) was added and the samples were centrifuged at 10 000 g for 5 min. The supernatants were processed following the manufacturers' instructions in the protocol for isolation of genomic DNA from small volumes of blood (Qiagen) for CSF and in the protocol for isolation of nucleic acids from bacteria and yeasts (Roche) for blood, spinal cord and brain homogenate. Samples were stored at 4°C until needed. These DNA extractions followed the principles of fungal DNA extraction recommended by the European *Aspergillus* PCR Initiative (White *et al.*, 2010), which indicated that bead disruption of samples improved PCR positivity rates.

Quantitative PCR (qPCR). A Taqman probe qPCR assay targeting the internal transcribed spacer 1 (ITS1)/5.8S rRNA gene region (Springer *et al.*, 2011) was used to quantify fungal DNA. In brief, 21 μl reactions contained 0.3 μM primer Asp fum_F (5'-GCAGT-CTGAGTTGATTATCGTAATC-3'), 0.6 μM primer Fungi 5.8_R (5'-CAGGGGGCGCAATGTGC-3'), 0.15 μM hydrolysis probe ITS-PF (5'-FAM-CAGCGAAATGCGATAAGTAATGTGAATTGCA-TAM-3'), 10 μl Taqman Gene Expression Mastermix (Applied Biosystems) and 10 μl template DNA. Amplification was carried out in a StepOnePlus machine (Applied Biosystems) with the following conditions: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s, 54°C for 30 s and 72°C for 30 s. The PCR primers Asp fum_F and Fungi 5.8_R used in this study have been used previously to quantify *A. fumigatus* DNA in murine serum samples with a detection limit equivalent to 0.3 *A. fumigatus* genomes ml^{-1} (Morton *et al.*, 2010). This assay had a sensitivity of 94 % and specificity of 100 % in spiked clinical samples (detection limit 20 c.f.u. ml^{-1} in donor blood), with cross-reactions observed with *Aspergillus terreus* and *Aspergillus flavus* but not with *Candida* species (Springer *et al.*, 2011). The number of gene copies was measured by comparing the sample C_q (qPCR threshold cycle) values with a standard curve. The ITS PCR standard was produced by cloning the PCR product using the pGEM-T system (Promega); the resultant plasmid was quantified with a Nanodrop spectrophotometer and used as the target for the standard curve.

GM assay. The presence of GM was detected in brain homogenates using an adaptation of a previously described method for lung samples (Vallor *et al.*, 2008). The amount of GM in supernatants from centrifuged brain homogenates was measured using a Platelia *Aspergillus* EIA kit (Bio-Rad) in accordance with the manufacturer's instructions.

Histology. Mouse brains for histopathological examination were fixed in 10 % neutral buffered formalin and processed for embedding in paraffin wax (Chiller *et al.*, 2002). Sections were stained with haematoxylin and eosin, periodic acid-Schiff (PAS) and Gomori's silver stain, and examined by light microscopy.

Statistical analysis. Two independent experiments were carried out showing reproducible results and the data presented are from a single representative experiment. Data for qPCR experiments were compared by one-way analysis of variance for each dataset followed by Dunn's multiple comparison post test to examine response over time, and by Mann-Whitney *U* test to compare tissue types at each time point, using Graphpad Prism (Graphpad Software). c.f.u. burdens were compared using a Mann-Whitney *U* test.

RESULTS

Murine cerebral aspergillosis

Mice showed clinical signs of progressive CNS disease, with ruffled fur, weight loss and head cant as the primary clinical signs. Some mice showed spinning or occasional paresis prior to euthanasia.

Detection of fungal c.f.u., DNA and GM in brain tissue

Quantification of *A. fumigatus* c.f.u. in brain tissue confirmed that all mice had burdens of $\log_{10} > 3$ from 4 to 120 h p.i., which showed a trend towards a gradual decrease over time (Fig. 1). The quantitative data at 4 h p.i. indicated the initial inoculum in the brain tissue to which later time points could be compared. There was a fluctuation in the amount of fungal DNA detected that was not observed for c.f.u. counts (Fig. 1). Culture of blood and other tissues from uninfected control mice did not yield any fungal growth. The GM data (Fig. 1) indicated that the initial inoculum of conidia did not release GM and that it was only released by actively growing fungi as the infection progressed. The amount of detectable GM showed the largest increase between 24 and 72 h p.i.,

which was similar to the PCR data. The uninfected control mice gave no GM signal.

qPCR detection of *A. fumigatus* in CNS samples and blood

A. fumigatus DNA was detected in 96 % of brain samples, 92 % of spinal cord samples (Fig. 2a), 88 % of blood samples and 52 % of CSF samples (Fig. 2b). *A. fumigatus* DNA was detected in uninfected control mouse samples (mean $\pm$ SEM values were 5 ± 5 gene copies in 200 μ l blood, 30 ± 10 gene copies in 500 μ l brain homogenate, 38 ± 21 gene copies in 10 μ l CSF and 383 ± 211 gene copies in 50 μ l spinal cord homogenate). Tissue-specific mean values were subtracted from infected mouse samples before the data were plotted. The mean level of contamination varied considerably among sample types. As an *A. fumigatus* genome contains 38–90 copies of the ribosomal operon (Herrera *et al.*, 2009), this suggested contamination of approximately $\leq$ one conidium in uninfected blood, brain and CSF samples, with potentially ten conidia in uninfected spinal cord samples. These values could be used to establish and apply a cut-off value for a routine diagnostic assay depending on the sample type, as has been suggested for the development of diagnostic PCR assays (Pham *et al.*, 2003). Blood contained the lowest amount of

Fig. 1. Comparison of the detection of *A. fumigatus* by PCR, c.f.u. and GM ELISA in the brains of intracranially infected neutropenic mice. There was a trend towards a decrease in c.f.u. counts (c.f.u. at 4 and 8 h p.i. > c.f.u. at 120 h p.i.; $P < 0.05$) and a fluctuation in the amount of detectable DNA, whereas GM was detectable only after 24 h. Five mice were examined at each time point after inoculation. The qPCR data are expressed as conidial equivalents (CE), which are equivalent to one *A. fumigatus* genome. The graphs show the number of *A. fumigatus* CE ($\log_{10}$ scale), c.f.u. ($\log_{10}$ scale) and GM index score detected in brain homogenate from each mouse; the median value for each time point (horizontal bar) is shown to highlight trends in the data. PCR samples at each time point were compared by analysis of variance and Dunn's post test. Asterisks indicate where the quantified DNA burden differed significantly between time points ($P < 0.05$).

Fig. 2. Detection of *A. fumigatus* DNA by PCR on samples from intracranially infected neutropenic mice. (a) *A. fumigatus* DNA was detected in 96 % of brain samples and 92 % of spinal cord (SC) samples. There was significantly more fungal DNA in brain than in spinal cord, except at 24 h p.i. ($P < 0.05$). (b) *A. fumigatus* DNA was detected in 88 % of blood samples and 52 % of CSF samples, and the quantified amounts of DNA did not differ significantly between these two tissues. Tissue types at each time point were compared by Mann–Whitney *U* test. Asterisks indicate where the quantified DNA burden differed significantly between sample types: *, $P < 0.05$; **, $P < 0.01$.

contaminating DNA, suggesting that the fungal DNA in uninfected CNS samples may have been due to increased sample handling, e.g. brain and spinal cord need to be

dissected from the head, leading to possible spore deposition from fur. Contamination is a constant problem with *Aspergillus* PCR, and some sources are unavoidable

such as contamination of blood collection tubes (Harrison *et al.*, 2010), reagents (Fredricks *et al.*, 2005; Loeffler *et al.*, 1999) and the host's food (Millon *et al.*, 2010). Establishing a tissue-specific background level is one of the few methods of accounting for this problem.

There was variability in the amount of PCR product detected in specimens at each time point, with brain samples showing the greatest amounts of fungal DNA (Fig. 2). Although the brain samples were larger than the spinal cord samples, it was notable that there was not a significant difference between the sample types at 24 h p.i. Some of the variability was probably attributable to the response of individual mice and further confounded by the small sample volumes for CSF and spinal cord. As blood and CSF are dynamic liquid systems, it can be expected that the yield of fungal DNA would vary between samples, but it was clear from the data that blood gave more consistent results (Fig. 2b). This could be attributable to sample volume, which is an important consideration when choosing a diagnostic sample.

Each sample type showed a similar trend, with a decrease in DNA levels at 24 h p.i., followed by an increase at 72 and 120 h p.i. The variability in the amounts of DNA detected by PCR indicated that this was not significant ($P>0.05$) for blood, spinal cord and CSF (Fig. 2). However, there was a significant increase in brain fungal DNA between 24 and 120 h p.i. (Fig. 1).

Histology of murine CNS aspergillosis

The brains of mice that were inoculated intracranially with *A. fumigatus* showed signs of necrosis in the white brain

matter after 72 h (Fig. 3a). Examination of the necrotic lesions showed focal demyelination and stasis of the capillary blood vessels (Fig. 3b), but there was no cellular reaction besides a mild microglial reaction and no neutrophilic reaction (Fig. 3). Hyphae, showing branching at $\leq 45^\circ$ angles, which is characteristic of *Aspergillus*, were only observed at 72 h p.i. (Fig. 3). There was no detectable communication of necrotic areas with the CSF.

DISCUSSION

Cerebral aspergillosis in humans has a high rate of mortality (Lin *et al.*, 2001) that could be reduced through improved diagnosis. GM detection in CSF has shown potential for early diagnosis of aspergillosis, with reported detection in one patient 45 days before cultures were positive (Klont *et al.*, 2004). PCR has a similar potential for early diagnosis: a nested PCR assay detected *A. fumigatus* in the CSF of leukaemic patients with cerebral aspergillosis (Hummel *et al.*, 2006). However, as diagnosis of *A. fumigatus* CNS infection is not optimal, we sought to test the diagnostic potential of a modified qPCR procedure.

This study showed that a single-round PCR and 10 μ l sample volume, which is approximately 20 % of the total volume of CSF in mice (Wenk *et al.*, 2004) and would be equivalent to a 20 ml sample from a human, can detect the presence of *A. fumigatus* DNA in CSF (52 % of samples). Other PCR studies have used larger sample volumes, such as 1.5–2 ml (Hummel *et al.*, 2006) and 200 μ l CSF for spiking experiments (Schabereiter-Gurtner *et al.*, 2007). These data from minute amounts of murine CSF demonstrated that even

Fig. 3. Histopathological examination of brains from intracranially infected mice. Hyphae were not detected in brain samples until 72 h p.i. (a) PAS-stained section from the brain of a mouse 72 h after infection; the circled area shows focal demyelination and stasis of capillary blood vessels. Magnification $\times 100$. (b) Higher-magnification ($\times 630$) image of the PAS-stained section in (a), showing no cellular reaction besides a mild microglial reaction. There was, however, capillary stasis and focal oedema. The arrows indicate *A. fumigatus* hyphae.

suboptimal quantities of sample material can yield usable results. Brain and spinal cord are not commonly used as diagnostic samples but were relevant to this model in order to monitor the fate of the *A. fumigatus* inoculum. Blood and CSF are potential diagnostic samples, with CSF of more interest for cerebral aspergillosis. The data for detection of fungal DNA in CSF (Fig. 2b) suggested that the majority of the inoculum remained in the CNS tissues or was cleared from the CSF, similar to the situation in humans where *A. fumigatus* is only rarely recovered from the CSF (Kami *et al.*, 1999). CSF samples not yielding a PCR-positive result (48%) may have been a result of the high turnover of CSF in rodents (Johanson *et al.*, 2008), as rodents can turn over their entire volume of CSF 11 times a day. This would lead to the rapid clearance of conidia from the CSF at the site of infection and, together with the low sample volumes studied, could have created possible false-negative results for some CSF samples. Detection of *Aspergillus* by qPCR in the blood samples (Fig. 2b) suggested that blood is a route of dissemination for fungal elements, i.e. hyphal fragments or conidia; bead beating to process for extraction of fungal DNA is recommended for the isolation of cellular genomic DNA rather than extracellular DNA (Klingspor & Loeffler, 2009). In a previous model of cerebral invasive aspergillosis, conidia could be detected in the kidneys at 4 h after infection, indicating that they are actively cleared from the CSF, most likely via the arachnoid granulations and access into the venous sinuses, a pathway that helps to explain the presence of fungal DNA in blood (Chiller *et al.*, 2002).

PCR results indicated that during the initial 24 h p.i., there was an initial decline in fungal DNA from the brain, confirmed by comparison with spinal cord at 24 h p.i. where there was no longer a significant difference between the two tissues (Fig. 2), followed by an increase in fungal DNA in brain and spinal cord at 72 h p.i., indicating proliferation of the organism in the CNS tissues (Figs 1 and 2a). This apparent disparity between c.f.u. and qPCR data has been observed previously (Bowman *et al.*, 2001; Singh *et al.*, 2005) and has been reviewed elsewhere (Clemons & Stevens, 2009). It may stem from the fact that c.f.u. counts represent viable fungal elements, whilst PCR measures gene copies that do not have to originate from viable fungus. The difficulty in accurately measuring viable fungal burden is also a product of fungal morphology, as hyphae do not possess a traditional cellular structure: a hyphal tip that would give rise to 1 c.f.u. could contain several nuclei, which would lead to PCR giving a higher estimate of fungal burden (Clemons & Stevens, 2009). Hyphae were not observed until 72 h p.i. in mouse brain tissue, which supports this hypothesis (Fig. 3). The emergence of hyphae was further supported by the GM measurements, which were negative at 4 and 8 h p.i. but increased from 24 h p.i. (Fig. 1). This indicated that the brain contained actively growing fungus, which indicates that the PCR and c.f.u. from 24 h p.i. were derived from hyphae.

Our adaptation of the extraction protocol to maximize yield allowed PCR detection in small sample volumes and

different sample types. In this model of cerebral aspergillosis, the qPCR data suggested that a portion of the initial inoculum, both viable and dead fungal elements, was cleared from the brain and CSF into the blood, thus leading to a reduced fungal burden after 24 h. The subsequent increase in fungal DNA in CNS tissues after 72 h was probably the result of the establishment of an infection through live hyphal growth, which was confirmed by microscopy and GM detection. In the presence of viable fungus, qPCR and GM detection were complementary, and it has been proposed that combining diagnostic tests such as antigen detection assays and qPCR is a potential diagnostic solution for invasive aspergillosis (Aquino *et al.*, 2007; Botterel *et al.*, 2008).

The capacity to detect fungal DNA from extremely small specimens could be important in a clinical setting where samples for multiple assays are required or when samples are needed from infants. We have provided data that also suggest that CSF may be a useful marker for parenchymal CNS infection by *A. fumigatus* using real-time PCR.

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