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Comparison of three methodologies for the determination of pulmonary fungal burden in experimental murine aspergillosis

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ABSTRACT

Quantitative culture, quantitative PCR and the galactomannan enzyme immunoassay (EIA) were compared for their ability to determine the pulmonary fungal burden in a murine model of invasive aspergillosis. Quantitative culture of specimens containing hyphae under-represented the absolute fungal burden in established infection when compared with the two other methods. The best correlation was observed between the two non-culture methods. Higher variability was observed with the galactomannan EIA when compared with quantitative PCR. Collectively, these data suggest that quantitative PCR is the preferred method for determination of the pulmonary fungal burden in experimental aspergillosis.

Keywords Aspergillosis, fungal burden, galactomannan, murine model, PCR, quantitative culture

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INTRODUCTION

The ability to accurately quantify the infectious burden during experimental infection is critical in the evaluation of novel therapeutics and diagnostics. Quantitative culture has been the reference standard for determining the fungal burden in organs during infection by a variety of organisms, including such fungal pathogens as *Candida albicans* and *Cryptococcus neoformans*. Recently, concern has been raised regarding the accuracy of quantitative culture for the determination of fungal burden in experimental infection with filamentous fungi such as *Aspergillus fumigatus* [1]. Since these organisms form aggregates of cells within tissue by continuous extension of existing hyphae during infection [2,3], shearing of hyphae during mechanical disruption of tissues may reduce the fungal viability, resulting in an artificially low organ fungal burden. Furthermore, incomplete homogenisation of these aggregates may result in a failure to disperse the fungal lesion into single organisms, leading to an under-representation of the fungal burden by quantitative culture. Thus, erroneously low quantitative culture results have been observed *in vitro* with *A. fumigatus* because of clumping of cells in suspension [4]. Conversely, shearing of a single filamentous organism into multiple fragments could potentially cause an increase in the apparent fungal burden. Indeed, it has been suggested that therapy with caspofungin acetate, which has been associated with hyphal fragmentation, leads to spuriously high quantitative culture results [5].

Non-culture-based methods, including quantitative PCR [6] and determination of galactomannan levels by commercial enzyme immunoassays (EIAs) [6,7], provide an alternative to quantitative culture. These newer methods avoid many of the problems associated with quantitative culture from organs infected with *A. fumigatus*. However, while both quantitative PCR and the galactomannan EIA have been compared individually with quantitative culture [1,8–12], these two methods

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have not been compared directly with each other. Therefore, the present study compared quantitative culture with quantitative PCR and galactomannan EIA for the determination of pulmonary fungal burden in a murine model of invasive aspergillosis.

MATERIALS AND METHODS

Organisms

A. fumigatus AF293 (a generous gift from P. Magee, University of Minnesota, USA) was used for all studies. For infection, AF293 was grown for 10 days on Sabouraud dextrose plates at 37°C. Conidia were harvested by flooding the plates with sterile phosphate-buffered saline supplemented with Tween-80 0.1% v/v, and were enumerated using a haemacytometer.

Animal model

To perform the most stringent evaluation of the performance of each method, a low-dose inhalational model of murine pulmonary aspergillosis was used. Immunosuppressed mice were infected by aerosol chamber as described previously [3]. Briefly, 2 days before infection, female Balb/c mice, weighing 18–20 g (National Cancer Institute, Bethesda, MD, USA), were immunosuppressed with 250 mg/kg of both cyclophosphamide (Western Medical Supply, Arcadia, CA, USA) and cortisone acetate (Sigma-Aldrich, St Louis, MO, USA), followed by a second dose of cyclophosphamide (200 mg/kg) and cortisone acetate (250 mg/kg) 3 days after infection. The mice were infected for 1 h in an inhalation chamber by aerosolising 12 mL of a suspension of *A. fumigatus* conidia containing 1×10^9 conidia/mL. Groups of eight mice were then killed humanely immediately post-infection (day 0), and at days 3, 5 and 7 post-infection. At each time-point, two immunosuppressed but uninfected mice were included as negative controls. The lungs were removed by sterile dissection, weighed, added to 5 mL of NaCl 0.85% w/v and homogenised by blunt crushing in whirlbags (Fisher Scientific, Pittsburgh, PA, USA). An aliquot from each specimen was processed immediately for quantitative culture. Additional aliquots were frozen for subsequent quantitative PCR and galactomannan testing (see below). All procedures involving mice were approved by the institutional animal use and care committee, according to the National Institutes of Health guidelines for animal housing and care.

Quantitative culture

Aliquots of lung homogenates were diluted serially in NaCl 0.85% w/v. Each dilution (1 mL) was then overlaid with Sabouraud dextrose agar supplemented with chloramphenicol 50 mg/L. Plates were incubated for 48 h at 37°C, and the number of colonies was then counted.

Quantitative PCR

Each lung homogenate (0.1 mL) was digested and the DNA was purified using the MasterPure Yeast kit (Epicentre, Madison, WI, USA) and inhibitor removal resin from the SoilMaster kit (Epicentre). The alcohol-precipitated DNA was resuspended in 0.1 mL of 0.1 mM Tris-EDTA and then frozen until used for PCRs. Quantitative PCR was performed as described previously [6], except that an *Aspergillus* TaqMan probe was employed (TTTCTATGATGACCCGCTCGGCA) with a 5′-fluorescein label and a 3′-TAMRA quencher. This assay uses broad-range primers to amplify a 220-bp segment of the fungal 18S rRNA gene, with detection of *A. fumigatus* based on hybridisation and cleavage of the TaqMan probe. Purified DNA (5 μL) was added to a 50-μL PCR reaction containing Universal Master Mix (Applied Biosystems, Warrington, UK). All samples were run in duplicate, with the mean result being reported. Template and digest controls were run in each batch of assays to monitor contamination. The 45-cycle PCR comprised 15 s at 95°C, 30 s at 55°C and 30 s at 65°C. Known quantities of purified genomic DNA from *A. fumigatus* were added to PCRs to generate a standard curve. In separate tubes, lung homogenate DNA (5 μL) was added to 1000 pg of *A. fumigatus* DNA in order to determine whether PCR inhibitors were present in the lung samples. The amount of *A. fumigatus* DNA was assessed by comparing the amplification threshold cycle of the unknown sample with the threshold cycle values of the standards.

Galactomannan enzyme immunoassay

Aliquots of the lung homogenate were vortexed and centrifuged (1000 *g*) to remove large fragments. Galactomannan was quantified in 300-μL aliquots of homogenate using Platelia Galactomannan EIA kits (Bio-Rad, Edmonds, WA, USA) according to the manufacturer's instructions [7].

Statistics

The Wilcoxon rank sum test was used to compare the differences in fungal burden between time-points for a single test method. Spearman's rank correlation was used to analyse the relationship between results obtained with the different methods. For both tests, $p \leq 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

The mice were infected with conidia by inhalation. Therefore, the organisms isolated from the lungs immediately after infection (day 0) were conidia. The conidia had germinated by the subsequent time-points, so that essentially all of the organisms were hyphae and virtually no conidia were present [4]. As expected from previous experiments [4], almost all the mice survived for at least 7 days after inoculation. Only a single animal died before the planned sampling time, and this animal was excluded from the analysis. *A. fumigatus* was not detected from any uninfected mice by the quantitative culture, PCR or quantitative galactomannan assays.

When the pulmonary fungal burden was analysed by quantitative culture, the median fungal

burden was 1.2×10^4 CFU/g lung tissue immediately after inoculation (Fig. 1). The pulmonary fungal burden decreased significantly after 3 days of infection, and remained relatively stable for the next 4 days. This apparent drop in organ fungal burden between day 0 and subsequent days was probably caused by inadequate dispersion of clumps of hyphae into single cellular elements by the tissue homogenisation process. By virtue of their small size, conidia are dispersed more easily, leading to the detection of higher CFU counts. Another potential cause of the apparent decrease in pulmonary fungal burden at the later time-points is that the homogenisation procedure reduced the viability of hyphal elements through shearing during homogenisation, although this is less likely.

When the pulmonary fungal burden was measured by quantitative PCR, the initial organ fungal burden was low at day 0, but increased progressively throughout the experiment (Fig. 1). Because hyphae are multicellular structures, this rise in organ fungal burden probably reflects an increase in both the number of hyphae and the number of nuclei within the hyphae.

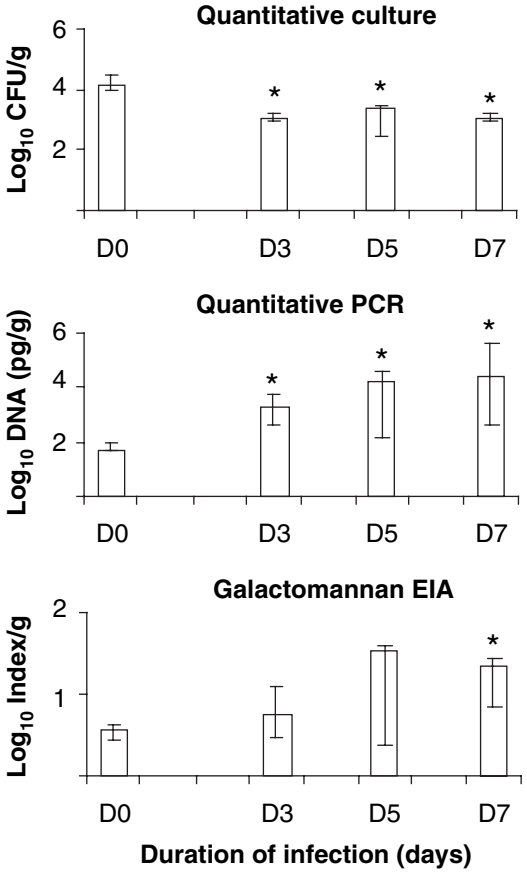

Fig. 1. Time course of fungal burden as determined by three methods. Results are presented as median ± interquartile range.
*Significantly different ($p < 0.05$) from day 0 results by the Wilcoxon rank sum test. *n* = eight mice for each time-point.

The pulmonary fungal burden was also low at day 0 when it was assessed using the galactomannan EIA (Fig. 1). Of the 32 specimens, 25 had a galactomannan index above the 0.5 cut-off index used for serum samples, with one negative sample immediately post-infection, and two negative samples at each of the other time-points. However, all of these values were >0.35, and the cut-off index of 0.5 has not been evaluated for tissue specimens. Although there was a trend towards an increase in lung fungal burden at day 5, this increase was not significant (p 0.19) because of the substantial mouse-to-mouse variability in the results. However, the increase in organ fungal burden achieved statistical significance at 7 days after infection.

Next, the results of all three methods for each individual sample were compared directly. Since the time course results demonstrated clearly that quantitative culture of hyphae and conidia was not comparable, separate analyses were performed for the results of each method immediately post-infection, i.e., when the organisms remained as conidia (Fig. 2A), and at later time-points, when the organisms had germinated to produce hyphae (Fig. 2B). There was poor correlation among the values obtained immediately post-infection with all of the three assays, although the number of samples was small in this subset analysis. The best correlation was observed between the quantitative PCR and the galactomannan EIA, although this correlation was not significant (rho 0.55; p 0.15). Interestingly, at later time-points, when the organisms were growing as hyphae, the relationship between quantitative culture and either quantitative PCR or galactomannan EIA was much more robust. Despite the observed drop in absolute fungal burden with quantitative culture (Fig. 1), this method continued to allow discrimination between high and low fungal burdens in individual samples (Fig. 2). Thus, the degree of under-representation seems to be preserved between individual samples, thereby providing a reproducible assessment of relative, although not absolute, fungal burden. Similar results were seen

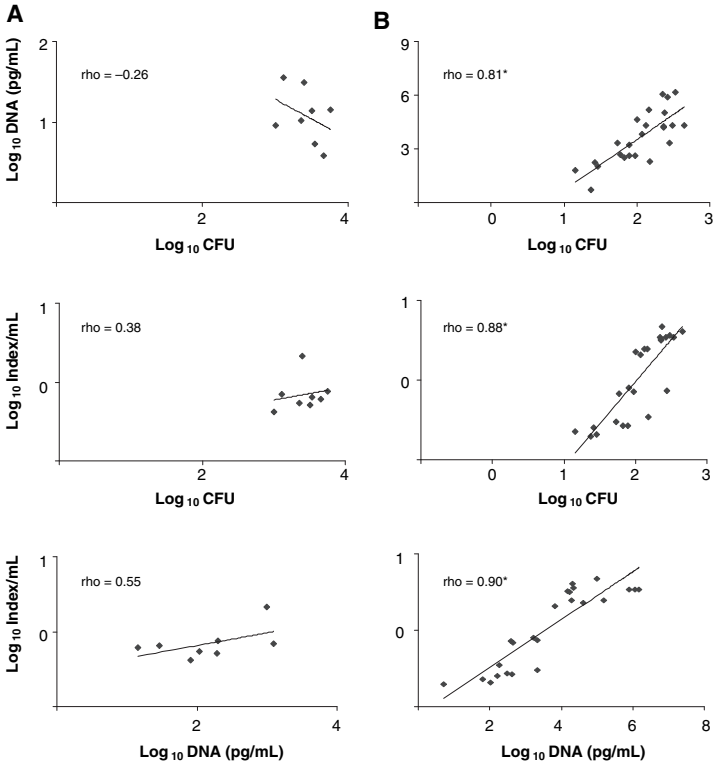

Fig. 2. Correlation of the results of tissue fungal burden determination by different methods. Top row—quantitative PCR vs. quantitative culture; middle row—galactomannan EIA vs. quantitative culture; bottom row—galactomannan EIA vs. quantitative PCR. (A) Comparison of three methods immediately post-infection when organisms remain as conidia. n = eight mice. (B) Comparison at all later time-points when organisms have formed hyphae.
*Significant ($p < 0.05$) correlation between results by the Spearman rank correlation test. n = 24 mice.

when comparing the galactomannan EIA results with quantitative culture. Again, as with the earlier (conidial) time-points, the highest correlation was found between quantitative PCR and the galactomannan EIA. This strong correlation exists because both methods allow the detection of an increasing fungal burden during the course of infection.

The results of this study provide an insight into the utility of quantitative culture for the determination of the relative pulmonary fungal burden during experimental invasive aspergillosis. While this technique clearly remains useful, and results in less variability than either quantitative PCR or galactomannan EIA, two important limitations have emerged. First, quantitative culture underrepresents the absolute fungal burden when performed after an infection is established and the organisms have germinated. Second, the best correlation was observed between the two non-culture methods, particularly at time-points of infection. In the absence of a true reference standard, the interpretation of such comparisons is challenging, particularly since non-culture methods may not be able to distinguish between live and dead organisms. However, the continued increase in fungal burden, indicated by quantitative PCR and galactomannan EIA, is much more consistent with the histopathological course of infection in this model, in which fungal lesions are first detected after 7 days of infection, and continue to increase in size until the recovery from neutropenia [3]. Furthermore, it is probable that the vast majority of organisms were viable, even after 7 days of infection, because the mice remained profoundly neutropenic at this time-point. Almost no leukocytes were present at foci of pulmonary infection until after day 8 following infection [3]. Thus, given the concordance of these two methods, each based on the detection of a different substrate, they seem likely to provide the best estimate of fungal burden over time. In this study, quantitative PCR was associated with less sample-to-sample variability than was the galactomannan EIA, suggesting that it is the preferred method for the determination of fungal burden after organisms have undergone germination and are growing as hyphae.

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