Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*

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Aspergillus fumigatus is an increasingly prevalent opportunistic fungal pathogen of various immunocompromised individuals. It has the ability to form filaments within the lungs, producing dense intertwined mycelial balls, which are difficult to treat. The aim of this study was to develop a suitable model of *A. fumigatus* to examine the effects of antifungal challenge on these intertwined filamentous communities. *A. fumigatus* NCPF 7367 growth conditions were optimized on both Thermanox coverslips and on flat-bottomed microtitre plates to establish optimal conidial seeding densities. Isolates were treated with itraconazole, voriconazole, amphotericin B and caspofungin and their overall killing efficiency was measured using an XTT formazan metabolic dye assay. This was compared with the CLSI (formerly NCCLS) methodology of broth microdilution of moulds (standard M38-A). It was shown that 1×10^5 conidia ml^{-1} in RPMI 1640 was the optimum concentration of spores for biofilm formation. Filamentous growth characteristics were not observed until 10 h incubation, followed by an exponential increase in the biofilm biomass (hyphae and extracellular material) and cellular activity (metabolism). When susceptibility testing of biofilms was compared with that of planktonic cells by CLSI broth microdilution testing, all antifungal drugs were at least 1000 times less effective at reducing the overall metabolic activity of 90 % of the cells. Overall, this study showed that *A. fumigatus* has the ability to form coherent multicellular biofilm structures that are resistant to the effects of antifungal drugs.

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INTRODUCTION

Aspergillus species are opportunistic filament-forming moulds. The genus comprises over 180 species, with *Aspergillus fumigatus* causing the majority of human aspergillus infections (Hope *et al.*, 2005). *A. fumigatus* is a ubiquitous saprophytic fungus with a worldwide distribution due to the production of small spores called conidia, which have a mean size of 2–3.5 μm, resulting in the conidia being dispersed into the air and remaining in the atmosphere for time periods that depend on the conditions of the external environment, such as temperature, humidity and seasonal variations (Rivera *et al.*, 2006).

In recent times, the frequency of disseminated fungal infections has increased dramatically. Overall, *A. fumigatus* is now the second most common cause of fungal infection found in hospitalized patients, after *Candida albicans* (Ellis *et al.*, 2000). *Aspergillus* conidia are usually eliminated efficiently by the innate and acquired immune systems. However, in immunocompromised patients, such as transplant, leukaemia and human immunodeficiency virus (HIV)-positive patients, *A. fumigatus* can cause a range of systemic diseases with mortality rates ranging from 30 to 90 % (Brakhage, 2005; Denning *et al.*, 1998; Herbrecht *et al.*, 2002). Pulmonary infection may also occur in other patients such as those with cystic fibrosis (CF). In these patients, infection with *A. fumigatus* may cause allergic bronchopulmonary aspergillosis (ABPA), a mycetoma (fungus ball) or invasive aspergillosis (IA) (de Almeida *et al.*, 2006; Shibuya *et al.*, 2004).

The initial establishment of chronic *A. fumigatus* infection involves the germination of conidia and subsequent hyphal invasion of the lung tissue (Filler & Sheppard, 2006). Histological and microscopic examination of bronchopulmonary lavage samples has revealed the presence of

Abbreviations: ABPA allergic bronchopulmonary aspergillosis; AmpB, amphotericin B; CF, cystic fibrosis; CLSM, confocal laser scanning microscopy; IA, invasive aspergillosis; PMIC, planktonic cell MIC; SMIC, sessile cell MIC; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide.

numerous *A. fumigatus* hyphae in the form of a complex multicellular structure (mycetoma), which is similar to the biofilms formed by *Candida* species (Ramage *et al.*, 2001c, 2005). In contrast to the biofilms formed by *Candida* species, very limited information is currently available on the development and behaviour of *A. fumigatus* adherent multicellular communities and their response to antifungal treatment. To date, there are only two reports suggesting that *Aspergillus* species are able to grow and form biofilms (Beauvais *et al.*, 2007; Villena & Gutierrez-Correa, 2006).

The purpose of this study was to investigate the growth characteristics of filamentous *A. fumigatus* multicellular communities through the development of an *in vitro* model that could be utilized to screen the growth characteristics of clinical isolates or mutants, and to examine the antifungal susceptibility profiles of complex biofilm-like structures (Ramage *et al.*, 2002a, c).

METHODS

Organisms. *A. fumigatus* NCPF 7367 and four clinical isolates (YHCF1, YHCF2, YHCF3 and YHCF4) obtained from the Royal Hospital for Sick Children (Yorkhill Division, NHS Glasgow, UK) were used throughout this study. All isolates were stored on Sabouraud dextrose agar slopes (Oxoid) at 4 °C.

Growth conditions and standardization of conidial inoculum. *A. fumigatus* was grown on Sabouraud dextrose agar at 37 °C for 72 h. Conidia were harvested by flooding the surface of the agar plates with 5 ml PBS (Oxoid) containing 0.025 % (v/v) Tween 20 and rocking gently. The conidial suspension was recovered and dispensed into a 5 ml sterile glass bottle. The conidia were counted using a Neubauer haemocytometer and adjusted to the required concentration in RPMI 1640 (Sigma) buffered to pH 7.0 with 0.165 M MOPS. All procedures were carried out in a HERASafe laminate flow cabinet (model K515; Kendro).

Biofilm formation. *A. fumigatus* biofilms were formed on commercially available, pre-sterilized, polystyrene, flat-bottomed, 96-well microtitre plates (Corning). Biofilms were formed by adding 200 µl of a standardized cell suspension in MOPS-buffered RPMI 1640 to each well for selected time periods (4, 8, 12, 24 and 48 h), and incubating statically at 37 °C. A minimum of 12 replicates was performed for each experimental parameter, plus suitable controls. At each selected time point, the medium was aspirated and the biofilms were washed thoroughly three times with sterile PBS by repeated pipetting to remove non-adherent cells.

Confocal laser scanning microscopy (CLSM). *A. fumigatus* biofilms were formed as described above on the surface of 13-mm-diameter sterile Thermanox plastic cell culture coverslips (Nunc) in 24-well tissue culture plates (Nunc). After incubation at 37 °C for various time periods (0, 2, 4, 8, 10, 12, 16, 18 and 24 h), the coverslips were washed in sterile PBS and stained using the LIVE/DEAD fluorescent stain (Molecular Probes), according to the manufacturer's instructions. The FUN1 component of the kit was used, which is a bright green fluorescent intracellular stain. This was applied to the washed biofilms for 20 min in the dark. The biofilms were then washed in PBS and mounted on a slide. The fluorescent filamentous biomass was examined using a Zeiss Axiovert LSM510 confocal microscope attached to an LSM510 laser scanning system with a 488 argon ion laser at ×200 magnification. Sections of the *xy* plane were taken at 1 µm intervals along the *z*-axis to determine the depth of the biofilms and overall physical ultrastructure. Three-dimensional images were obtained using computer software.

Biofilm quantification. Biofilm biomass was assessed using a modified version of a protocol first developed by Christensen *et al.* (1985) and subsequently modified by O'Toole & Kolter (1998). At each time interval, the spent culture medium was removed from each well and the adherent cells were washed three times with PBS. These were air-dried and 100 µl of 0.5 % (w/v) crystal violet solution was added for 5 min. The solution was then removed by carefully rinsing the biofilms under running water until excess stain was removed. The biofilms were destained by adding 100 µl 95 % ethanol to each well. The ethanol was gently pipetted to completely solubilize the crystal violet for 1 min, the ethanol was transferred to a clean 96-well microtitre plate and the A_{570} was read (FLUO Star Optima fluorescence microplate reader; BMG Labtech). The absorbance values are proportional to the quantity of biofilm biomass, which comprises hyphae and extracellular polymeric material (the greater the quantity of biological material, the higher the level of staining and absorbance).

2,3-bis(2-Methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) reduction assay. A semi-quantitative measure of each biofilm was calculated using an XTT reduction assay, adapted from previous studies (Hawser *et al.*, 1998). This is a metabolic reduction assay that measures the activity of cells and can be used to compare untreated cells with cells treated with antimicrobial agents, and has been used previously in studies to evaluate the antifungal sensitivities of filamentous fungi (Antachopoulos *et al.*, 2006; Meletiadis *et al.*, 2001b, c). Briefly, XTT (Sigma) was prepared in a saturated solution at 0.5 g l^{-1} in PBS. The solution was filter-sterilized through a 22 µm pore size filter, aliquoted and stored at −80 °C. Prior to each assay, an aliquot of stock XTT was thawed and menadione (10 mM prepared in acetone; Sigma) was added to a final concentration of 10 µM. A 100 µl aliquot of XTT/menadione solution was added to each well and to appropriate control wells to measure background XTT reduction levels. The plates were incubated in the dark for 3 h at 37 °C and the colour change was measured using a 490 nm filter in a microplate reader (FLUO Star Optima; BMG Labtech). The colorimetric change in the XTT reduction assay directly correlates with the metabolic activity of the biofilm.

End-point susceptibility testing

M38-A broth microdilution testing. Planktonic cell MICs (PMICs) were evaluated using the CLSI (formerly NCCLS) M38-A standard methodology (NCCLS, 2002). The antifungal agents itraconazole (Sigma), voriconazole (Pfizer), amphotericin B (AmpB; Bristol Myers Squibb) and caspofungin (Merck) were prepared as stock solutions in DMSO (Sigma) and diluted in MOPS-buffered RPMI 1640 to working concentrations. Microtitre plates containing 100 µl of each antifungal were serially diluted twofold to produce a concentration range of 0.03–16 mg l^{-1}. A range of conidial suspensions was then prepared in MOPS-buffered RPMI 1640 to a final concentration of $0.4–5 \times 10^4$ conidia ml^{-1} and 100 µl was added to each well. The plates were incubated for 48 h at 35 °C. The PMIC for each antifungal was defined as the lowest concentration that produced complete visible inhibition of growth. Testing of these isolates was performed in quadruplicate.

Modified M38-A broth microdilution testing. For antifungal susceptibility testing of biofilms [sessile cell MICs (SMICs)], conidia were prepared as described above and standardized to a density of 1×10^6 conidia ml^{-1} in MOPS-buffered RPMI 1640. Biofilms were formed by pipetting standardized conidial suspensions into selected wells of a microtitre plate and incubating for 24 h at 35 °C, as

described above. After growth, the medium was aspirated and non-adherent cells were removed by thorough washing of the cells (three times) using sterile PBS and gentle pipetting. Residual PBS from each well was removed by blotting with paper towels. All antifungals (itraconazole, voriconazole, AmpB and caspofungin) were prepared as described above to provide a working concentration of 512 mg l^{-1} in MOPS-buffered RPMI 1640. These were serially diluted twofold (1–256 mg l^{-1}) directly into adjacent wells and the challenged cells were incubated statically for a further 48 h at 35 °C. A number of antifungal-free wells and biofilm-free wells were also included to serve as positive and negative controls, respectively. SMICs were determined as 50 and 90 % reduction in metabolism compared with the untreated control using the XTT reduction assay described above. Testing of these isolates was performed in quadruplicate.

Statistical analysis. The absorbance values of individual biofilms were compared by one-way analysis of variance and using the Bartlett's test for homogeneity of variances and the Bonferroni's multiple comparison post-test. A value of $P < 0.05$ was considered to be significant. Analyses were performed using SPSS 13.0 for Windows.

RESULTS AND DISCUSSION

A. fumigatus is now a leading fungal pathogen and one of the most significant opportunistic fungi in bone-marrow-transplant patients (Singh, 2005). *A. fumigatus* is also found in a range of other patient groups, including CF patients, HIV-positive patients and other immunocompromised individuals (Cimon *et al.*, 2001). The presence of mycetomas in the upper airways, the pulmonary epithelial cells of the alveoli or in the maxillary sinuses provides compelling evidence that *A. fumigatus* biofilms are more prolific and problematic than once thought (Filler & Sheppard, 2006; Mensi *et al.*, 2004). These infections are typified by intricate networks of hyphae that develop from inhaled conidia (Shibuya *et al.*, 2004). Morphologically, these structures resemble other fungal biofilms, such as those of *C. albicans* (Ramage *et al.*, 2001c), which are clinically important due to their role in pathogenesis and resistance to antifungal agents (Ramage *et al.*, 2002c, 2005). Therefore, we hypothesized that similar pathogenic traits might be exhibited by this fungus, meriting further investigation.

Standardizing an *in vitro* model

To date, there have been no published accounts establishing that filamentous *A. fumigatus* grows as a biofilm. Histopathological evidence has indicated the presence of mycelial plugs and proliferation of hyphae with acute-angle dichotomous branching (Shibuya *et al.*, 2004), which are complicit with the definition of a biofilm. There are many forms of aspergillosis, ranging from ABPA to IA, and despite different clinical presentations, morphological characteristics remain relatively similar and are typified by intricate mycelial networks. Formation of *A. fumigatus* mycelial aggregates that exhibit classic biofilm characteristics is reported here, i.e. an adherent microbial population, adherent to each other and/or surfaces or interfaces (Costerton *et al.*, 1995). To our knowledge, this study is the first to examine submerged cultures of *A. fumigatus in vitro* in this growth modality.

A key factor that we noticed early in our studies was the critical importance of conidial seeding density. Clearly, the structural morphology and integrity of these multicellular structures was dependent on the concentration of conidia (ml medium)$^{-1}$ (Figs 1 and 2), a phenomenon previously identified with *C. albicans* biofilm development (Ramage *et al.*, 2001c). To produce adherent aggregated multicellular populations (biofilms) with a similar morphology to those seen during *in vivo* aspergillosis lung infection, we developed a model, initially examining conidial seeding density of *A. fumigatus* NCPF 7367 and four clinical isolates. First, we examined a serial dilution of conidial densities, ranging from 10 to 1×10^6 conidia ml^{-1}, and examined the resultant multicellular structures after a 24 h inoculation using semi-quantitative metabolic and biomass assays. Fig. 1 illustrates that both the metabolic activity and the biomass of the biofilms exhibited a positive correlation with the conidial seeding density, although at the highest concentrations of conidia the biomass appeared to decline. We subsequently used CLSM, a non-invasive technique that enabled three-dimensional structural imaging and depth measurements of intact multicellular structures on

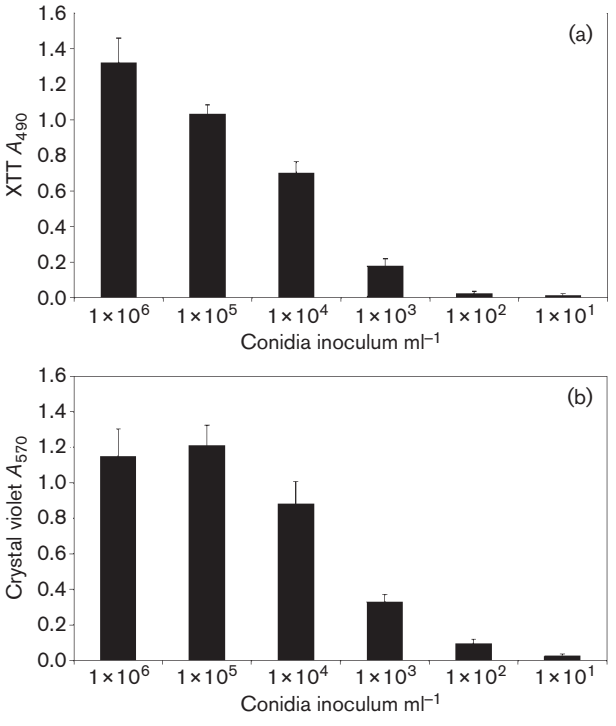

Fig. 1. Effect of conidial concentration on *A. fumigatus* NCPF 7367 biofilm development after 24 h. Metabolic activity of the biofilm (a) and the biomass (b) increased with the concentration of conidia seeded. Note how there is a limit to the development of the biofilm, as indicated from the biomass generated from an inoculum of 1×10^6 conidia ml^{-1}.

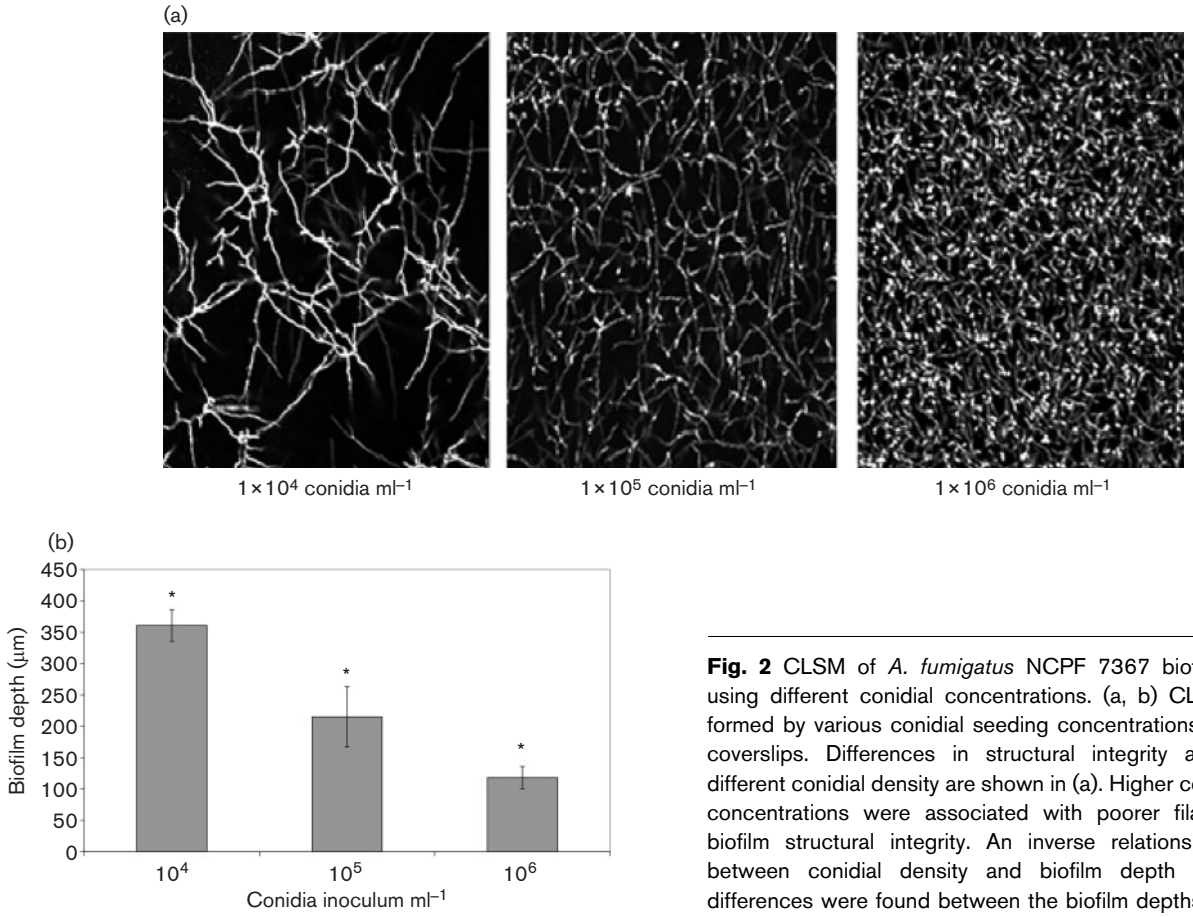

Fig. 2 CLSM of *A. fumigatus* NCPF 7367 biofilms generated using different conidial concentrations. (a, b) CLSM of biofilms formed by various conidial seeding concentrations on Thermanox coverslips. Differences in structural integrity associated with different conidial density are shown in (a). Higher conidial inoculum concentrations were associated with poorer filamentation and biofilm structural integrity. An inverse relationship was found between conidial density and biofilm depth (b). Significant differences were found between the biofilm depths (*, $P < 0.05$).

Thermanox coverslips. The mature biofilms formed using a series of standardized conidial suspensions (1×10^4, 1×10^5 and 1×10^6 conidia ml^{-1}) were evaluated by microscopy to investigate the optimal conidial inoculum concentration. Fig. 2(a) illustrates complex multicellular aggregates formed from three different conidial seeding densities after growth for 24 h, which ranged in depth from 117 to 360 μm. All of the filamentous multicellular structures exhibited acute-angle dichotomous branching to a varying extent. However, the conidial seeding density played an important role in the overall structural integrity of the biofilm structure. Biofilm stability was assessed by shear mechanical force by serially pipetting the biofilms with PBS during the washing procedure (results not shown). Either increasing or decreasing the conidial seeding density 10-fold led to significant differences in the depth of the resultant biofilms ($P > 0.05$). At the highest conidial concentration (1×10^6 conidia ml^{-1}), the biofilms were relatively thin (117 μm) and development of filamentous growth was severely restricted, resulting in less overall biomass (Fig. 2). Conversely, biofilms formed by fewer seeded conidia (1×10^4 conidia ml^{-1}) were observed to be thicker (360 μm) and have longer mycelial frameworks. These were easily disrupted and removed by mechanical forces and were therefore not reproducible. The conidial concentration of 1×10^5 conidia ml^{-1} produced robust filamentous structures that were resistant to mechanical disruption (Fig. 2). Therefore, this concentration of conidia was selected, as the resultant biofilms exhibited reproducible characteristics that were amenable to the high-throughput testing required for screening of clinical isolates and defined mutants, or for testing the susceptibility of antifungal agents.

Kinetics of biofilm development

The growth and development of *A. fumigatus* in the lung, from initial inhalation of conidia to filamentous forms associated with mycetomas, is a key factor in pathogenesis. Clearance of inhaled conidia via innate immune mechanisms in immunocompetent individuals is the key defence against aspergillosis (Latge, 2001). The progression of disease in susceptible patient groups is dependent on the ability of the conidia to germinate and form mycelial masses (mycetomas), which then penetrate the pulmonary epithelium prior to angioinvasion and systemic spread (Filler & Sheppard, 2006; Kamai *et al.*, 2006). In our studies, we examined the kinetics of multicellular development. Initial adherence, conidial germination, and filamentous growth and differentiation of *A. fumigatus*

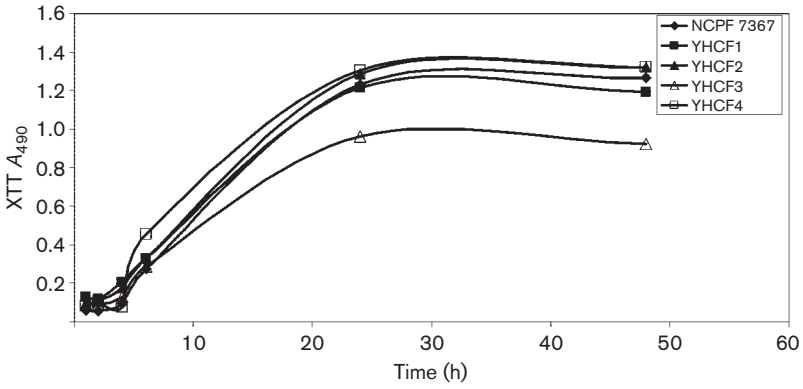

Fig. 3. Growth kinetics of *A. fumigatus* NCPF 7367 and four CF isolates over a 48 h period (seeding density 1×10^5 conidia ml^{-1}).

NCPF 7367 and four clinical isolates were analysed on the surface of polystyrene wells over 48 h (1×10^5 conidia ml^{-1}), as assessed by metabolic activity (XTT) and biomass (crystal violet) measurements (Fig. 3). A correlation between metabolism, biomass and hyphal development was demonstrated. The metabolic activity and the biomass of the biofilms were shown to increase over time. Microscopic analysis revealed that *A. fumigatus* did not form hyphae until approximately 8 h, in agreement with the results of Meletiadis *et al.* (2001a), who demonstrated germination at approximately 6 h using a similar microtitre plate method while examining filamentous growth characteristics of *A. fumigatus*. When the kinetics of biofilm formation was measured, their results were comparable to those in this study, with a lag phase of approximately 10 h. Hyphae began to intertwine forming a monolayer (10–16 h), followed by increased structural complexity over the subsequent 4–8 h (Fig. 4a). This was shown more clearly from the depth measurements taken over a 24 h period. Fig. 4(b) showed an exponential rise in the depth of the biofilm between 10 and 18 h, which then reached a plateau as development ceased and a steady state occurred. These biofilm development characteristics were observed for all isolates tested in this study. The development of the biofilm was slower than that of *C. albicans* biofilms; nevertheless, the overall characteristics of development were similar (Chandra *et al.*, 2001; Ramage *et al.*, 2001c). The slower initial development of mycelia may be related to the overall density of the cells, indicating a potential role for quorum sensing in multicellular *A. fumigatus* populations. This phenomenon has been demonstrated in *C. albicans* with the secreted molecule farnesol, which prevents hyphal development and subsequent biofilm formation (Ramage *et al.*, 2002b).

Antifungal susceptibility testing

Currently, antifungal therapy remains the main way of controlling the progression of aspergillosis. It is clear from our results that the activity of antifungal agents *in vitro* is considerably different when biofilms are compared with planktonic cells, and that a number of currently prescribed antifungal agents may be ineffective in the treatment of established biofilm-associated infections. The methodologies used to examine PMICs and SMICs are quite disparate, i.e. there was a 3 log difference in the number of conidia used in the initial inoculum between these assays. This factor may account, in part, for the disparity between the PMICs and SMICs reported here. For example, itraconazole and caspofungin were ineffective against multicellular structures, exhibiting over 1000-fold more resistance than their planktonic counterparts (Table 1).

The current gold standard for choosing the most appropriate treatment for aspergillosis infections is the CLSI standard M38-A broth microdilution *in vitro* antifungal susceptibility testing assay (NCCLS, 2002). This assay measures inhibition of growth and provides an accurate method for the prediction of fungistatic antifungal drugs. However, in this study we were unable to quantify the effects of the antifungal drugs using this criterion on multicellular structures, which are no longer actively dividing yet are representative of an established infection. ABPA infections with a characteristic mycetoma are typified by greater cellular burdens than are used in planktonic assays and exhibit biofilm characteristics. We used a metabolic dye that exhibited a direct correlation with cellular viability. Viable cell counting was not used due to the inability to correlate colony counts with individual cells. The assay that we described here provides an accurate means of predicting *in vivo* activity due to the reduced metabolic activity of the cells tested. XTT has been used in other mycological studies to monitor antifungal exposure (Antachopoulos *et al.*, 2006; Hawser *et al.*, 2001; Ramage *et al.*, 2001a). For example, Hawser *et al.* (2001) previously described an XTT-based methodology for measuring the minimum effective concentrations of antifungal agents against *A. fumigatus*. In addition, Antachopoulos *et al.* (2006) recently demonstrated how this simple technology could be used to assess antifungal activity against zygomycetes, and demonstrated a reproducible correlation between XTT-deduced MICs and the CLSI methodology. Ramage and colleagues also showed its utility against both fungal (*C. albicans*) and bacterial (*Pseudomonas aeruginosa*) cells, planktonic and sessile cells, and with

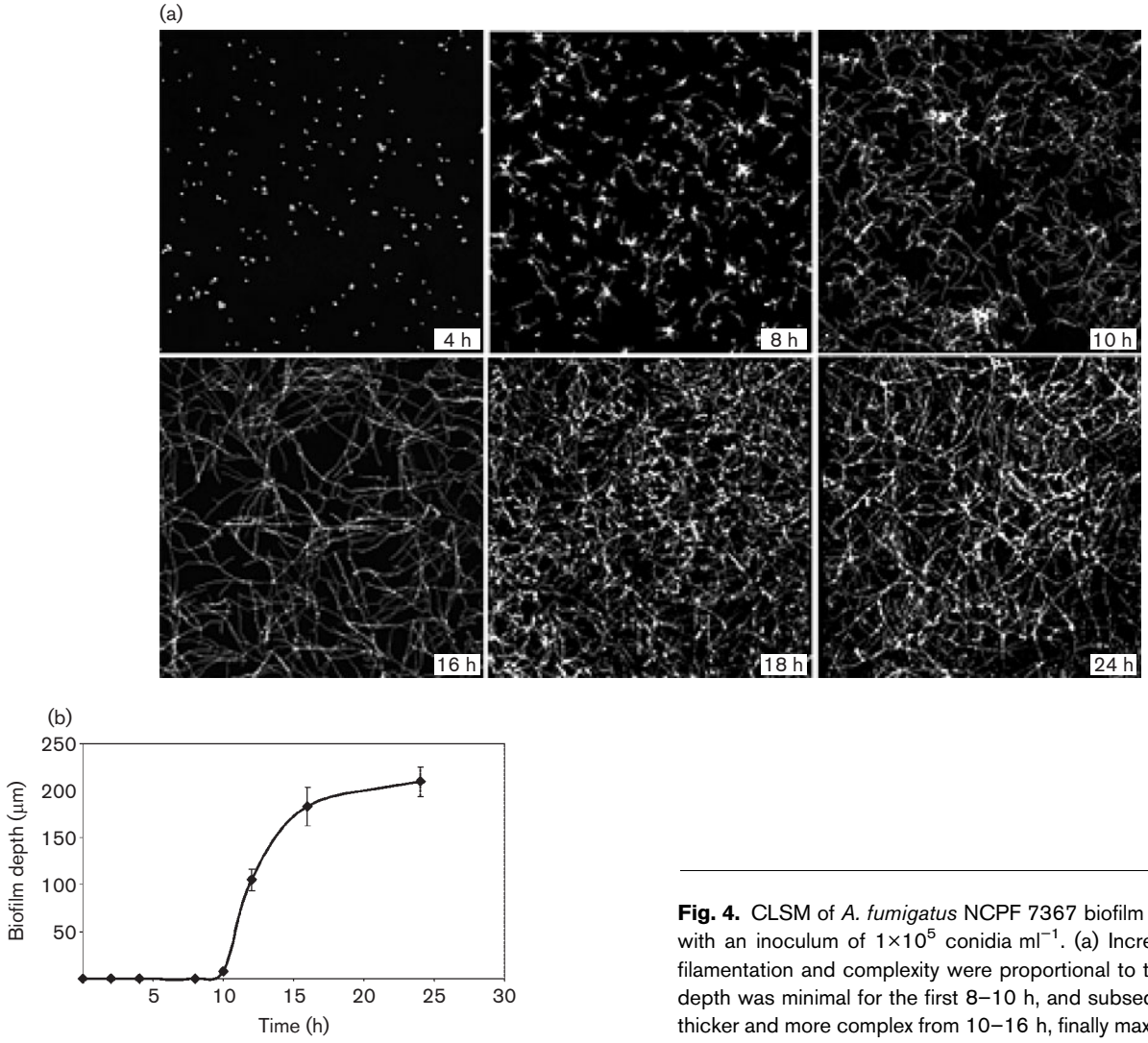

Fig. 4. CLSM of *A. fumigatus* NCPF 7367 biofilm growth kinetics with an inoculum of 1×10^5 conidia ml^{-1}. (a) Increasing levels of filamentation and complexity were proportional to time. (b) Biofilm depth was minimal for the first 8–10 h, and subsequently became thicker and more complex from 10–16 h, finally maximizing at 24 h.

various antimicrobial agents (Ramage *et al.*, 2001a; Tunney *et al.*, 2004). There may be limitations to this assay, such as viable but non-culturable organisms. However, it is unlikely that a high proportion of the biofilm organisms are composed of these viable but non-culturable cells. Overall, the assay enables the user to assess rapidly, either by naked eye or by spectroscopic analysis, the concentration of antifungal agent required to kill or inhibit *A. fumigatus*.

Table 1. Biofilm susceptibility testing

Concentrations are given as mg l^{-1}.

Isolate	Itraconazole			Voriconazole			AmpB			Caspofungin		
	PMIC	$SMIC_{50}$	$SMIC_{90}$	PMIC	$SMIC_{50}$	$SMIC_{90}$	PMIC	$SMIC_{50}$	$SMIC_{90}$	PMIC	$SMIC_{50}$	$SMIC_{90}$
NCPF 7367	0.5	>256	>256	0.25	16	>256	1.0	0.125	32	0.25	128	>256
YHCF1	0.5	>256	>256	0.25	16	>256	1.0	0.125	16	0.25	128	>256
YHCF2	0.5	>256	>256	1.0	128	>256	0.25	<0.125	8	0.25	64	>256
YHCF3	0.5	>256	>256	0.5	32	>256	1.0	<0.125	32	0.25	64	>256
YHCF4	0.25	>256	>256	0.25	16	>256	0.5	1	16	0.25	64	>256

In this study, five strains of *A. fumigatus* were examined for their susceptibility to a range of antifungal agents when grown as a complex mycelial structure, using a methodology previously employed for *C. albicans* biofilms (Ramage *et al.*, 2001a). These agents included two azoles (itraconazole and voriconazole), a polyene (AmpB) and an echinocandin (caspofungin), which were serially diluted and used to challenge planktonic and sessile cells. Table 1 illustrates the results from both conventional planktonic CLSI M38-A susceptibility testing and the modified sessile susceptibility testing. The results indicated that caspofungin had a PMIC of 0.25 mg l^{-1}, itraconazole 0.25–0.5 mg l^{-1}, voriconazole 0.25–1 mg l^{-1} and AmpB 0.25–1 mg l^{-1}. Testing of the sessile cells showed that AmpB was the most effective antifungal agent, with $SMIC_{50}$ values ranging from <0.125 to 1 mg l^{-1} and $SMIC_{90}$ values from 8 to 32 mg l^{-1}. The other azole, voriconazole, exhibited increased efficacy ($SMIC_{50}$ of 16–128 mg l^{-1}). Caspofungin showed poor overall activity against adherent multicellular *A. fumigatus*, with $SMIC_{50}$ and $SMIC_{90}$ values of 64–128 and >256 mg l^{-1}, respectively. Itraconazole was ineffective against all adherent multicellular populations ($SMIC_{50}$ and $SMIC_{90}$ values of >256 mg). Overall, AmpB was the most effective against sessile cells at the lowest concentrations, followed by voriconazole, caspofungin and itraconazole. It has been shown previously that azole antifungals were ineffective against *C. albicans* and *Candida dubliniensis* biofilms, whereas echinocandins were the most effective (Ramage *et al.*, 2001a, b, c). Hawser *et al.* (2001) reported that *A. fumigatus* isolates were more susceptible to echinocandins using conidial concentrations equivalent to the CLSI M38-A method combined with XTT measurements. However, in this study, with an increased cell biomass, caspofungin was ineffective. The inability to successfully treat IA patients with caspofungin has been reported elsewhere, with only 41 % responding during salvage therapy (Maertens *et al.*, 2004). We note, however, that both voriconazole and AmpB had the ability to reduce cellular viability by over 50 % at relatively low concentrations (Table 1). Therefore, although total death was not achieved, there was a certain degree of efficacy against these tenacious multicellular structures, which has been reported previously for other *in vitro* fungal biofilms (Ramage *et al.*, 2002c). This may be an important observation when these agents are used empirically, as they may have a role in preventing the establishment of a mature biofilm. More work will be required to elucidate this.

How do these results relate to clinical practice? A recent review of empirical antifungal therapy in neutropenic patients compared 13 studies. The success rates of the treatments reported showed variations from 31 to 86 % (Martino & Viscoli, 2006). This variability in outcome may be as much to do with the patient population, the weakness of the indications for treatment and the consequent difficulty in establishing objective and reproducible end points for comparisons as the effectiveness of the antifungal agents. The time that treatment is started in relation to the development of the mature biofilm may also impact on the outcome. The optimal time for starting antifungal therapy in neutropenic patients remains undetermined, although most experts recommend waiting until day 5 or 7 of persistent fever (Bennett *et al.*, 2003). It may be more prudent to start treatment before the multicellular structure has established, but this will require further *in vitro* studies. In CF, there is recognition that *A. fumigatus* in sputum cultures in the absence of ABPA may be a pathogen that can directly cause respiratory exacerbations. Antifungal therapy should be considered when deteriorating respiratory function is not responding to antibacterial therapy. Treatment with antifungal agents has been evaluated in these patients and an improvement in clinical condition observed (Shoseyov *et al.*, 2006). Nevertheless, more studies are required before the effectiveness of antifungal agents in this group of patients can be evaluated fully.

Overall, this study demonstrated that *A. fumigatus* grows as a complex, multicellular biofilm and that the concentration of antifungal drug required for the effective treatment of these biofilm-related infections is distinct from assessment by the standard CLSI M38-A assay. This standard method of susceptibility testing does not give a true evaluation of the susceptibility of the disease-causing organism to a given antifungal agent. This may have implications for the diagnosis and management of patients with both invasive and non-invasive infections with this organism. Future studies to examine changes in gene expression of these biofilm-associated organisms may provide ways of elucidating new therapeutic options for controlling *A. fumigatus* biofilms in immunocompromised individuals.

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