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Correlation between *in vitro* growth rate and *in vivo* virulence in *Aspergillus fumigatus*

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We describe a kinetic microbroth method of measuring the growth rate of *Aspergillus fumigatus* spectrophotometrically. Using this method, growth rates were determined for nine *A. fumigatus* isolates for which an LD_{90} value in immunosuppressed CD-1 mice had previously been obtained. Comparison of the growth rates and LD_{90} values of these isolates suggests that a correlation exists between the two parameters.

Keywords fitness, amphotericin, resistance, genome

Introduction

Previous studies have suggested that reduced virulence in *Aspergillus fumigatus* is associated with reduced growth rate. For example, it was found that double mutation of the *chsC* and *chsG* chitin synthase genes in *A. fumigatus* led to a reduced colony radial growth rate as well as a decrease in mortality and a delay in onset of illness in a murine model of invasive pulmonary aspergillosis [1]. In separate studies, *A. fumigatus* mutants deficient in either chitin synthase gene *chsD* or *chsE* were generated. Despite having morphological abnormalities that included hyphal swelling, both mutant strains showed no changes in colonial growth rate accompanied with normal virulence in a similar murine model of invasive pulmonary aspergillosis [2,3]. A tentative correlation between growth rate and virulence in *A. fumigatus* has therefore previously been demonstrated.

Numerous direct and indirect methods have been employed to measure the growth rate of filamentous fungi including determination of hyphal length and branching [4,5] dry weight, chitin, ergosterol, protein and ATP determination [6,7].

The spectrophotometric measurement of turbidity has also been employed as it allows the non-destructive direct estimation of biomass levels to be made repeatedly over time in the same sample [8]. However, the application of spectrophotometry to the measurement of growth of filamentous fungi has been regarded as unsatisfactory because the morphology of these species provides no identifiable growth unit. Advances in spectrophotometry that enable small changes in absorbance of microcultures in a 96-well plate format to be detected have, however, facilitated the improvement of this methodology. After demonstrating a correlation between absorbance of microbroth suspensions and mycelial weight for a variety of filamentous fungi, Granade *et al*. described a method of spectrophotometric measurement of filamentous fungal growth in a 96-well plate microbroth format [9]. More recently, Meletiadis *et al.* used a similar method to analyse the growth characteristics of three species of filamentous fungi with the intention of optimising methodologies for antifungal susceptibility testing [10,11].

Here we describe a modified microbroth method for kinetic measurement of *A. fumigatus* growth. We selected nine *A. fumigatus* isolates for which virulence data, in the form of the LD_{90} dose in a CD1 mouse model of invasive aspergillosis, was already known. Our microbroth method was used to measure the growth rates of these isolates allowing us to demonstrate a correlation between *A. fumigatus* growth rate and virulence.

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Materials and methods

Isolates

Nine clinical isolates of the filamentous fungus *A. fumigatus* were selected, Af10, Af65, Af71, Af72, Af90, Af91, Af210, Af293 and Af294. Isolates were revived from liquid nitrogen storage by subculturing on Sabouraud dextrose agar (Sab) (Oxoid, Basingstoke, UK) for 7 days at 37°C. Conidia and spores were then collected into PBS/0.05% Tween-20 using a cotton swab. Concentrations of the suspensions were determined using a haemocytometer and viable counts confirmed by plating serial dilutions for each suspension.

Measurement of growth rates

Spore suspensions were inoculated into Sab liquid medium (Oxoid), RPMI-1640 medium (RPMI) (Sigma, Poole, UK) or yeast peptone dextrose (YPD) broth (Becton Dickinson, Oxford, UK) to produce the spore concentration required. The spore suspension in broth was pipetted into 96-well microtitre plates in quadruplicate. Plates were then sealed using Breathe Easy gas-permeable sealing membranes (Diversified Biotech, Boston, MA). The optical density (*OD*) at 405 nm was determined for each well using a Thermomax microplate spectrophotometer (Molecular Devices, Menlo Park, CA). Readings were taken automatically every 5 min for a period of 24 h (289 measurements). Plates were incubated at 37°C throughout and shaken for 5 s before every measurement. Soft Max Pro software (Molecular Devices) was used to automatically generate growth curves for each well by plotting OD_{405} versus time in seconds.

The natural log of the OD_{405} measurements were taken at 30-min intervals during the logarithmic growth phase (11.5–17.5 h after the experiment was started) and plotted versus the time in hours. Excel software was used to calculate the slope of a regression line plotted using this data, giving the specific growth rate in h^{-1}.

In-vivo *virulence studies*

The selected nine isolates had all been previously studied in the temporarily neutropenic murine model [12–17]. All isolates had been studied for the impact of treatment on survival and organ cultures, with the exception of AF293. The LD90 was determined using a range of inocula over approximately a 20-fold range. Subsequent treatment experiments included a control (untreated) group, which allowed confirmation of the inoculum.

Results

Development of microbroth method for growth-rate measurement

We wanted to assess the growth characteristics of a variety of *A. fumigatus* isolates by using a microbroth kinetic method based upon absorbance measurement at 405 nm over a 24-h period. To determine the most suitable microbroth conditions for this, growth curves were obtained for the isolate Af293 under a variety of different conditions (Inoculum: 10^4, 10^5 or 10^6 total spores per well; final volume per well: 50, 75, 100 or 125 μl; media: RPMI, Sab or YPD) and compared.

Growth in all volumes of RPMI with each concentration of inoculum was minimal. Both Sab and YPD gave more conventionally shaped growth curves (i.e. lag phase, first transition period, log phase, second transition period, stationary phase) but only when a volume of 50 μl per well was used. Growth curves in Sab appeared more uniform than those in YPD, with the second transition period in particular being more regular in shape (see Fig. 1). Specific growth rate (h^{-1}) was calculated for each of the growth curves obtained. Values for Sab medium spore concentrations of 10^4 and 10^5 spores per well were greater than at a concentration of 10^6 spores per well. We thus decided to routinely measure growth using 10^4 spores in 50 μl Sab medium (see Fig. 1, expanded panel) as these conditions gave us uniformly shaped growth curves and allowed the measurement of specific growth rate values with a high level of sensitivity.

Correlation of A. fumigatus *growth rates with LD90 in mice*

Using the microbroth conditions described above we measured the growth characteristics of nine isolates of *Aspergillus fumigatus* for which the LD_{90} dose in CD1 mice had previously been determined. Growth was measured in quadruplicate in three separate experiments and the average specific growth rate across these experiments calculated (see Table 1). This enabled the isolates to be compared and ranked according to their growth rate (e.g. highest specific growth rate first, see Table 1). Isolates were also ranked according to their LD_{90} value (e.g. lowest LD_{90} ranked first, see Table 1).

The rank order of LD_{90} value was plotted against the rank order of average specific growth rate and regression analysis performed (Fig. 2). The correlation between these two parameters for the nine isolates was poor ($r^2 = 0.1841$). However, removal of Af65

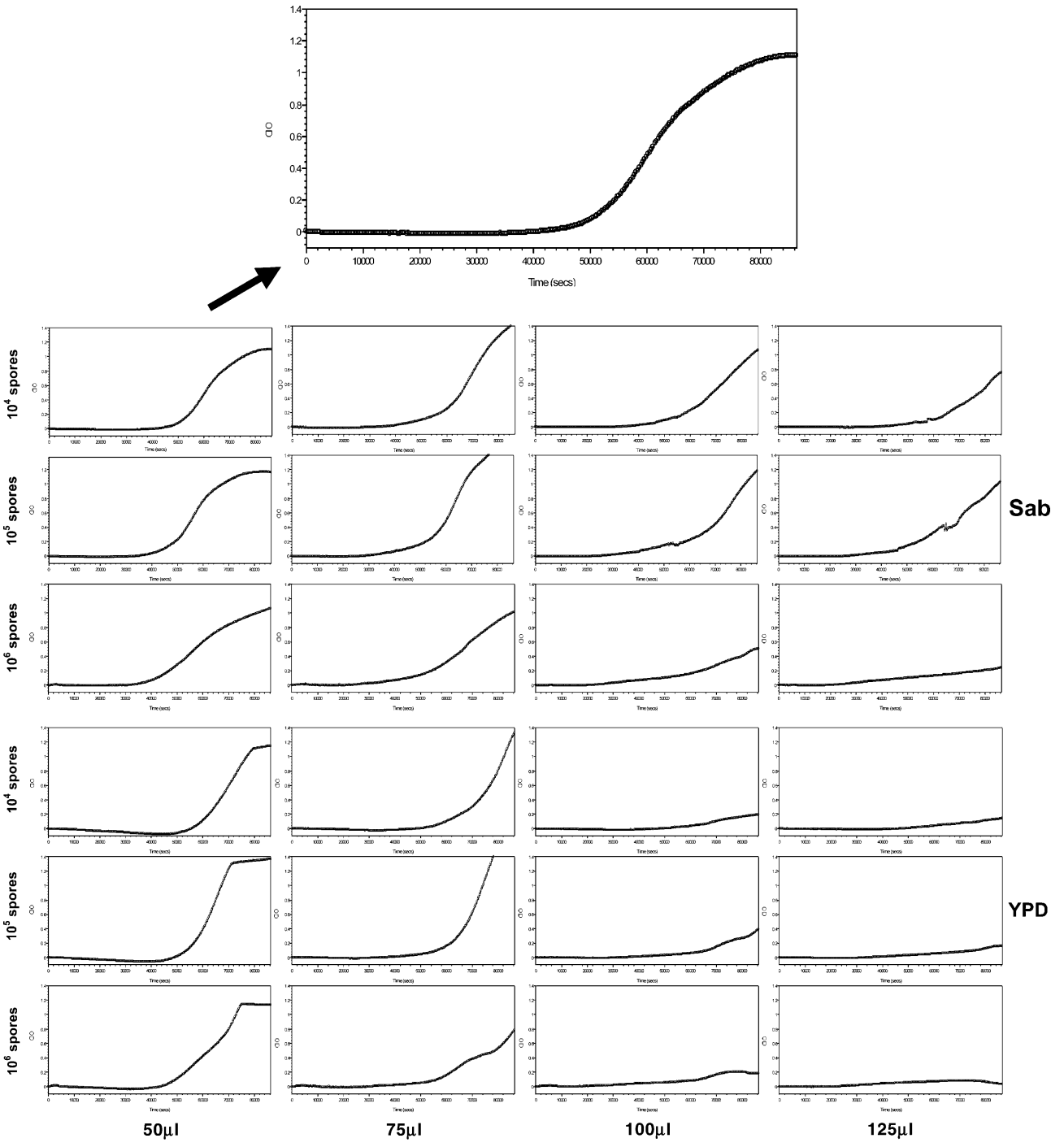

Fig. 1 $OD_{405\ nm}$ versus time (s) for *Aspergillus fumigatus* 293 grown in combinations of different growth media (Sab, YPD), suspension volumes (50, 75, 100 and 125 µl) and spore concentrations (10^4, 10^5 and 10^6 total spores). Conditions of 10^4 spores grown in 50 µl Sab medium (plot highlighted and expanded above) allowed uniform growth and were selected for further experiments.

(amphotericin B resistant) prior to ranking and regression analysis of the isolates (Table 1, Fig. 2) substantially improved the correlation between rank order of LD_{90} and rank order of average specific growth rate ($r^2 = 0.6315$).

Discussion

We describe here a method for measuring the growth of *A. fumigatus* using spectrophotometric absorbance measurements of microbroth cultures in a 96-well plate format.

Table 1 Growth rate measurement and LD_{90} values of selected *Aspergillus fumigatus* isolates. Isolates were assigned a rank order for both parameters with and without the inclusion of isolate Af65

Organism	Af 71	AF 65	Af 210	Af 72	Af 294	Af 293	Af 90	Af 10	Af 91
LD90	3×10^5	4×10^6	3×10^5	4×10^5	4×10^5	5×10^6	2.2×10^6	1×10^6	2.2×10^6
Average growth rate, 405 nm (h^{-1})	0.3375	0.3224	0.3085	0.3009	0.2696	0.2657	0.2628	0.257	0.2349
±SD	0.0147	0.0117	0.0094	0.0210	0.0101	0.0085	0.0313	0.0158	0.0122
Rank order average growth rate	1	2	3	4	5	6	7	8	9
Rank order LD_{90}	1 =	8	1 =	3 =	3 =	9	6 =	5	6 =
Rank order average growth rate (−Af 65)	1		2	3	4	5	6	7	8
Rank order LD90 (−Af65)	1 =		1 =	3 =	3 =	8	6 =	5	6

Indirectly measuring the growth of filamentous fungi by spectrophotometry has been regarded as unsatisfactory because the morphology of these species provides no identifiable growth unit. Studies by Meletiadis *et al*. have, however, described a correlation of a spectrophotmetric method of growth measurement, with direct assessment of growth by measuring hyphal extension [10,11]. The method used in this instance is comparable to that described here.

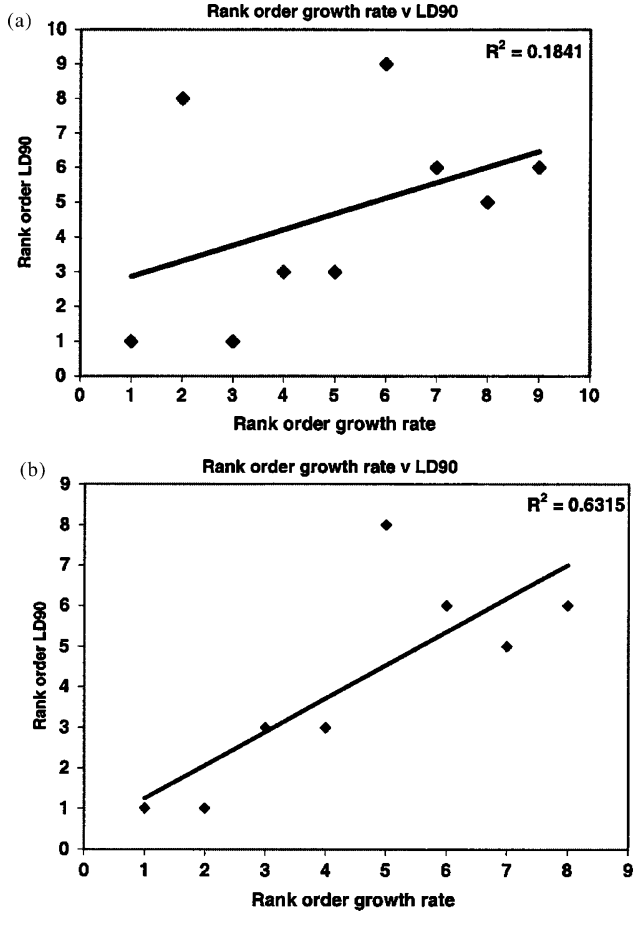

Fig. 2 Rank order of LD_{90} versus rank order of growth rate for *Aspergillus fumigatus* isolates (a) including AF65 (second fastest grower *in vitro*, *x*-axis) and (b) excluding Af65. Regression analysis was performed on both plots, demonstrating an improved correlation between the parameters following the removal of Af65.

Using this method, we have demonstrated a correlation between the specific growth rate (h^{-1}) and the virulence (as defined by the LD_{90}) of eight different *A. fumigatus* isolates.

The correlation was substantially improved following the removal of Af65 prior to ranking and regression analysis of the isolates. Despite being amongst the least virulent of the isolates examined, Af65 was found to have the second highest average specific growth rate. It has been demonstrated that drug resistance in fungi can be associated with a reduction in fitness when compared with drug-sensitive organisms. In the yeast *Candida albicans*, populations evolved in the presence of fluconazole were found to diverge in fitness with a number of drug-resistant populations showing a significant reduction in fitness [18]. Furthermore, for a variety of a number of bacteria and viruses, drug resistance has been associated with a reduction in virulence in animal infection models as well as in other measures of fitness [19–21]. It is possible that the absence of a correlation between the growth rate and virulence of Af65 observed here could be related to a reduction in the 'fitness' of this isolate resulting from resistance to amphotericin B.

The finding that reduced virulence of *A. fumigatus* is associated with reduced growth rate means that genes involved in promoting the growth of this organism may be important virulence determinants. Some evidence for this exists. For example the *rhbA*, a gene implicated in nitrogen sensing, has been found to affect the growth rate of *A. fumigatus* and is also a virulence-determining factor [22]. Cyclic Amp signalling has also been shown to affect *A. fumigatus* growth rate and virulence [23,24].

Endogenous hypercortisolaemia or pharmacological doses of corticosteroid have been identified as a risk factor for disseminated aspergillosis [25,26]. This was thought to be due to inhibitory effects of corticosteroid upon the anti-fungal actions of monocytes [27] and

tissue macrophages [28]. However, Ng *et al*. have reported that corticosteroid promotes the growth of *A. fumigatus in vitro* [5]. The suggestion that increased growth rate is associated with increased virulence, could therefore provide an alternative hypothesis for the mechanism by which corticosteroids increase the risk of contracting disseminated aspergillosis. This in turn may present a novel target for the future development anti-*Aspergillus* agents.

The growth rate of *A. fumigatus* thus appears to be an important parameter influencing the pathogenesis of what is now one of the most prevalent airborne fungal pathogens. The link between growth rate and virulence thus warrants further investigation.

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